ABSTRACT

MIXSON, STEPHANIE MARIE. *Dunaliella* spp. Under Environmental Stress: Enhancing Lipid Production and Optimizing Harvest. (Under the direction of JoAnn M. Burkholder.)

Agricultural crops including corn, sugar cane, and oil palm have been investigated as potential sources for biofuel; however, they produce only a fraction of the oil percent biomass as compared to that of microalgae. Growth and lipid production by microalgae is regulated by a variety of environmental factors, including light intensity, availability of nutrients, temperature regime and salinity. We assessed 14 strains of the saltwater algae *Dunaliella* spp. (Teodoresco) in unialgal cultures within four species to determine a best strain or strain(s) as potential feedstock for biofuels. The taxonomy of these 14 strains was elucidated by comparing both physiological characteristics and the ITS2 and 18S regions. After careful analysis, the data suggest that the 14 strains grouped within four species: *D. tertiolecta*, *D. pseudosalina*, *D. salina*, and *D. viridis*. In addition, the isolation and accurate quantification of neutral lipids in *Dunaliella* was developed from existing techniques. Nile Red was optimized as a qualitative stain to rapidly screen and visualize neutral lipids. Direct transesterification was determined to be the best quantitative method because it yielded high amounts of neutral lipids with precise and reproducible results when compared to conventional extraction methods. Seven strains were selected for further efforts to enhance lipid production using salinity stress, nutrient limitation, pH stress, continuous light, and bubbling with carbon dioxide (CO_2). High salinity yielded the maximum total fatty acid (FA) content (up to 65% by dry weight) in comparison to controls (~10-25% total FAs). High pH x low salinity, low pH, and continuous light x CO_2 yielded near maximum FA content (56%,

43%, and 42%, respectively). Nitrogen and/or phosphorus limitation and 12:12 (light:dark photoperiod) x CO_2 did not significantly enhance FA production (23% and 31%, respectively). Results were strain-specific with high intraspecific variation observed within each environmental stressor. Glycerol production, a known mechanism of osmoregulation in *Dunaliella*, was measured in a short-term salinity stress experiment and found to significantly increase 30 min to 24 hr after exposure. In addition, the glycerol biosynthesis gene, glycerol-3-phosphate dehydrogenase or *GPDH*, was significantly expressed 30 min to 2 hr in response to hyperosmotic stress. The data suggest that *Dunaliella* strains may incorporate a proportion of glycerol as triacylglycerol (TAG) under short-term, high-salinity stress. High lipid-producing strains were grown in mass culture, but at this time the commercialization of harvesting has not been proven economically feasible. Auto-flocculation, electro-flocculation, and hollow-fiber filtration were compared as potential harvesting mechanisms for the mass culture of *Dunaliella* spp. Hollow-fiber filtration (>99% biomass recovery) as harvesting mechanism offers many attractive advantages (i.e. reuse of filtrate as culture medium) when compared to auto-flocculation and indirect electro-flocculation (>95% biomass recovery). This research provides evidence that *Dunaliella* can be used as a source of biofuel because these strains can be mass-cultured; their lipids enhanced through a simple high-salinity adjustment; and commercially harvested.

Dunaliella spp. Under Environmental Stress: Enhancing Lipid Production and Optimizing Harvest

by

Stephanie Marie Mixson

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Biology

Raleigh, North Carolina

2013

APPROVED BY:

_____	_____
JoAnn Burkholder	Amy Grunden
Chair of Advisory Committee	Committee Member – Minor
_____	_____
Heike Winter-Sederoff	Parke Rublee
Committee Member	Committee Member

DEDICATION

I dedicate my dissertation work to my family and my many friends who have supported me along this journey. A special thanks to my parents, James and Lori Mixson, and to my brother, Robert, whose words of wisdom and encouragement helped me throughout the years. I also dedicate this work to Nathan Byrd for his love and support and always being there for me.

BIOGRAPHY

Stephanie M. Mixson was born in Raleigh, North Carolina on May 6, 1986. She attended middle school and high school in Bucks County, Pennsylvania and then moved back to North Carolina to further her education. Stephanie graduated *cum laude* from Wake Forest University in 2004 with a Bachelor of Science degree in Biology. Wishing to further her education, Stephanie attended North Carolina State University from 2008-2013. She was first accepted as a Master's student in the Department of Plant Biology and transferred to the Doctor of Philosophy program in the same department in 2009. She also attended classes to receive a minor in Biotechnology. For her first three semesters, Stephanie taught Introductory Biology laboratory sections as a Teaching Assistant. For the remainder of her time at North Carolina State University, Stephanie was a Research Assistant at the Center for Applied Aquatic Ecology. During this time, she wrote and received a grant from the Charles A. and Anne Morrow Lindbergh Foundation. Stephanie was also a Graduate Research Assistant for a National Science Foundation – Emerging Frontiers in Research and Innovation grant from 2009-2013. She pursued her research under the direction of Dr. JoAnn Burkholder and received her Ph.D. in Plant Biology with a minor in Biotechnology from North Carolina State University in 2013.

ACKNOWLEDGMENTS

I would first like to thank my entire committee; Dr. JoAnn Burkholder, Dr. Park Rublee, Dr. Amy Grunden, and Dr. Heike Winter-Sederoff for their support and encouragement. I especially thank my advisor and mentor, JoAnn, for her unwavering support of my work throughout these five years. I would also like to thank the co-authors on these manuscripts, Dr. Larry Stikeleather, Dr. Otto (Chip) Simmons, Cameron Wilson, Jake Dums, and Dr. Lisa Dean. I would be remiss if I did not thank all of the members at the Center for Applied Aquatic Ecology who have helped me numerous times throughout the years. A special thanks to Ms. Elle Allen and Ms. Elisabeth Alexander for their help and being there for me to bounce ideas for this work. Thank you to Stacie Flood and Huma Ahmad for being fantastic lab mates. I would also like to thank the Charles A. and Anne Morrow Lindbergh Foundation for choosing my work to support through a grant. Their belief in using science and innovation to tackle real-world problems is inspiring.

I also thank my friends at North Carolina State University who have helped me through these five years of school. A special thanks to Jared Locklear for his help in editing and reading papers. Our times of laughter got me through a lot and I am so lucky to have you as a friend. I would also like to thank Renee Marchin, Stephanie Hollingsworth, Kim Bagian, and Robert San Miguel for their friendship and support. Thank you to Colleen Brannan, who was a great running/workout buddy and someone I could always turn to when times were difficult. I also thank Brian McCann, Robert Natelson, Andrew Campos, Wilson Wang, and Abhisheka Bhargava for their help with the engineering aspects of this research and turning my work into a reality.

I also thank my best friends, Nathan Byrd, Whitney Meyer, Mary Khodaparest, Sarah Scholl, and Jennifer Diaz, for their unwavering support and friendship. They have reminded me to not take life too seriously and always knew that I would complete my degree. I also thank my family, near and far, who always supported my dreams.

TABLE OF CONTENTS

LIST OF TABLES

COMPARISON OF QUALITATIVE AND QUANTITATIVE METHODS FOR ANALYSIS OF TOTAL FATTY ACIDS IN *DUNALIELLA* SPP.

COMPARISON OF MORPHOLOGICAL CHARACTERISTICS, POPULATION GROWTH, AND THE ITS REGION TO ELUCIDATE THE TAXONOMY OF FOURTEEN STRAINS OF *DUNALIELLA* (CHLOROPHYCEAE)

ENHANCED LIPID PRODUCTION IN *DUNALIELLA* SPP. EXPOSED TO SALINITY STRESS

EFFECTS OF ENVIRONMENTAL STRESSORS ON TOTAL FATTY ACID PRODUCTION IN *DUNALIELLA* SPECIES

AUTO-FLOCCULATION, ELECTRO-FLOCCULATION, AND HOLLOW-FIBER FILTRATION TECHNIQUES FOR HARVESTING THE SALTWATER MICROALGA *DUNALIELLA*

LIST OF FIGURES

COMPARISON OF QUALITATIVE AND QUANTITATIVE METHODS FOR ANALYSIS OF TOTAL FATTY ACIDS IN *DUNALIELLA* SPP.

COMPARISON OF MORPHOLOGICAL CHARACTERISTICS, POPULATION GROWTH, AND THE ITS REGION TO ELUCIDATE THE TAXONOMY OF FOURTEEN STRAINS OF *DUNALIELLA* (CHLOROPHYCEAE)

ENHANCED LIPID PRODUCTION IN *DUNALIELLA* SPP. EXPOSED TO SALINITY STRESS

LIST OF ABBREVIATIONS

CTAB: cetyl trimethyl ammonium bromide

DT: direct transesterification

FAs: fatty acids

FID: flame ionization detector

GC: gas chromatography

GPDH: glycerol-3-phosphate dehydrogenase

ITS: internal transcribed spacer

MEGA: Molecular Evolutionary Genetics Analysis

NR: Nile Red

PCR: polymerase chain reaction

Quantitative RT-PCR: reverse transcription quantitative PCR

SNPs: single-nucleotide polymorphism

TAG: triacylglycerol

TE: tris-ethylenediaminetetraacetic

1. COMPARISON OF QUALITATIVE AND QUANTITATIVE METHODS FOR ANALYSIS OF TOTAL FATTY ACIDS IN *DUNALIELLA* SPP.

Prepared for submission to *Journal of Applied Phycology*

1.1 Abstract

The isolation and accurate quantification of neutral lipids in small sample sizes of microalgae has become increasingly important for the renewable biofuel industry. Here, selected qualitative and quantitative techniques were optimized to determine the best method for analyzing total fatty acid (FA) content of *Dunaliella* spp. The wash method of 0.5 M ammonium formate and Karl Fischer titration yielded a wet-weight-to-dry-weight conversion factor of 95% for several *Dunaliella* strains. The fluorescent Nile Red (NR) dye preferentially stains neutral lipids, and was useful as a qualitative, rapid screening technique. NR was optimized with respect to solvent type and concentration for use with these *Dunaliella* strains. For quantitative analysis, gravimetric solvent-based methods were compared to direct transesterification (DT), which combines extraction and transesterification in one step. DT yielded the highest total neutral lipid content, with precise and reproducible results when compared to conventional extraction methods. Overall, for cultures of the saltwater microalgae *Dunaliella* spp., NR was a good qualitative method for visualizing neutral lipids, while DT was the optimal method for quantifying total neutral lipids.

1.2 Introduction

Development of a reliable and accurate method to quantify total lipids was first approached by Folch (Folch *et al.* 1957), who extracted and isolated total lipids into separate fractions (non-polar vs. polar) using a combination of organic solvents. To separate the various phases, this method, called the Folch procedure, implemented a specific ratio of the solvents methanol, chloroform, and water. However, large volumes of caustic solvents and extensive time were required. The best known modification of the Folch procedure was developed by Bligh and Dyer (Bligh and Dyer 1959). The main advantage of using this technique over Folch was the large reduction in solvent-to-sample ratio, so that less total solvent was required to extract total lipids. Many researchers have used this modified procedure, but few specify their exact modifications. Both the Folch (1957) and Bligh and Dyer (1959) methods were optimized for use with marine fish (Iverson *et al.* 1997, Smedes and Askland 1999), but not for use with microalgae. Furthermore, evaluations of the methods have been completed on samples containing less than 1.5% total lipid (Iverson *et al.* 2001), and is not useful for many microalgal samples which can contain upwards of 80% total lipid (Chisti 2008). While the extraction of microalgal lipids is usually completed using solvent-based techniques, extraction efficiency decreases as water content within the cells increases (Hidalgo *et al.* 2013).

Efforts to bypass the extraction step (MacGee and Allen 1974, Shimasaki *et al.* 1977) have been attempted. The most reproducible and accurate method combined extraction and transesterification into one step (Lepage and Roy 1984, Griffiths *et al.* 2010). This method, called direct transesterification (DT), simplified processing steps while reducing the potential

for FA loss. This process converts saponifiable lipids or triacylglycerides (TAGs) to fatty acid (FA) methyl esters (FAMEs), which can then be directly quantified using gas chromatography (GC). Traditionally, one catalyst is used for transesterification, but Griffiths *et al.* (2010) found that using a combination of acidic and basic catalysts was more efficient, especially on wet samples. This research assessed extraction methods from several gravimetric extraction techniques and compared them to DT to determine the best method for quantifying total FAs from *Dunaliella* samples.

In contrast to other techniques (Rodríguez-Ruiz *et al.* 1998, Fajardo *et al*. 2007), samples processed using the DT method do not need to be dried if a combination of acidic and basic catalysts are used (Griffiths *et al.* 2010, Hidalgo *et al.* 2013). However, in order to compare total FAs to other microalgal literature, we needed to convert total FAs calculated based on wet weight to a dry-weight basis. Therefore, an accurate wet-weight-to-dry-weight conversion had to be calculated from small-biomass samples (0.09 to 1 g). The dry weight of marine samples such as *Dunaliella* spp. can be affected by the amount of salts present on the cell surface. Washing is a conventional technique used to avoid error in weight measurements. Here, common preparation steps for saltwater samples including no washing versus washing to eliminate adsorbed salts (e.g. Zhu and Lee 1997, and methods therein) were compared to Karl Fischer titration (KF). KF titration can be used to directly assess water content (Bruttel and Schlink 2003) as long as the cells have released all of their intracellular water content.

A simple method for rapidly determining the presence of neutral lipids in microalgae is desirable because traditional gravimetric methods for lipid extractions are costly and time

consuming. Nile Red (NR; 9-diethylamino-5-benzo[α]phenoxazinone) preferentially stains neutral lipids or TAGs, and can be used as a qualitative assay to visualize intracellular lipids in microalgae (Cooksey *et al.* 1987, Lee *et al.* 1998, Chen *et al.* 2009). It was used as a screening technique to visually assess the presence/absence of neutral lipids in *Dunaliella* cells. Permeability of the stain into algal cells and the length of time required to analyze samples were instrumental in determining whether NR could additionally have value as a semi-quantitative method. *Dunaliella* lacks a cell wall, and the solvent used to dissolve NR causes detrimental changes in cell shape within a short time frame (~20 min). Therefore, we optimized NR solvent type, concentration, temperature, and fluorescence excitation and emission wavelengths for use as a qualitative analysis with *Dunaliella* spp.

The overall aims of this study were to purify and culture *Dunaliella* spp., determine a wet-weight-to-dry-weight conversion, compare quantitative methods (solvent-based extractions vs. DT) for analyzing total FA content, and optimize the qualitative method of NR to preferentially stain neutral lipids in *Dunaliella* samples.

1.3 Materials and Methods

1.3.a Culturing and purification of *Dunaliella*

Fourteen strains spanning five species of *Dunaliella* were obtained from the American Type Culture Collection (ATCC), the Culture Collection of Algae and Protozoa (CCAP), the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, formerly the National Center of Marine Phytoplankton, CCMP), and the Culture Collection of Algae at the University of Texas at Austin (UTEX) (Table 1.1).

These strains were labeled by the culture collections within five species of *Dunaliella*. However, in separate work, ITS2 sequence data for each of the 14 strains indicated that the strains actually fall within four distinct species (clades), and that 10 of the 14 strains had been labeled incorrectly (Chapter 2) (Table 1.1). Thus, here we refer to these strains by the species names indicated from the ITS2 sequence data, along with the original strain numbers.

In addition, strain UTEX 1644, which was sent to us 5 yr ago by the culture collection, was determined from ITS2 sequence data to be a unique strain of *D. viridis* that evidently was a culture contaminant in the collection. It is indeed unique from all of our other strains in major features including cell size, population growth as cell production, sustained growth in continuous light, and high lipid production throughout the cell cycle (see Chapter 4). Based on that information, the strain was not simply a contaminant from our other *Dunaliella* cultures. UTEX 1644 was ordered recently from the culture collection by colleagues, and was confirmed to be *D. salina* rather than this unique strain. Thus, the unique strain from the UTEX collection evidently was a contaminant that can no longer be obtained under the number, UTEX 1644. Here we refer to it as CONTAM.

All strains were first cultured in the media used by the commercial culture facilities (Table 1.1), except for *D. salina* ATCC 30861 which was provided in L1-Si medium at salinity 105. Once growth was established for strains in their source media (salinity 30 or 60), each culture was transferred into a series of increasing proportions of L1-Si and the source medium (after Lorenz *et al.* 2005) until the cells finally were acclimated to 100% L1-Si medium. In a second series of transfers, cultures were adjusted using NaCl (Fisher

Scientific, Fairlawn, New Jersey) by salinity ± 5 to 10 for each adjustment, and tested for population growth rates (as cell production) to determine the optimal salinity for each strain (Table 1.1). An analogous approach was used to identify the optimum pH for each strain, using incremental changes of 0.5 pH unit (PerpHecT LogR Meter 350, Boston Massachusetts) for an initial pH of 7.5 to 8.5.

After ~2-3 weeks of growth at 25 mL volume, strains were purified through a combination of flow cytometry and differential centrifugation to remove bacterial contamination (Guillard 2005, Kawachi and Noël 2005). Flow cytometry (Beckman Coulter EPICS ALTRA, Hialeah, Florida) obtained unialgal cells which were then grown in L1-Si medium. The auto-clone feature was implemented to achieve clonal populations by depositing a single algal cell per well into a 96 well plate (Applied Biosystems). Light microscopy (Olympus CK40, Japan) was used to confirm a cell was deposited in a well. In general, there was about a 5% success rate of autocloning per 96 well plate (Becton Dickinson, Franklin Lakes, New Jersey). After the unialgal culture was grown in 100 mL volume (L1-Si, salinity 30 or 60 achieved through adjustment with NaCl), differential centrifugation was used to further clean the culture of bacterial contamination (Guillard 2005, Kawachi and Noël 2005). Cultures were plated on ~25mL 1% Agar M (Sigma Aldrich®, St. Louis, Missouri), allowing for algal growth, or 1% Phytoblend (Caisson Laboratories, Logan, Utah), which promotes bacterial growth (200 µL of culture on each agar type) in petri dishes. All transfers were completed aseptically in liquid media (~every 2 weeks) as well as on agar plates (~ once a month). Once purified (i.e. no bacterial growth observed on Phytoblend agar plates), stocks for each strain (n = 3) were grown in 25 mL

volume under optimal salinity (30 or 60, depending on the strain) and pH (pH 8.0-8.4 for all strains) at 23˚C, and a light intensity of ~180 $\mu Em^{-2}sec^{-1}$ (light source, ProLume® compact fluorescent bulbs) under a 12-hr : 12 hr light : dark (L:D) photoperiod. Cultures were aseptically transferred to fresh L1-Si medium every ~2 to 3 weeks.

1.3.b Wet weight : dry weight determination

D. tertiolecta strains UTEX 999 and UTEX 1000, and *D. viridis* CONTAM were grown in 250 mL total volume under growth conditions described above. Pre-washed glass fiber filters (Whatman, UK, 0.2 µm pore size) were pre-weighed and 250 mL of dense culture was gently filtered (n = 3) with a 5-mL wash (deionized water [DI], filtered medium L1-Si at salinity 30 or 60, or 0.5 M ammonium formate (Sigma Aldrich) following each 50-mL addition of culture. Between each rinse the vacuum source was disconnected so that the wash covered the filter completely and was then rapidly removed, ensuring that the cells were not exposed to air (Strickland and Parsons 1968, Zhu and Lee 1997). Controls did not receive washes between culture additions. Wet weight was measured immediately after filtration (Sartorius BP211D, Germany). Filters with wet algae were then oven-dried (105˚C, Isotemp oven – Fisher Scientific, Pittsburgh, Pennsylvania) for 24 hr, placed in a vacuum to cool for ~1 hr to room temperature, and then re-weighed. This process was repeated after 24 hr and 48 hr of additional oven-drying to ensure that there was no change in dry weight over time (Wetzel and Likens 2001).

KF titration was performed using a Karl Fischer 701 Titrino (Metrohm Ltd., Riverview, Florida). This method is based on the oxidation of sulfur dioxide by iodine within a methanolic hydroxide solution in the presence of water. A solution containing iodine was

added until a slight excess of iodine was present. The amount of iodine converted was calculated through reading the change in volume (Bruttel and Schlink 2003, Zeiller *et al.* 2007). The equipment was calibrated using DI until the titrant number was consistent. A dense culture of *Dunaliella viridis* CONTAM was centrifuged (3.0 RPM, 10 min - CL2 Centrifuge, ThermoScientific, Milford, Massachusetts) and the pellets re-suspended in 1 mL of DI so that the cells ruptured and released intracellular content (Bruttel and Schlink 2003). A 20-µL sub-sample of algae was injected, and the percent water in the samples was calculated by the equipment software. This process was repeated with both fresh and frozen algae to ensure consistent readings.

1.3.c Qualitative lipid analysis

Several factors were optimized for NR use with *Dunaliella* spp., including solvent type and concentration, NR concentration, temperature, and fluorescence excitation and emission wavelengths. Several concentrations (25%, 50%, 75%, and 100%) of two solvents, methanol MeOH (Fisher Scientific) and dimethyl sulfide DMSO (Fisher Scientific), were tested in conjunction with NR (Sigma Aldrich) concentration. An initial NR concentration of 10 µg/mL was serially diluted to 0.001 µg/mL. Samples were compared with heat (slides slightly warmed on a hotplate) versus without heat treatment. Lastly, a range of excitation (ex; 450-500 nm) and emission (em; 500-700 nm) wavelengths were tested using a fluorometer (Tecan Safire, Invitrogen, Carlsbad, California) to optimize wavelength range.

Dunaliella cells were immobilized using 1.3% Type V agarose (Sigma Aldrich). Slides were placed on a hotplate set to low heat to warm the slides. Two drops of melted agarose and two drops of culture were placed on the center of a warmed slide and gently

mixed. Then, 10 µL of NR was added and allowed to penetrate the cells for several minutes. Micrographs were taken using either an Olympus AX70 microscope connected to an Olympus DP70 camera at 600x, or a Zeiss LSM 710 confocal microscope at 630x magnification.

1.3.d Quantitative lipid analysis

Unless otherwise specified, all chemicals were purchased from Sigma Aldrich, St. Louis, Missouri. The Bligh and Dyer method (1959) was slightly modified for saltwater microalgae samples in that, after the sample was pelleted and frozen, 1 mL of DI was added and the sample was transferred to a 15-mL stoppered glass centrifuge tube. Then, 3.75 mL of MeOH : chloroform (2:1, v/v, 1 mL internal standard – 0.5 mg/mL, tridecanoic acid in ethanol, EtOH, Pharmco-AAPR, Brookfield, Connecticut) was added. Tubes were mixed and left at room temperature for 2 hr with intermittent shaking (~every 15 min). Tubes were then centrifuged (1.0 RPM, 5 min – CL2 Centrifuge, ThermoScientific, Milford, Massachusetts) and the supernatant was collected in a clean 15-mL stoppered glass centrifuge tube. The pellet was re-suspended with 4.75 mL of MeOH : chloroform : water (2:1:0.8, v/v), mixed, and centrifuged again (1.0 RPM, 5 min). Supernatants were combined and 5 mL total of chloroform : DI (1:1, v/v) were added. This mixture was inverted and centrifuged (1.0 RPM, 5 min). Samples were dried under nitrogen gas. The lipid residue was dissolved in MeOH : chloroform (1:1, v/v), centrifuged and chloroform was added to a final desired volume. The chloroform phase was then removed, methylated, and analyzed for total FAs using gas chromatography-mass spectrophotometry (GC-MS).

For the Folch method (1957), a 15-mL stoppered glass centrifuge tube was pre-weighed. Then, 1 mL of 0.9% NaCl was added to the frozen, pelleted algal sample, and this mixture was added to the 15 mL tube and re-weighed. To this, 10 mL of MeOH : chloroform (1:2, v/v, with 1 mL internal standard – 0.5 mg/mL, tridecanoic acid in EtOH). Tubes were mixed and left at room temperature for 2 hours with intermittent shaking (~ every 15 min). Tubes were centrifuged (1.0 RPM, 5 min) and the supernatant collected in a clean 15-mL stoppered glass centrifuge tube. The pellet was then re-suspended with MeOH : chloroform : DI (1:2:0.8, v/v) and mixed and centrifuged again (1.0 RPM, 5 min). To the combined supernatants, a total volume of 5 mL of chloroform : 0.9% NaCl solution (1:1, v/v) was added, mixed, and centrifuged (1.0 RPM, 5 min). Samples were dried under nitrogen and the lipid residue dissolved in MeOH : chloroform (1:1, v/v), centrifuged and lastly, chloroform was added to a final desired volume. The chloroform phase was removed, methylated, and analyzed for total FAs using gas chromatography-mass spectrophotometry (GC-MS).

For processing samples using DT (Griffiths *et al.* 2010) a combination of acidic and basic transesterification catalysts was used. Internal standard (1 mL – 0.5 mg/mL, tridecanoic acid in EtOH) was added to a clean 10-mL glass tube. Then, 1 mL of 0.5 M methanolic KOH was added to each sample, homogenized, transferred to the glass tube containing the internal standard, and heated at 85˚C for 5 min. Next, 1 mL of boron triflouride (BF_3, 14% in MeOH) was added and the samples were heated at 85˚C for 10 min. DI (1 mL) was then added and the samples were vortexed. Lastly, 1 mL of hexane was added and the samples were vortexed for at least 30 sec. The samples were separated into layers by first sonicating (Fisher Scientific, Pittsburgh, Pennsylvania) and then centrifuging the samples (1.0 RPM, 2 min).

GC was then used to quantify the recently converted FAMEs, hereafter referred to as total fatty acids (FAs), present in the less dense hexane layer. This layer was carefully removed and directly analyzed with a GC HP5890 series II equipped with a FID (Hewlett Packard, refurbished by Primera Scientific LLC, Princeton, New Jersey) and a 7673A autosampler (Alpha Omega Technologies, Inc., refurbished by Primera Scientific LLC, Princeton, New Jersey). Separation was achieved in an Rtx-2330 capillary column (Hewlett Packard). The temperature was programmed to include an initial 3 min at 60˚C, and then was increased to 230˚C at a rate of 4˚C / min. Both injector and detector temperatures were set at 265˚C. Injections were performed under the splitless mode (Sparkman *et al.* 2011). Data acquisition and analysis were completed using the GC Chemstation Rev. A.08.03 (847) software. Calculations for total FAs were completed using the relative response factor (RRF) method (Sparkman *et al.* 2011) and area ratios were compared to the internal standard (KEL-FIM-FAME-5 Mixture, Matreya, LLC, Pennsylvania, USA).

Three conventional solvent-based extraction methods (Folch 1:2, MeOH : chloroform – Iverson *et al.* 2001; Bligh and Dyer 2:1, MeOH : chloroform – Bligh and Dyer 1959; and Bligh and Dyer with a 0.9% NaCl solution – Iverson *et al.* 2001) were compared to DT. To test which method was most desirable for total FA analysis in *Dunaliella* (n = 2 for each method), ~3.5 L of *Dunaliella viridis* CONTAM was grown under conditions described above. When cultures reached early senescence, this flask was evenly distributed into eight sterile 50-mL tubes (Corning® Centristar^{TM}) by concentrating ~450 mL culture by centrifuging (3,000 RPM, 10 min - CL2 Centrifuge, ThermoScientific, Millford, Massachusetts). Samples were held frozen at -80˚C until analysis of FAs (within 7-10 days).

1.4 Results and Discussion

1.4.a Wet weight : dry weight determination

Best methods for determining a wet-weight-to-dry-weight conversion factor for three strains of *Dunaliella* (*D. tertiolecta* strains UTEX 999 and UTEX 1000, and *D. viridis* CONTAM) were found to be the use of ammonium formate (0.5 M) wash or KF titration. Each filter held ~250 mL of dense culture before becoming clogged. Using filtered medium or no wash yielded a 90% conversion factor. The higher dry weight yielded from samples washed with filtered medium was likely due to NaCl being retained on the filter paper or in intercellular spaces. Use of DI as a wash yielded a 98% conversion factor. The significantly lower weight yielded by samples rinsed with deionized water can be explained by the bursting of cells due to the severe difference in osmotic potential. Both 0.5 M ammonium formate and KF titration yielded a 95% wet-weight-to-dry-weight conversion factor. Zhu and Lee (1997) suggested using ammonium bicarbonate as an alternative to ammonium formate because this chemical is readily available and more cost-effective. Both solutions are isotonic and have been demonstrated as effective washing agents for marine microalgae samples (Zhu and Lee 1997). KF titration is a specific determination method because only water content will be calculated. This method is highly reproducible and precise over a wide range of concentrations from <1% to 100% (Bruttel and Schlink 2003), and yielded a 95% conversion factor. The calculation used for converting total FAs (wet weight basis) to total FAs (dry weight basis) is as follows:

$$\text{Total FAs (\% by dry weight)} = [(\text{Total FAs (\% by wet weight)})/5]$$

Percent total FAs (by wet weight) was calculated from the GC software Chemstation Rev. A. 08.03 (847) using the RRF method (Sparkman *et al.* 2011) and area ratios were compared to the internal standard (KEL-FIM-FAME-5 Mixture, Matreya, LLC, Pennsylvania). The "5" in the equation represents the wet-weight-to-dry-weight conversion factor (100 – 95) as previously determined. Calculations for total FAs were completed using the relative response factor (RRF) method (Sparkman *et al.* 2011).

1.4.b Qualitative lipid analysis

The fluorescent dye NR preferentially stains neutral lipids and has been frequently used as a qualitative method for microalgae (Cooksey *et al.* 1987, Elsey *et al.* 2007, Chen *et al.* 2009). Staining chlorophytes with NR has been difficult due to the presence of a thick, rigid cell wall (Graham *et al.* 2009, Chen *et al.* 2009). *Dunaliella* lacks a cell wall, which facilitates rapid staining, but this also means that the cells are sensitive to the solvents used. In fact, the cells burst within twenty minutes of staining (Fig. 1.1A, white arrow). The optimal set of conditions for NR qualitative analysis were as follows: Add 10 µL NR (0.625 µg/mL dissolved in 100% MeOH) to slightly heated slides containing 2 drops of sample immobilized on 1.3% Type V agarose. Carefully analyze a spectrophotometric pre-scan to determine the optimal excitation (488 nm) and emission (545-655 nm) spectra for the combination of solvents and chemicals used. In conjunction, conduct a check with confocal microscopy (here, a Zeiss LSM 710 – Zeiss Corporation) to ensure that the NR has preferentially stained the neutral lipids in *Dunaliella* (Fig. 1.1B, white arrows). This wavelength spectrum cannot be generalized for all microalgae, because it is specific not only to *Dunaliella* but also to solvent type, concentration, and NR concentration (Chen *et al.*

2009). As a quantitative method, further refinements would need to be implemented, since there is little information about calibrating total neutral lipids from conventional extraction (Chen *et al.* 2009) or DT methods to lipid standards. In addition, many factors would need to be considered, including cell wall composition, chlorophyll content, or membrane lipids that may interfere with fluorescence background (Chen *et al.* 2011). In this research, NR was used only as a rapid, visual screening method for the presence of neutral lipids within *Dunaliella* cells.

1.4.c Quantitative lipid analysis

The gravimetric solvent-based extraction methods of Folch (1956) and Bligh and Dyer (1959) were not only time-consuming (6-8 hr required to process six samples), but also required large amounts of solvents per sample. Additional MeOH was required for rinsing glass stoppered tubes before use. These methods also required a final step of drying with nitrogen gas and a methylation step after extraction. DT, on the other hand, required less than one-third the volume of the chemicals per sample, and at least 24 samples were processed within 4 hr. In DT, extraction and methylation are combined in a one-step procedure, thus reducing potential sample loss. In addition, the final layer of hexane containing the FAMEs can be directly injected into the GC for analysis of total FA.

DT yielded less variability among samples as well as comparable (slightly higher) amounts of total FAs when compared to the three conventional extraction methods (Table 1.2). Use of a 0.9% NaCl solution instead of DI did not significantly impact analysis for the Bligh and Dyer method (there was a slight decrease in standard deviation – Table 1.2). The Bligh and Dyer method, with and without NaCl solution, yielded the largest variation. The

Folch method was a more effective extraction method when compared to Bligh and Dyer; however, when compared to DT, both traditional extraction methods may have been incomplete.

These findings were similar to those found by Griffiths *et al.* (2010), who compared DT to the extraction methods of Folch (1957), Bligh and Dyer (1959), and Smedes and Askland (1999) with three species of chlorophytes: *Chlorella vulgaris, Nannochloropsis* spp., and *Scenedesmus* spp. Griffiths *et al.* (2010) found that DT yielded higher total FA content than the traditional extraction methods. In fact, processing the remaining biomass from each extraction method yielded additional FA content. The sum of the extraction yield and the additional yield recovered from DT almost equaled that yielded by DT alone (Griffiths *et al.* 2010). In this study, DT was found not only to be more convenient in terms of total volumes of solvents used and time required, but was also more accurate than solvent-based extraction methods in quantifying total FA content in *Dunaliella* spp.

Advantages of using DT compared to conventional extraction methods include combining lipid extraction and purification steps and the ability to use this method on small sample amounts. DT has recently been suggested as a cost-effective technique for directly processing microalgal samples for the final product of biodiesel (Hidalgo *et al.* 2013). Additionally, all steps are completed in one tube, thus minimizing potential sample or chemical loss. By sequentially adding a basic (KOH) and an acidic (BF_3) catalyst, the DT method has been further improved, particularly for use with samples containing small amounts of water (Griffiths *et al.* 2010; Hidalgo *et al.* 2013). This improvement is especially advantageous when the microalgal samples are not dried before processing, as in this study.

Thus, we evaluate DT as the preferential method for quantifying total FAs from small samples of saltwater *Dunaliella*.

1.5 References

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

Bruttel, P., Schlink, R., 2003. Water determination by Karl Fischer Titration. Met. Mono. 1, 1-80.

Chen, W., Zhang, C., Song, L., Sommerfeld, M., Hu, Q., 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods 77, 41-47.

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126-131.

Cooksey, K.E., Guckert, J.B., Williams, S.A., Callis, P.R., 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J. Microbiol. Methods 6, 333-345.

Elsey, D., Jameson, D., Raleigh, B., Cooney, M.J., 2007. Fluorescent measurement of microalgal neutral lipids. J. Microbiol. Methods 68, 639-642.

Fajardo, A.R., Cerdán, L.E., Medina, A.R., Fernández, F.G.A., Moreno, P.A.G., Grima, E.M., 2007. Lipid extraction from the microalga *Phaeodactylum tricornutum*. Eur. J. Lipid Sci. Technol. 109, 120-126.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Føyn, B., 1934. Lebenszyklus, Cytologie und Sexualiät der Chlorophycee *Cladophora suhriana* Küzing. Arch. Protistenk. 83, 1-56.

Graham, L.E., Graham, J.M., Wilcox, L.W., 2009. Algae. 2nd Edition. Pearson Education, Incorporated, San Francisco (California). 616 pp.

Griffiths, M.J., van Hille, R.P., Harrison, S.T.L., 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 45, 1053-1060.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York (New York). pp. 29-60.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234-236.

Guillard, R.R.L., 2005. Purification Methods for Microalgae. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Academic Press, Burlington (Massachusetts). pp.117-132.

Hidalgo, P., Toro, C., Ciudad, G., Navia, R. 2013. Advances in direct transesterification of microalgal biomass for biodiesel production. Rev. Environ. Sci. Biotechnol. 12, 179-199.

Iverson, S.J., Frost, K.J., Lowry, L.F., 1997. Fatty acid signatures reveal fine scale structure of foraging distribution of harbor seals and their prey in Prince William Sound, Alaska. Mar. Ecol. Prog. Ser. 151, 255-271.

Iverson, S.J., Lang, S.L.C., Cooper, M.H., 2001. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36, 1283-1287.

Kawachi, M., Noël, M.-H., 2007. Sterilization and Sterile Technique. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts). pp. 65-81.

Lee, S.J., Yoon, B.-D., Oh, H.-M., 1998. Rapid method for determination of lipid from the green alga *Botryococcus braunii*. Biotechnol. Tech. 12, 553-556.

Lepage, G., Roy, C.C., 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J. Lipid Res. 25, 1391-1396.

Lorenz, M., Friedl, T., Day, J.G., 2005. Perpetual Maintenance of Actively Metabolizing Microalgal Cultures. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts). pp. 145-156.

MacGee, J., Allen, K.G., 1974. Preparation of methyl esters from the saponifiable fatty acids in small biological specimens for gas-liquid chromatographic analysis. J. Chromatogr. 100, 35-42.

Rodríguez-Ruiz, J., Belarbi, E.-H., Sánchez, J.L.G., Alonso, D.L., 1998. Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. Biotechnol. Tech. 12, 689-691.

Shimasaki, H., Phillips, F.C., Privett, O.S., 1977. Direct transesterification of lipids in mammalian tissue for fatty acid analysis via dehydration with 2,2-dimethoxypropane. J. Lipid Res. 18, 540-543.

Smedes, F., Askland, T.K., 1999. Revisiting the development of the Bligh and Dyer total lipid determination method. Mar. Pollut. Bull. 38, 193-201.

Sparkman, O.D., Penton, Z.E., Kitson, F.G., 2011. Gas Chromatography and Mass Spectrometry: A Practical Guide, 2nd Edn. Elsevier Incorporated, Burlington (Massachusetts). 611 pp.

Strickland, J.D.H., Parsons, T.R., 1968. A practical handbook of seawater analysis. J. Fish. Res. Board Can. 55, 181-184.

Tompkins, J., DeVille, M.M., Day, J.G., Turner, M.F., 1995. Culture Collection of Algae and Protozoa. Catalog of Strains. Ambleside (United Kingdom). 204 pp.

Wetzel, R.G., Likens, G.E., 2001. Limnological Analyses, 2nd Edition. W.B. Saunders, New York (New York). 429 pp.

Wetzel, R.G., 2001. Limnology: Lake and River Ecosystems 3rd Edition. Academic Press, San Diego (California). 1006 pp.

Zeiller, E., Benetka, E., Schorn, R., 2007. Dry mass determination: what role does it play in combined measurement uncertainty? Accredit. Qual. Assur. 12, 295-302.

Zhu, C.J., Lee, Y.K., 1997. Determination of biomass dry weight of marine microalgae. J. Appl. Phycol. 9, 189-194.

Table 1.1 – Species, strain source and identification number, source culture medium, and optimum salinity in L1-Si medium for the 14 strains of *Dunaliella* examined in this study (see text). *D. tertiolecta* strains UTEX 999 and UTEX 1000, and *D. viridis* CONTAM were used in wet-weight-to-dry-weight conversion experiments.

Species Name[1]	Commercial Source and Strain Number	Source Culture Medium	Optimal Salinity (L1-Si)
Dunaliella pseudosalina	CCAP 19/18	2ASW	60
Dunaliella pseudosalina	UTEX 1983	Erdschreiber's	30
Dunaliella pseudosalina	UTEX 200	2X Erdschreiber's	60
Dunaliella salina	ATCC 30861	L1-Si	60
Dunaliella tertiolecta	CCAP 19/9	f/2-Si	30
Dunaliella tertiolecta	CCAP 19/24	f/2-Si	60
Dunaliella tertiolecta	CCAP 19/26	f/2-Si	30
Dunaliella tertiolecta	CCMP 364	L1-Si	30
Dunaliella tertiolecta	CCMP 1320	L1-Si	30
Dunaliella tertiolecta	UTEX 999	ASW	30
Dunaliella tertiolecta	UTEX 1000	Erdschreiber's	30
Dunaliella viridis	CCAP 19/3	2ASW	60
Dunaliella viridis	CCAP 19/10	2ASW	60
Dunaliella viridis	CONTAM	2X Erdschreiber's	60

[1] ITS2 sequence data for each of the 14 strains indicate that the strains fall into four distinct species (clades) as indicated, and that many of the strains were incorrectly designated by the commercial culture facilities (see Chapter 2). Note that strain UTEX 1644, sent to us ~5 yr ago, evidently was a culture contaminant in the strain received. It is no longer available from the culture collection under that strain number. Therefore, we have deposited it at the NCMA (see text).

Table 1.2 – Comparison of total FA yield (mg/g) using the three conventional extraction methods and DT (means ± 1 standard deviation [SD]).

Method	**Total FAs (mg/g, means ± 1 SD)**
Folch	22.86 ± 4.56
Bligh and Dyer	22.89 ± 13.55
Bligh and Dyer + Salt	22.11 ± 10.78
DT	23.03 ± 2.38

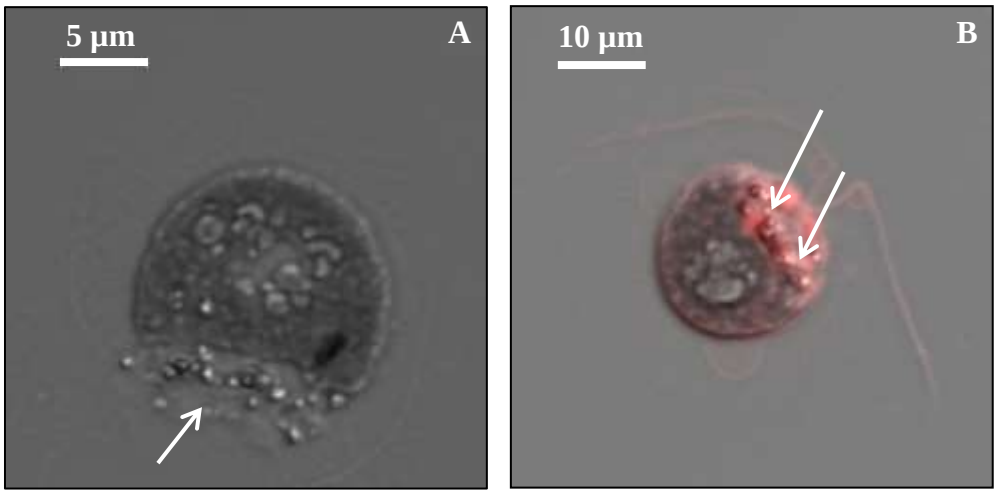

Figure 1.1 – Confocal micrographs of *D. tertiolecta* CCMP 364: A) Bursting (white arrow) of the cell after being stained too long (DCIM, 630x; scale bar = 5µm) and, B) Epifluorescence of a cell stained with Nile Red, showing neutral lipids (white arrows; 488 nm excitation, 545-655 nm em, 400x; scale bar = 10µm).

2. COMPARISON OF MORPHOLOGICAL CHARACTERISTICS, POPULATION GROWTH, AND THE ITS REGION TO ELUCIDATE THE TAXONOMY OF FOURTEEN STRAINS OF *DUNALIELLA* (CHLOROPHYCEAE)

Prepared for submission to *Phycologia*

2.1 Abstract

The original taxonomy of *Dunaliella* (Chlorophyceae) has been based upon physiological characteristics such as morphology and biochemistry. The objective of this research was to elucidate the taxonomy of *Dunaliella* strains by analyzing the ITS and 18S regions as well as investigating individual strain morphological characteristics. Fourteen strains were purified and grown under optimal salinity and pH, and comparable other environmental conditions, to determine physiological characteristics such as maximum cell production and biovolume. In addition, the ITS and 18S regions were sequenced to elucidate the phylogenetic relationships among all 14 strains. The ITS2 sequences indicated that of the 14 strains of *Dunaliella* studied, there are only four distinct species. After careful analysis of both sequence and physiological characteristics, the data suggest that the 14 strains grouped within four species: *D. tertiolecta, D. pseudosalina, D. salina*, and *D. viridis*. The ITS2 sequences provided valuable information for creating accurate phylogenies as well as determining or verifying culture identifications. Future studies can build upon this research by investigating biochemical characteristics such as the capability of producing β-carotene and additional genes, in order to provide a more comprehensive phylogenetic analysis of *Dunaliella*.

2.2 Introduction

Defining phytoplankton species is a long-standing struggle in phycology, as historically based morphospecies are reconsidered using molecular data or a combination of morphological and molecular traits while also attempting, where possible, to adhere to the biological species concept (organisms with the ability to sexually reproduce together; Guiry 2012). Species descriptions are further complicated by typically high intraspecific variation among populations within the same microalgal species (Burkholder and Glibert 2006, Guiry 2012), and by rapid changes in culture (Lakeman and Cattolico 2007, Lakeman et al. 2009).

Here we investigated differences in the morphological and sequencing data of 14 strains within the genus *Dunaliella*. Until relatively recently, the taxonomy of the Chlorophyceae has been based solely on morphological characteristics under light microscopy, together with certain biochemical features (Ben-Amotz *et al*. 2009, Graham *et al.* 2010). Use of only morphological and biochemical traits to distinguish among different strains of *Dunaliella* (Teodoresco) can be unreliable, however, as well as difficult to interpret (Ben-Amotz *et al.* 2009, Assunção *et al.* 2012). Massjuk (1973) identified 28 species within the genus *Dunaliella*, but many of these strains were incorrectly named and, since that work, new species names have been introduced unnecessarily (Olmos-Soto *et al.* 2002). Additionally, complications arise due to the enormous variability within as well as among strains of one *Dunaliella* species (Gómez & González 2004, Assunção *et al.* 2012). The development of biotechnology tools such as polymerase chain reaction (PCR) allow for the detection and molecular characterization of strain-specific differences within a given genome. For example, the ribosomal internal transcribed spacer (ITS) or 18S regions and

microsatellite motifs have been used to provide insights about variability within a microalgal population (Lakeman and Cattolico 2007). In considerations about *Dunaliella*, because various strains have been successfully mass-cultured for β-carotene production, studies mainly have focused on molecular identification of strains that can accumulate high levels of β-carotene (Olmos-Soto *et al*. 2002). A major difficulty in performing a phylogenetic analysis of *Dunaliella* is the fact that culture collections and GenBank commonly contain misidentified species, and this misinformation is widely repeated and, thus, perpetuated in the literature (Borowitzka & Siva 2007, Assunção *et al.* 2012).

The phylogeny of *Dunaliella* is poorly understood, but helpful insights can be gained through use of the ITS and 18S regions of its nuclear ribosomes. ITS1 and ITS2 are non-coding introns between the 18S, 5.8S, and 26S rRNA portions (Müller *et al.* 2007). In general, rRNA gene sequences have highly conserved flanking regions that can be easily accessed using universal primers (Meyer *et al.* 2010). Genetic mutations occur naturally within populations and can be used to resolve intraspecific and interspecific relationships within *Dunaliella* species. The 18S rRNA region is highly conserved and is part of the functional ribosomal core (Meyer *et al.* 2010). The primary structure of the ITS region is not conserved and differences in the sequence can indicate interspecific variation among microalgal populations (Gómez & González 2004, Assunção *et al.* 2012). The ITS2 has evolutionarily conserved secondary structure and a frequency of mutation that allows for strain level differentiation (Keller *et al.* 2010, Assunção *et al.* 2012). An in-depth analysis of the ITS2 secondary structure was completed by Assunção *et al.* 2012 and is the most inclusive phylogenetic analysis of *Dunaliella* to date.

A combination of sequence data in conjunction with morphological and physiological characteristics of species is often used to further resolve species names. Physiological differences among strains or species of *Dunaliella* is complicated because these cells are able to tolerate a wide range of salinities, light intensities, and temperatures (Ginzburg 1987, Ben-Amotz *et al.* 2009). Such variation is common among phytoplankton (Wood and Leatham 1992, Lakeman *et al.* 2009). One main feature distinguishing *Dunaliella* from many unicellular chlorophytes is the lack of a rigid polysaccharide cell wall, which allows cell shape to vary significantly depending upon culture conditions (Ben-Amotz *et al.* 2009). Borowitzka & Siva (2007) completed an in-depth taxonomy of *Dunaliella* using morphological and biochemical based traits. In order to improve *Dunaliella* taxonomy, simultaneous comparison of morphological and molecular features was suggested (Borowitzka & Siva 2007).

In this research we analyzed the ITS and 18S regions in combination with certain morphological features to gain insights about the taxonomic relationships among 14 *Dunaliella* strains. Higher variation within the ITS region was expected, and the ITS2 region was emphasized in phylogenetic analysis. Morphological attributes such as biovolume and cell size, and population growth were compared to provide further understanding of *Dunaliella* intraspecific variation.

2.3 Materials and Methods

2.3.a Culturing and purification of *Dunaliella*

The 14 *Dunaliella* strains were obtained from the American Type Culture Collection (ATCC), the Culture Collection of Algae and Protozoa (CCAP), the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, formerly the National Center of Marine Phytoplankton, CCMP), and the Culture Collection of Algae at the University of Texas at Austin (UTEX) (Table 2.1). Preliminary work indicated that the *Dunaliella* strains grew well in Erdschreiber's (Føyn 1934), Artificial Seawater (ASW, made using f/2-Si nutrients; Guillard 1975, Tompkins *et al.* 1995), and L1-Si medium (Guillard and Hargraves 1993), at salinity 30 or 60 depending on the strain. Erdschreiber's and ASW media contained soil water with undefined constituents, and nutrient components of f/2-Si were similar to L1-Si. Therefore, L1-Si was selected for use in this study. Once growth was established for strains in their source media (Table 2.1; salinity 30 or 60), each culture was transferred into a series of increasing proportions of L1-Si and the source medium (after Lorenz *et al.* 2005) until the cells finally were acclimated to 100% L1-Si medium. Cell production for each strain was compared in its source medium versus L1-Si medium, and all strains attained comparable or higher maximum cell production in L1-Si (see Chapter 3). In a second series of transfers, cultures were adjusted using NaCl (Fisher Scientific, Fair Lawn, New Jersey), by salinity ± 5 to 10 for each adjustment (YSI 3200 Conductivity Instrument, Yellow Springs, Ohio), and tested for growth rates (as cell production) to determine the optimal salinity for each strain (see Chapter 3). An analogous approach was used to identify the

optimum pH for each strain, using incremental changes of 0.1 pH unit (PerpHecT LogR Meter 350, Boston Massachusetts) from an initial pH of 7.5 to 8.0.

Strains were purified using a combination of differential centrifugation and flow cytometry (Beckman Coulter® EPICS Altra, Hialeah, Florida) to remove bacterial contamination (Guillard 2005, Kawachi and Noël 2005, Chapter 1 and methods therein). Cultures were plated on 1% Agar M (Sigma Aldrich®, St. Louis, Missouri) or 1% Phytoblend (Caisson Laboratories, Logan, Utah) to promote algal growth or bacterial growth, respectively. Subsequent plating was completed aseptically on a monthly basis until each strain was purified. Stock culture for each strain (n = 3) was grown under the additional conditions of 23˚C and a light intensity of ~180 $\mu E\ m^{-2}\ sec^{-1}$ (light source, ProLume® compact fluorescence bulbs) under a 12-hr : 12-hr light : dark (L:D) photoperiod.

Population growth rates (as cell production) were assessed by growing each strain in 100 mL volume (n = 3) as unialgal culture until late senescence (~35 days). Subsamples were taken between 0800 and 0900 every other day, preserved in acidic Lugol's solution (1% final concentration; Vollenweider *et al.* 1974), and quantified within 1-2 days using Palmer-Maloney chambers (Wetzel and Likens 2000) under light microscopy at 200x magnification using an Olympus BH-2 light microscope (Germany). Population growth curves (as cell production) were generated for each strain.

The mean biovolume for each strain was determined as follows: Cells were immobilized using 1.3% Type V agarose (Sigma Aldrich). Two drops of agarose and two drops of culture were placed in the center of a warmed slide and gently mixed using a pipette. After the agarose had solidified, at least 30 micrographs of each *Dunaliella* strain were taken

under 600x magnification (No. 1.5 25mm^{-2} coverslip, Corning®), using an Olympus AX70 microscope connected to an Olympus DP70 camera. Biovolumes were calculated using the formula for a prolate spheroid (Hillebrand *et al.* 1999; Table 2.1).

$$V = (\frac{\pi}{6} * d^2 * h)$$

2.3.b DNA Extraction

Total DNA was extracted from 50 mL of exponentially growing cells (~10^6 cells/mL). Cells were first pelleted (3,000 RPM, 10 min) and then DNA was extracted using a modified CTAB extraction method (Stewart & Via 1993, Walker *et al.* 2005) for each of the 14 strains. Briefly, an equal volume of Solution A (Walker *et al.* 2005) and chloroform: isoamyl alcohol (Sigma Aldrich; 24:1) was added to the samples, and the mixture incubated at 65˚C for 10 min. Samples were centrifuged (14000 RPM, 5 min), the aqueous phase collected, and RNaseA (Qiagen; 1 mg/mL, final concentration) was added. After incubation at 37˚C for 20 min, an equal volume of chloroform: isoamyl alcohol (24:1) was added, the samples centrifuged, and the supernatant collected. An equal volume of isopropanol (Sigma Aldrich) was added to precipitate the DNA and samples were centrifuged at maximum speed. The DNA was re-suspended in TE (Fisher Scientific; 10:1) buffer.

2.3.c Amplification of the ITS and 18S regions

Amplification of the ITS (González *et al.* 1999) and 18S (Olmos-Soto *et al.* 2002) regions were completed using a GeneMate Genius (Kaysville, Utah) and Taq polymerase (Invitrogen, Carlsbad, California) following manufacturer's instructions. Table 2.2 shows

ITS and 18S primer set sequences (Invitrogen). For ITS amplification, an initial denaturation cycle of 95 ˚C for 5 min was followed by two annealing cycles to account for the different lengths and T_m of the forward (TW81) and reverse (AB28) primers. Thermocycler conditions were as follows: five cycles of 90˚C for 1 min, 50˚C for 2 min followed by one cycle of 68˚C for 1 min, and 30 cycles of 90˚C for 1 min, 60˚C for 1 min, 68˚C for 1 min, and a final annealing stage of 68˚C for 10 min. 18S R Universal-C and 18S R Universal-T primers were mixed at equal concentrations and used as the reverse primer for 18S amplification. Thermocycler conditions for amplifying the 18S region included an initial denaturation period of 2 min at 95˚C, then 35 cycles of 95˚C for 30 sec, 60˚C for 30 sec, 68˚C for 2 min 30 sec, and a final extension time of 68˚C for 7 min. All products were confirmed by 1% agarose (Sigma Aldrich) gel electrophoresis and visualized with GelStar® (Lonza, Rockland, Maine) on a Gel Doc XR + UV transilluminator using Image Lab Software (NCSU).

PCR products were purified using a Qiagen QIAquick PCR purification kit (Qiagen, Maryland), following manufacturer's instructions. Purified products were prepared according to GENEWIZ, Inc. instructions and sequenced (GENEWIZ, Inc., South Plainfield, New Jersey). Consensus sequences were designed by aligning the forward and reverse strands and comparing all nucleotides to the original chromatograms. Clustal Omega (1.1.0) was used to align multiple sequences using default parameters. Lastly, MEGA5 (maximum likelihood statistical method; 100 bootstrap replications; nearest-neighbor-interchange) was used to create a phylogenetic tree for the ITS2 region (Tamura *et al.* 2011). Final analyses were completed by comparing the strain sequences to available sequences on GenBank. (http://www.ncbi.nlm.nih.gov/genbank/).

2.4 Results

The cell size, biovolume, cell production, and sequencing data suggest that the 14 strains of *Dunaliella* were actually less diverse than their original identifications would suggest. All strains were grown in the same culturing conditions as described above, and all were imaged at the same time of day to minimize potential cell cycle size differences. Biovolume calculations were completed from at least 30 micrographs for each strain and means ranged from 43 to 646 μm^{-3} (Table 2.1). Micrographs were arranged into four groups of species as suggested by biovolume (Fig. 2.1 A-N), cell production (Fig. 2.2 A-D), and sequencing data (Assunção *et al.* 2012, Figs. 2.3 and 2.4, Table 2.3). Seven strains grouped together within the *D. tertiolecta* clade and, in general, can be characterized as having the second smallest biovolume (120 to 168 μm^{-3}; Fig. 2.1 A-G) and second highest cell production (3.2 to 6.6 x 10^6 cells/mL; Fig. 2.2 A). The *D. pseudosalina* clade grouped three strains together due to having the second largest biovolume (183 to 240 μm^{-3}; Fig. 2.1 H-J), but the second lowest cell production (1.9 to 3.4 x 10^6 cells/mL; Fig. 2.2 B). The smallest cells (43 to 74 μm^{-3}; Fig. 2.1 K-M) had the highest cell production (1.9 to 10.0 x 10^6 cells/mL; Fig. 2.2 C) and grouped in the *D. viridis* clade. Lastly, the *D. salina* clade contained the culture with the largest cell (646 μm^{-3}; Fig. 2.1 N) having the lowest cell production (0.3 x 10^6 cells/mL; Fig. 2.2 D). In general, the smallest cells exhibited the highest cell production, whereas larger cells peaked at a lower density. The *D. parva* CCAP 19/10 cultures were outliers in the data because these cells had low cell production (1.9 x 10^6 cells/mL; Fig. 2.2 C) despite their small size (46 μm^{-3}; Table 2.1).

A ~1600 base pair (bp) product was sequenced for the 18S region for each *Dunaliella* strain and was found to be highly conserved. Of the strains examined, *D. parva* CCAP 19/10, *D. salina* CCAP 19/3, and *D. salina* UTEX LB 1644 had the same 6 bp differences when compared to the other strains. All other nucleotides within the 18S region were conserved among all 14 strains. Analysis of the 18S region was inconclusive in providing information about relationships among these strains due to the high level of conservation (Fig. 2.3).

A ~227 bp product was sequenced for the ITS2 region for each strain (Table 2.3). ITS2 alignment displayed many bp differences, indicative of significant interspecific variation. Analysis of the ITS2 region suggested that the 14 *Dunaliella* strains grouped into four distinct clades (Fig. 2.4). Further comparison of the sequences revealed that there were only four unique sequences among the set of 14 strains (Appendix A) with each sequence corresponding to one clade (Fig. 2.4). When the 14 sequences were merged into the comprehensive phylogenetic tree from Assunção *et al.* (2012), it was clear that the strains were grouped in four clades (Appendix B). *D. parva* strains CCAP 19/9, CCAP 19/26, *D. primolecta* UTEX 1000, and *D. tertiolecta* strains CCAP 19/24, CCMP 364, CCMP 1320, and UTEX 999 clustered within the *D. tertiolecta* clade. *D. parva* UTEX 1983 and *D. salina* strains CCAP 19/18 and UTEX 200 grouped within the *D. pseudosalina* clade. The strain designated as *D. bardawil* ATCC 30861 grouped within the *D. salina* clade. Lastly, *D. parva* CCAP 19/10 and *D. salina* strains CCAP 19/3 and UTEX 1644 grouped within the *D. viridis* clade. GenBank Accession information for each of the 14 strains sequenced can be found in Table 2.3.

2.5 Discussion

A major difficulty in analyzing the phylogeny of *Dunaliella* is the misinformation and misidentification of species available in both culturing facilities and GenBank. In fact, studies have determined that many species are misidentified (González *et al.* 2001, Borowitzka & Siva 2007), but *Dunaliella* species names continue to be associated with the collector's or culturing facility's original identifications. The number of total *Dunaliella* species is likely to be less than what has been previously suggested or indicated.

We assessed the taxonomic relationship of 14 strains of *Dunaliella* by analyzing characteristics such as biovolume and maximum cell production in conjunction with ITS2 and 18S sequence data. The use of both genetic and biological data was important in determining the approximate phylogenetic identity of each of the 14 strains. As to the accuracy of the strain names, the biological data alone were too variable to yield effective conclusions. The 18S region was highly conserved and could not be used to differentiate among strains of *Dunaliella*. The integration of the genetic data from the ITS2 region clarified the biological data, and the original population of 14 strains fell within only four clades.

Confusion among different *Dunaliella* strains has been documented previously (Borowitzka & Siva 2007). Morphological data in *Dunaliella* from different strains are highly variable, making it difficult to identify a strain based solely on its label. Intraspecific variation in a population can greatly exacerbate the difficulty in using morphological features to define *Dunaliella* species. The lack of a rigid polysaccharide wall means that *Dunaliella* cells are highly flexible and can change morphology rapidly (Borowitzka & Siva 2007, Ben-

Amotz *et al.* 2009). Variations in conditions such as temperature, nutrient availability, photoperiod, light intensity, and salinity contribute to this polymorphism. These environmental variables can also impact growth rates, as can be seen from the error bars of the growth curves in Fig. 2.2.

In this study, when the 18S sequences for the 14 *Dunaliella* strains were compared to available sequences in GenBank, the most conserved matches were all *Dunaliella* strains. As expected, the 18S region was conserved among all 14 strains. Only *D. parva* CCAP 19/10, and *D. salina* strains CCAP 19/3 and UTEX 1644 showed any sequence differences, differing from the others by the same 6 bp. The 18S region is commonly used as a potential identifier at the genus level (Nakada *et al.* 2008, Kim *et al.* 2010). For example, Nakada *et al.* 2008 completed an exhaustive analysis on the Volvocales (Chlorophyceae, Chlorophyta) using the 18S rDNA sequences available on GenBank. Unfortunately, however, the analysis represented many species by a single sequence, and the authors concluded that further work would need to be completed to create a phylogenetic tree for this order (Nakada *et al.* 2008). More recently, Olmos *et al.* (2009) used an 18S rDNA fingerprint to differentiate strains of *Dunaliella* strains that produce large amounts of β-carotene. However, the analysis was not successfully applied to identify a potential new strain of *Dunaliella*; more work was needed to accurately name that strain (Olmos *et al.* 2009).

The analysis of the ITS2 sequences from the 14 cultures showed the presence of only 4 unique sequences (Appendix A). While the phylogeny based on the ITS2 region for *Dunaliella* is not entirely clear, there is sufficient variation to determine relationships among different strains (Assunção *et al.* 2012). The fact that there were only four unique ITS2

sequences suggests that those cultures sharing an identical sequence are also identical cultures. Possible mistakes in culturing, identifications, and labeling can potentially explain why 4 unique strain sequences were found among the 14 strains. Many of these cultures had been maintained for numerous years and a simple placement of the wrong strain into a culture labeled as another strain could easily result in duplication or elimination of cultures. Additionally, several of the cultures have an uncertain history because the cultures were provided by other laboratories. These simple mistakes in culture maintenance can be easily missed because many of the different strains superficially look identical when grown under optimal conditions. In addition, biochemical data are often needed to differentiate species. For example, the morphology- and biochemistry-based taxonomy of Borowitzka and Siva (2007) requires data under a variety of conditions (i.e. salinity range experiments, temperature ranges, and high light to determine carotenogenic potential – see Borowitzka and Siva (2007)) in order to determine what section a *Dunaliella* species belongs (Phylum: Chlorophyta; Class: Chlorophyceae; Order: Dunaliellales; Family: Dunaliellaceae; Section: *Tertiolectae*, *Dunaliella*, *Virides*, or *Peirceinae* – see Borowitzka and Siva (2007)). This requirement makes the determination of a species difficult, exacerbated by attempts to determine and understand intraspecific strain differences.

The combination of both genetic (ITS2 region) and morphological/physiological (biovolumes/cell production) data helped in the determination of species of each of the 14 cultures. Unfortunately, GenBank does not contain the ITS2 sequences for *D. parva* strains CCAP 19/10 and CCAP 19/26, or for *D. tertiolecta* CCAP 19/24 even though these strains are readily available at culturing facilities. If sequences had been available, comparison to the

sequences generated in this study could have determined whether these cultures have been incorrectly labeled in our culture collection. Such a comparison cannot be made although the growth curves and the biovolumes for each culture are accurate. This study shows that members of the *D. tertiolecta* clade can have similar biovolumes and mean cell production as the cultures mislabeled as *D. parva* strain CCAP 19/26 and *D. tertiolecta* CCAP 19/24 (Table 2.1, Appendix B). The biovolume and mean cell production data of the mislabeled *D. parva* strain CCAP 19/10 culture can also be applied to the *D. viridis* clade (Table 2.1, Appendix B).

The *D. bardawil* ATCC 30861 culture groups near *D. bardawil* ATCC 30861 phylogenetically (Appendix B, AF313431.1) although there is a four SNP difference. The ITS2 sequence is, however, identical to that of *Dunaliella* sp. ABRIINW U1/1 which clusters closely to *D. bardawil* ATCC 30861 (Appendix A, AF313431.1) within the *D. salina* clade of the *Dunaliella* phylogenetic tree (Appendix B). There is ongoing debate as to whether the species name *bardawil* should be accepted (González *et al.* 2001, Borowitzka & Siva 2007). The ATCC 30861 strain always groups phylogenetically with *D. salina* strains (González *et al.* 1999, González *et al.* 2001) and it also accumulates β-carotene (Ben-Amotz 1987). This culture had an uncertain history due to its passing through several laboratories; however, due to its morphological and physiological characteristics, we believe our culture to be very closely related to *D. bardawil* ATCC 30861. Thus, the biovolume and mean cell production data (Table 2.1) of the potentially mislabeled *D. bardawil* ATCC 30861 culture can also be compared to *D. salina* strains that are closely related.

The five cultures that clustered within the *D. tertiolecta* clade, *D. parva* CCAP 19/9, *D. primolecta* UTEX 1000, and *D. tertiolecta* strains CCMP 364, CCMP 1320, and UTEX 999, were identical in ITS2 sequence to *D. tertiolecta* CCMP 1320 (Appendix A, AF313433.1) with no SNP differences. *D. tertiolecta* strain UTEX 999 had a 2 SNP difference compared to the *D. tertiolecta* UTEX 999 (AF313435.1) sequence available in GenBank. In addition, these strains had similar biovolumes as well as cell production (Table 2.1). It is therefore likely that these cultures are *D. tertiolecta* as they group within the *D. tertiolecta* clade (Appendix B), and the ITS2 sequence region is identical to a known *D. tertiolecta* CCMP 1320 strain (Appendix A). While these cultures do cluster in the *D. tertiolecta* clade, it is difficult to determine whether they are truly similar because the ITS2 sequences of *D. primolecta* UTEX 1000, and *D. tertiolecta* strains CCMP 364 and CCMP 1320 have been previously determined to be identical (Appendix A, Appendix B). However, the biovolumes and mean cell production data for these three stains are not definitive to determine whether these cultures are unique, which is why we suggest defining them as members of the *D. tertiolecta* clade rather than by potential strain name (Table 2.1).

D. salina CCAP 19/3 and UTEX 1644 cultures grouped within the *D. viridis* clade and are most closely related to *D. parva* UTEX 1983 (Appendix A, AF313441.1) with three SNP differences in the ITS2 sequence. *D. parva* UTEX 1983 has been known to have been misnamed and should carry the species name of *viridis*. These cells were the smallest in biovolume and sustained the highest cell production; they can undergo multiple cell divisions per day depending upon culture conditions. These two cultures, along with the mislabeled *D.*

parva strain CCAP 19/10 culture, provide both biovolume and cell production data that can be compared to other members of the *D. viridis* clade (Table 2.1).

Lastly, the *D. parva* UTEX 1983 and *D. salina* strains CCAP 19/18 and UTEX 200 cultures clustered within the *D. pseudosalina* clade and have an identical ITS2 sequence to *D. parva* SAG 19-1 (Appendix A, Appendix B, DQ377091.1). It is interesting to note that the ITS2 sequences from these cultures have only a one SNP difference from *D. salina* strain UTEX 200 (Appendix A, AF313423.1). The possibility exists that due to long culturing conditions, a mutation could have occurred in our culture distinguishing it from the official UTEX culture. However, we cannot answer this definitively since the history of our culture is in question. Therefore, these three cultures represent members of the *D. pseudosalina* clade and the biological data gathered on these cultures can be compared to other members of the *D. pseudosalina* clade (Table 2.1).

The use of the molecular sequences of the 18S and ITS regions in this study, together with the morphological characteristics of size and biovolume, and the trait of population growth as cell production, demonstrated the large variation that occurs when attempting to discern phytoplankton strains. To gain further insights about taxonomic relationships among and within *Dunaliella*, additional genes of interest (GOI) should be sequenced, for example the genes involved in major metabolic pathways. The resulting phylogenetic trees could then be compared to the overall ITS2 tree (Appendix B). A multi-gene molecular analysis would further clarify the relationship among these 14 strains of *Dunaliella*. Importantly, such an approach would also help to explain the considerable intraspecific variation known within *Dunaliella* species.

The design of appropriate universal primers for the GOI will require sequencing information be available to the public. In addition, sequencing of the full genome is needed for *Dunaliella* species. Considerable effort is required to sequence eukaryotic genomes (Rismani-Yazdi *et al.* 2011). Unfortunately, at present very few microalgal genomes have been completed (see the DOE Joint Genome Institute; www.jgi.doe.gov). On the other hand, transcriptome sequencing can provide valuable information regarding functional genomics and is a more efficient sequencing process. This type of sequencing facilitates *de novo* assembly for microalgal species when the full genome sequence is not yet available (Rismani-Yazdi *et al.* 2011; Wong *et al.* unpublished data).

The choice of GOI that provide the most insights about relatedness among microalgal strains should be based on conservation of significant metabolic pathways, availability of gene sequence information, and the relative ease of developing universal primers. For example, during hyperosmotic stress, the osmolyte glycerol is produced within *Dunaliella* cells to balance the osmotic potential (Ben-Amotz *et al.* 2009). The metabolic pathways involved in glycerol synthesis have been examined and are highly conserved in both prokaryotes and eukaryotes (Brisson *et al.* 2001, Venugopal *et al.* 2009). The inter-conversion of the intermediate product dihydroxyacetone phosphate (DHAP) to the final product, glycerol, is mediated by four enzymes. Glycerol-3-phosphate dehydrogenase or *GPDH* is one of the primary enzymes that catalyzes the reversible conversion of DHAP to glycerol-3-phosphate (Chen and Jiang 2009). This gene is highly conserved at the protein level (Brisson *et al.* 2001), but not at the nucleotide level, which is required to design universal primers.

GPDH has been used as a taxonomic indicator for 36 drosophilid species (Diptera: Drosophilidae) (Katoh *et al.* 2007), but has not been similarly applied in microalgae. We conducted a preliminary experiment to compare the *GPDH* protein and nucleotide sequences in our 14 *Dunaliella* strains, after piecing together transcriptome information for the plastidic *GPDH* that is available thus far for four strains (Wong *et al.*, unpublished data; http://www.onekp.com/index.html). While the protein sequences were found to be highly conserved, unfortunately there was not enough information on the nucleotide sequences, so that we were unable to retrieve the nucleotide sequences for the entire *GPDH* gene. Where nucleotide sequence information overlapped for multiple strains, the nucleotide sequences were not conserved as determined by Clustal Omega (1.1.0). Until additional sequence and/or transcriptome information is available, universal primers will be difficult to design for certain genes such as *GPDH*, due to the high intra- and interspecific variation observed among phytoplankton.

In addition to consideration of the ITS and 18S regions, which are both nuclear, genes from the chloroplast and mitochondrion can be sequenced as taxonomic identifiers. Sequence variation within chloroplast DNA (cpDNA) has been investigated to assist in assessing relationships among higher plant species (Taberlet *et al.* 1991). Intergenic spacers present in the chloroplast genome should be taken into consideration as taxonomic identifiers as they have been shown to provide a comparison to the ITS region (Taberlet *et al.* 1991, Kim *et al.* 1999). Mitochondrial DNA (mtDNA) is also useful for reconstructing phylogenies because while mt genes are largely conserved, they are generally more variable than nuclear genes such as small subunit (SSU) rDNA (Saccone *et al*. 2000). The genome of the mitochondria

consists of a limited set of genes that encode for proteins and RNAs (Gray *et al.* 1999). Lane *et al.* (2006) used a combination of genes from the nuclear genome (ITS and large subunit or LSU rDNA), the chloroplast genome (RUBISCO operon, including the intergenic spacer), and the mitochondrial genome (NADH dehydrogenase subunit 6) alone and in combination to elucidate the taxonomy among three families in the Laminariales. They reported that the ITS data demonstrated high divergence values compared to the LSU (Lane *et al.* 2006), similar to our findings. Multiple markers will help to clarify the phylogenetic trees that accurately depict the relationships among *Dunaliella* species and strains. Other GOI could include genes that regulate cell size or the rate of cell division because these two characteristics were highly variable among the 14 strains of *Dunaliella* investigated.

This study indicates that culture clearinghouse identifications were incorrect for the majority of the 14 strains of *Dunaliella* that were examined. By considering morphological data, population growth data, and molecular (ITS2) sequence data, we were able to draw general conclusions about where each culture clustered within the *Dunaliella* phylogenetic tree. These data collectively suggest that the 14 strains cluster within four distinct clades: *D. tertiolecta, D. pseudosalina, D. salina*, and *D. viridis*. The findings underscore the importance of using molecular data to support insights from morphological traits and population growth in assessing taxonomic relationships among microalgal species.

2.6 References

Assunção, P., Jaén-Molina, R., Caujapé-Castells, J., de la Jara, A., Carmona, L., Freijanes, K., Mendoza, H., 2012. Molecular taxonomy of *Dunaliella* (Chlorophyceae), with a special focus on *D. salina*: ITS2 sequences revisited with an extensive geographical sampling. Aquat. Biosyst. 8, 2.

Ben-Amotz, A., 1987. Effects of irradiance and nutrient deficiency on the chemical composition of *Dunaliella bardawil* Ben-Amotz and Avron (Volvocales, Chlorophyta). J. Plant Physiol. 131, 479-487.

Ben-Amotz, A., Polle, J.E.W., Rao, D.V.S., (Eds.), 2009. The Alga *Dunaliella*: Biodiversity, Physiology, Genomics and Biotechnology. Science Publishers, Enfield (New Hampshire). 555 pp.

Borowitzka, M.A., Siva, C.J., 2007. The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. J. Appl. Phycol. 19, 567-590.

Brisson, D., Vohl, M.-C., St-Pierre, J., Hudson, T.J., Gaudet, D. 2001. Glycerol: a neglected variable in metabolic processes? BioEssays 23, 534-542.

Burkholder, J.M., Azanza, R.V., and Sako, Y. 2006. The ecology of harmful dinoflagellates, In: Turner, G.E. (Ed.), The Ecology of Harmful Algae. Springer-Verlag, New York (New York). Pp. 53-66.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York (New York). pp. 29-60.

Chen, H., Jiang, J.-G., 2009. Osmotic responses of *Dunaliella* to the changes of salinity. J. Cell. Physiol. 219, 251-258.

Føyn, B., 1934. Lebenszyklus, Cytologie und Sexualiät der Chlorophycee *Cladophora suhriana* Küzing. Arch. Protistenk. 83, 1-56.

Ginzburg, M., 1987. *Dunaliella*: a green alga adapted to salt. Advances in Botanical Research 14, 93-183.

Gómez, P., González, M., 2004. Genetic variation among seven strains of *Dunaliella salina* (Chlorophyta) with industrial potential, based on RAPD banding patterns and on nuclear ITS rDNA sequences. Aquaculture 233, 149-162.

González, M., Gómez, P., Montoya, R., 1999. Comparison of PCR-RFLP analysis of the ITS region with morphological criteria of various strains of *Dunaliella*. J. Appl. Phycol. 10, 573-580.

González, M. A., Coleman, A.W., Gómez, P.I., Montoya, R., 2001. Phylogenetic relationship among various strains of *Dunaliella* (Chlorophyceae) based on nuclear ITS rDNA sequences. J. Phycol. 37, 604-611.

Graham, L.E., Graham, J.M., Wilcox, L.W., 2009. Algae. 2nd Edition. Pearson Education, Incorporated, San Francisco (California). 616 pp.

Gray, M.W., Burger, G., Lang, B.F. 1999. Mitochondrial Evolution. Science 283, 1476-1481.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York (New York). pp. 29-60.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234-236.

Guillard, R.R.L. 2005. Purification Methods for Microalgae. In: Anderson, R.A. (Ed.) Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts). pp. 117-132.

Guiry, M.D. 2012. How many species of algae are there? J. Phycol. 48, 1057-1063.

Hillebrand, H., Dürselen, C.-D., Kirschtel, D., Pollingher, U., Zohary, T., 1999. Biovolume calculation for pelagic and benthic microalgae. J. Phycol. 35, 403-424.

Katoh, T., Nakaya, D., Tamura, K., Aotsuka, T., 2007. Phylogeny of the *Drosophila immigrans* species group (Diptera: Drosophilidae) based on *Adh* and *Gpdh* sequences. Zoological Science 24, 913-921.

Kawachi, M., Noël, M.-H., 2007. Sterilization and Sterile Technique. In: Anderson, R.A. (Ed.) Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts). pp. 65-81.

Keller, A., Förster, F., Müller, T., Dandekar, T., Shultz, J., Wolf, M., 2010. Including RNA secondary structures improves accuracy and robustness in reconstruction of phylogenetic trees. Biology Direct 5, 4.

Kim, S.-C., Crawford, D.J., Jansen, R.K., Santos-Guerra, A. 1999. The use of a non-coding region of chloroplast DNA in phylogenetic studies of the subtribe *Sonchinae* (Asteraceae: Lactuceae). Pl. Syst. Evol. 215, 85-99.

Lakeman, M.B., Cattolico, R.A. 2007. Cryptic diversity in phytoplankton cultures is revealed using a simple plating technique. J. Phycol. 43, 662-674.

Lakeman, M.B., von Dassow, P., Cattolico, R.A. 2009. The strain concept in phytoplankton ecology. Harmful Algae. 8, 746-758.

Lane, C.E., Mayes, C., Druehl, L.D., Saunders. G.W. 2006. A multi-gene molecular investigation of the kelp (Laminariales, Phaeophyceae) supports substantial taxonomic re-organization. J. Phycol. 42, 493-512.

Lorenz, M., Friedl, T., Day, J.G., 2005. Perpetual Maintenance of Actively Metabolizing Microalgal Cultures. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts). pp. 145-156.

Massjuk, N.P., 1973. Morphology, taxonomy, ecology, and geographic distribution of the genus *Dunaliella* Teod and prospects for its potential utilization. *Naukova, Dumka*, pp. 242.

Meyer, A., Todt, C., Mikkelsen, N.T., Lieb, B. 2010. Fast evolving 18S rRNA sequences from *Solenogastres* (Mollusca) resist standard PCR amplification and give new insights into mollusk substitution rate heterogeneity. BMC Evolutionary Biology 10, 70.

Müller, T., Philippi, N., Dandekar, T., Shultz, J., Wolf, M., 2007. Distinguishing species. RNA 13, 1469-1472.

Nakada, T., Misawa, K., Nozaki, H. 2008. Molecular systematics of Volvocales (Chlorophyceae, Chlorophyta) based on exhaustive 18S rRNA phylogenetic analyses. Molecular Phylogenetics and Evolution 48, 281-291.

Olmos-Soto, J., Paniagua-Michel, J., Contreras, R., Trujillo, L., 2002. Molecular identification of β-carotene hyper-producing strains of *Dunaliella* from saline environments using species-specific oligonucleotides. Biotechnol. Letters 24, 365-369.

Olmos-J., Ochoa, L., Paniagua-Michel, J., Contreras, R. 2009. DNA fingerprinting differentiation between β-carotene hyperproducer strains of *Dunaliella* from around the world. Saline Systems 5.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Bibby, K., Peccia, J. 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: Pathway description and gene discovery for production of next-generation biofuels. BMC Genomics 12, 148.

Saccone, C., Gissi, C., Lanave, C., Larizza, A., Pesole, G., Reyes, A. 2000. Evolution of the mitochondrial genetic system: an overview. Gene 261, 153-159.

Stewart, C.N., Via, L.E., 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. BioTechniques 14, 748-749.

Taberlet, P., Gielly, L., Pautou, G., Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of the chloroplast DNA. Plant Molecular Biology 17, 1105-1109.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28, 2731-2739.

Tompkins, J., DeVille, M.M., Day, J.G., Turner, M.F., 1995. Culture Collection of Algae and Protozoa. Catalog of Strains. Ambleside (United Kingdom). 204 pp.

Venugopal, S.C., Chanda, B., Vaillancourt, L., Kachroo, A., Kachroo, P. 2009. The common metabolite glycerol-3-phosphate is a novel regulator of plant defense signaling. Plant Signaling and Behavior 4, 746-749.

Vollenweider, R.A., Talling, J.F., Westlake, D.F. 1974. A Manual on Methods for Measuring Primary Production in Aquatic Environments, 2nd Edition. Blackwell Scientific, Oxford. 225 pp.

Walker, T.L., Black, D., Becker, D.K., Dale, J.L., Collet, C., 2005. Isolation and characterisation of components of the *Dunaliella tertiolecta* chloroplast genome. J. Appl. Phycol. 17, 495-508.

Wetzel, R.G., Likens, G.E., 2001. Limnological Analyses, 2nd Edition. W.B. Saunders, New York (New York). 429 pp.

Wong, G. K.-S., Carpenter, E., Sauer, M.-L., Sederoff, H., Transcriptomes of four *Dunaliella* species in the 1KP project (unpublished data; http://www.onekp.com/index.html).

Table 2.1 – Strain names given by commercial culture collections, and strain numbers of each *Dunaliella* culture are listed as identified by each authority. Mean biovolume, maximum cell production, and suggested species name (based on ITS sequence data) for each mislabeled culture is indicated.

Species Name	Presumed[2] Source, Strain No.	Original Authority	Biovolume (mean μm^3 ± 1 SE; n = 30 cells)	Maximum Cell Production (x 10^6 cells/mL)	Clade Determined for Each Strain
D. parva	CCAP 19/9	Butcher	120 ± 6	6.2	*D. tertiolecta*
D. parva	CCAP 19/26	Lerche	120 ± 5	3.2	*D. tertiolecta*
D. tertiolecta	CCAP 19/24	Butcher	132 ± 6	3.4	*D. tertiolecta*
D. tertiolecta	CCMP 364	Butcher	135 ± 8	3.6	*D. tertiolecta*
D. tertiolecta	CCMP 1320	Butcher	136 ± 6	3.9	*D. tertiolecta*
D. primolecta	UTEX 1000	Butcher	168 ± 8	6.6	*D. tertiolecta*
D. tertiolecta	UTEX 999	Butcher	163 ± 9	4.4	*D. tertiolecta*
D. parva	UTEX 1983	Lerche	240 ± 11	3.4	*D. pseudosalina*
D. salina	CCAP 19/18	Kaethner	215 ± 10	1.9	*D. pseudosalina*
D. salina	UTEX 200	(Dunal) Teodoresca	183 ± 8	3.3	*D. pseudosalina*
D. parva	CCAP 19/10	Lerche	46 ± 2	1.9	*D. viridis*
D. salina	CCAP 19/3	(Dunal) Teodoresca	43 ± 3	7.7	*D. viridis*
D. salina	UTEX 1644	(Dunal) Teodoresca	74 ± 5	10.0	*D. viridis*
D. bardawil	ATCC 30861	Amotz and Avron	646 ± 218	0.3	*D. salina*

[2] Strain number is given as labeled by the culturing facility; this is "presumed" because many of these strains are mislabeled.

Table 2.2 – Primer sets for ITS and 18S. Sequences are given 5’ to 3’.

Gene Name	Sequence (5’ to 3’)
TW81	GGGATCCTTTCCGTAGGTGAACCTGC
AB28	GGGATCCATATGCTTAAGTTCAGCGGGT
18S F Universal	ACCTGGTTGATCCTGCCAG
18S R Universal-C	TGATCCTTCCGCAGGTTCAC
18S R Universal-T	TGATCCTTCTGCAGGTTCAC

Table 2.3 – ITS2 GenBank accession number for each strain, and indication of whether the strain was labeled in parallel with this study and the available GenBank information.

Species and Strain Name	ITS2 Accession Number	Note
CCAP 19/9 *D. parva*	KF229737	Mislabeled
CCAP 19/26 *D. parva*	KF229741	Unconfirmed[a]
CCAP 19/24 *D. tertiolecta*	KF229743	Unconfirmed[a]
CCMP 364 *D. tertiolecta*	KF229740	Agreement[b]
CCMP 1320 *D. tertiolecta*	KF229742	Agreement[b]
UTEX 1000 *D. primolecta*	KF229739	Agreement[b]
UTEX 999 *D. tertiolecta*	KF229738	Mislabeled[c]
UTEX 1983 *D. parva*	KF229736	Mislabeled
CCAP 19/18 *D. salina*	KF229735	Mislabeled
UTEX 200 *D. salina*	KF229734	Mislabeled[c]
CCAP 19/10 *D. parva*	KF229731	Unconfirmed[a]
CCAP 19/3 *D. salina*	KF229732	Mislabeled
UTEX 1644 *D. salina*	KF229730	Mislabeled
ATCC 30861 *D. bardawil*	KF229733	Mislabeled

[a]There is no GenBank information available for these strains so a comparison could not be made, see text

[b]These strains are known to have identical ITS2 regions to their respective strain sequences available in GenBank, see text

[c]These two strains (UTEX 999 and UTEX 200) had either 1 or 2 SNP differences from their respective strain sequences available in GenBank, see text

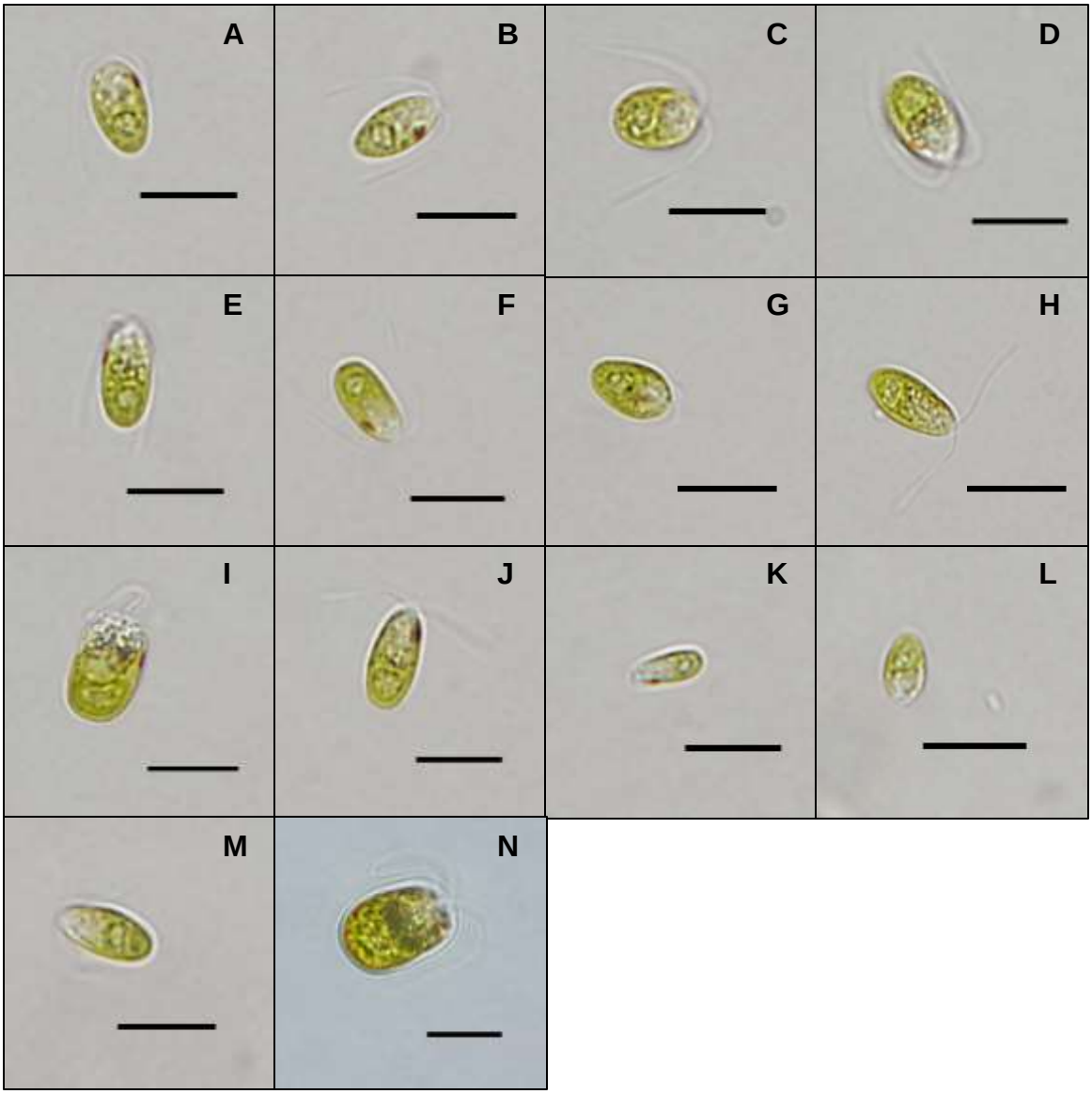

Figure 2.1 – Light micrographs depicting biovolume at 600x magnification, scale bar = 5µm. A) *D. parva* CCAP 19/9, B) *D. parva* CCAP 19/26, C) *D. primolecta* UTEX 1000, D) *D. tertiolecta* CCAP 19/24, E) *D. tertiolecta* CCMP 364, F) *D. tertiolecta* CCMP 1320, G) *D. tertiolecta* UTEX 999, H) *D. parva* UTEX 1983, I) *D. salina* CCAP 19/18, J) *D. salina* UTEX 200, K) *D. parva* CCAP 19/10), L) *D. salina* CCAP 19/3, M) *D. salina* UTEX 1644, and N) *D. bardawil* ATCC 30861.

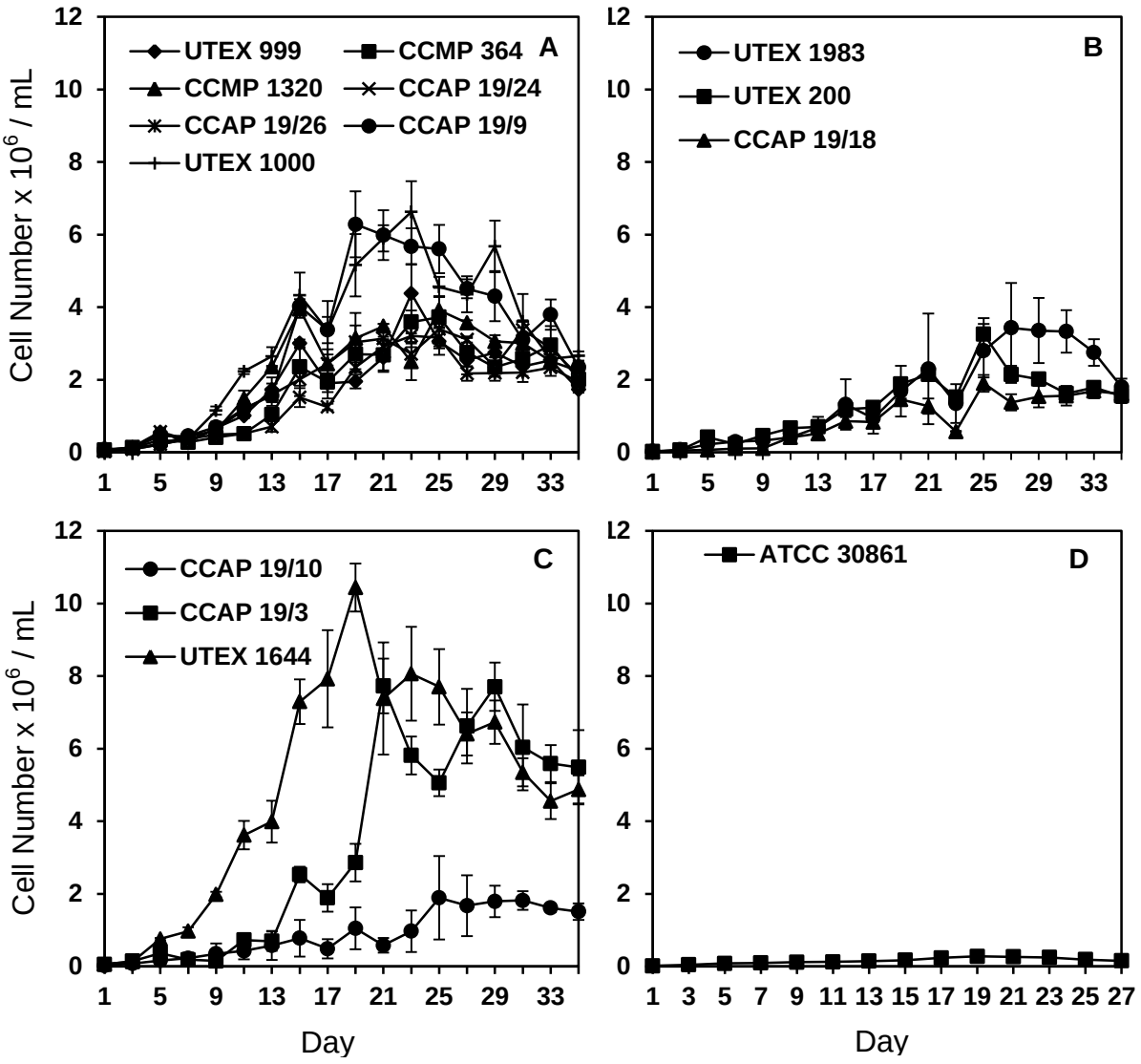

Figure 2.2 – Growth curves grouped by ITS2 sequence for A) *D. tertiolecta*, B) *D. pseudosalina*, C) *D. viridis*, and D) *D. salina*. *D. bardawil* ATCC 30861 was only grown for 27 days because cell production ceased.

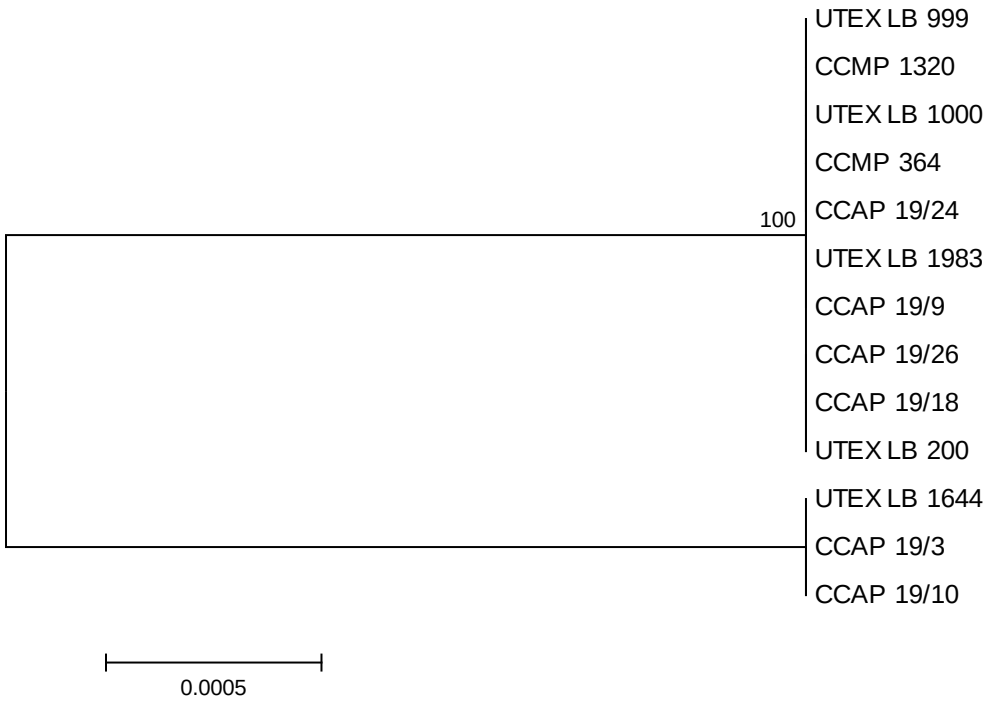

Figure 2.3 – Phylogenetic tree for the 14 *Dunaliella* strains, derived from sequences of the 18S rRNA region with supporting bootstrap values (100 replicates).

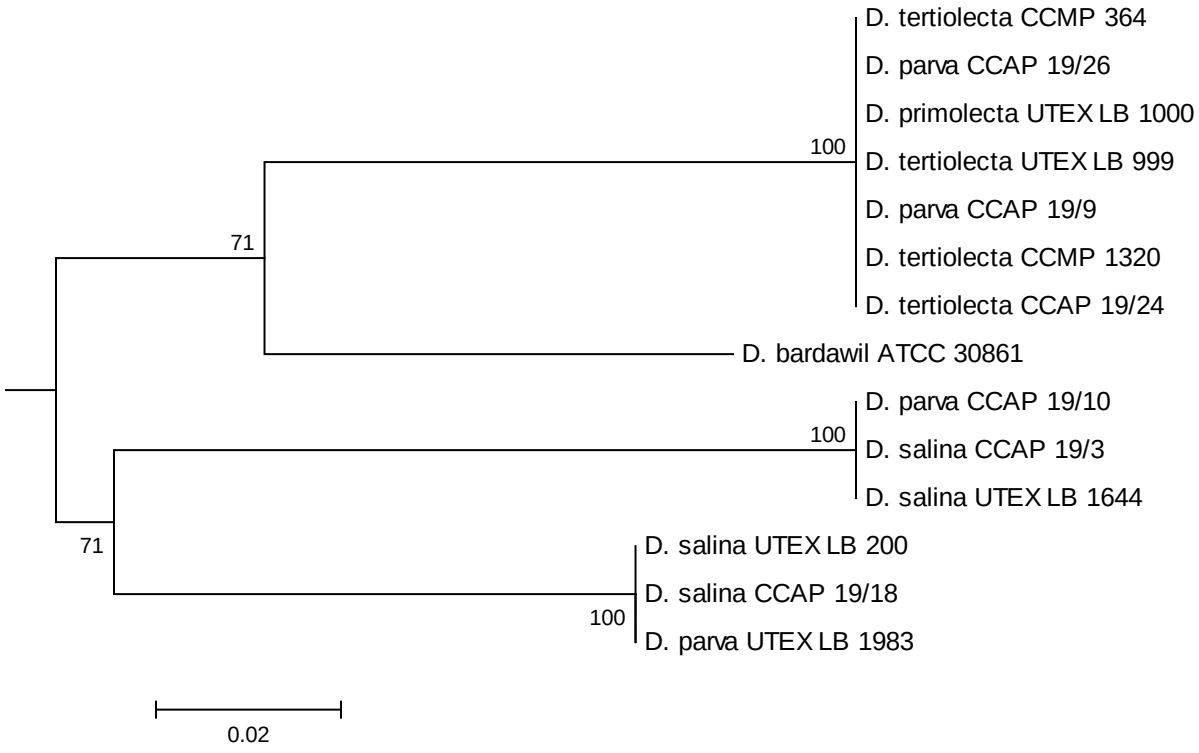

Figure 2.4 – Phylogenetic tree for the 14 *Dunaliella* strains, derived from the ITS2 region of the ITS rRNA sequences with supporting bootstrap values (100 replicates).

3. ENHANCED LIPID PRODUCTION IN *DUNALIELLA* SPP. EXPOSED TO SALINITY STRESS

Submitted to Journal of Experimental Marine Biology and Ecology

3.1 Abstract

We assessed 14 strains of the marine chlorophyte, *Dunaliella*, within four species for their potential utility in sustainable biofuel production by tracking lipid production under salinity stress. A modified technique with Nile Red stain was used to rapidly screen cultures for the presence of neutral lipid content. Promising strains with visually high lipid content and high growth as cell production were selected for further efforts to enhance lipid production using high salinity stress in short-term (sec to hr) and long-term (≥ 24 hr) bench-scale experiments (culture volume 0.1 to 3.5 L). Promising strains were also grown at mass culture scale (culture volume ~150 to 175 L) to ensure feasibility of scale-up. High salinity stress generally resulted in maximal total fatty acid (FA) content (up to 65% by dry weight) in comparison to controls (~10-25% total FAs by dry weight). Glycerol production, a known mechanism of osmoregulation in *Dunaliella*, was measured in a short-term salinity stress experiment on a promising strain and found to increase significantly 30 min to 24 hr after exposure to high salinity. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to evaluate the relative expression of glyceryl-3-phosphate dehydrogenase (*GPDH*), one of the primary glycerol biosynthesis genes for glycerol production, during a short-term experiment with high salinity stress. *GPDH* was significantly expressed (≥ 2-fold when compared to the endogenous gene *ACTIN*) 30 min after exposure and continued to be

expressed for 2 hr. In general, when cellular glycerol content was low, total FAs increased as an immediate or short-term response (30 sec to 30 min) to hyperosmotic stress. Responses were strain-specific and indicated both inter- and intraspecific variation. This study shows that a simple high salinity adjustment significantly increases lipid production in selected strains of *Dunaliella* spp. The data additionally suggest that these *Dunaliella* strains may incorporate a portion of the available glycerol as triacylglycerols (TAGs) or neutral lipids under short-term, high-salinity stress. Thus, glycerol not only acts as an osmolyte during hyperosmotic stress, but also provides the carbon structure to which FAs are covalently linked and form neutral lipids.

3.2 Introduction

Agricultural crops including corn, sugar cane, and oil palm have been investigated as potential sources of biofuel, but they produce only a fraction of the oil per unit biomass in comparison to microalgae (Chisti 2008, Schenk *et al.* 2008). This research focused on the halotolerant, saltwater microalgae, *Dunaliella* spp. In addition to not requiring freshwater for growth, this genus lacks a rigid cell wall (Ben-Amotz *et al.* 2009), a feature which facilitates lipid extraction. As a result of their tolerance to high salt, *Dunaliella* species can more easily out-compete potential bacterial contaminants (Avron and Ben-Amotz 1992, Hu *et al.* 2008). *Dunaliella* species accumulate neutral lipids in response to environmental stressors (Roessler 1990, Tagaki *et al.* 2006, Ben-Amotz *et al.* 2009) and also have high growth rates (as cell production), making them ideal candidates for mass culture as a renewable biofuel source (Hu *et al.* 2008, Rodolfi *et al.* 2009).

Dunaliella species are halotolerant, able to tolerate a wide range of salinities from < 5 to full saturation (Ginzburg 1987, Avron and Ben-Amotz 1992, Ben-Amotz *et al.* 2009). Under hyperosmotic stress when external salinity increases relative to the cellular osmotic pressure, *Dunaliella* can accumulate glycerol to high levels (Ben-Amotz and Avron 1973, Chitlaru and Pick 1991, Ben-Amotz *et al.* 2009, Chen and Jiang 2009). Depending upon the requirements of the cell, glycerol can either be synthesized or broken down into additional products such as starch, sugar, or pyruvate (Taiz and Zeiger 2006, Chen and Jiang 2009, Shariati and Hadi 2011)(Fig. 3.1). Glycerol is produced by CO_2 fixation or the degradation of starch (Avron and Ben-Amotz 1992, Ben-Amotz *et al.* 2009). Glycerol biosynthesis takes place primarily in the chloroplast, but also in the cytosol. Four enzymes are required for the inter-conversion of dihydroxyacetone phosphate (DHAP) to glycerol, including a key enzyme glycerol-3-phosphate dehydrogenase (*GPDH*) (Ben-Amotz *et al.* 2009, Chen and Jiang 2009). Metabolism of glycerol depends upon whether the cell is actively growing and requires energy for growth and division, versus if the cell is undergoing senescence, during which neutral lipids can be produced. In addition, glycerol provides the carbon structure or "backbone" for triacylglycerols (TAGs), formed by the condensation of glycerol with three fatty acid (FA) molecules (Miner and Dalton 1953). Thus, it is assumed that when neutral lipids are produced under conditions such as nutrient limitation (Chen *et al.* 2011), pH stress (see Chapter 4), and/or salinity stress (Takagi *et al.* 2006), a proportion of the glycerol pool will decrease as TAGs form from the incorporation of available glycerol. It is suggested that FA molecules will be covalently linked to some of the excess glycerol produced in response to hyperosmotic shock to form TAGs.

Dunaliella reacts to osmotic stress through an *immediate response* (30 sec to ≤ 5 min) wherein cell size, shape, structure, and ion concentration change; a *short-term response* (up to 2-3 hr) whereby the osmotic pressure between the outside environment and inside the cell is balanced by regulating the glycerol concentration; and a *long-term response* (≥ 24 hr) which includes stress-induced gene expression and accumulation of salt-induced proteins (Chen and Jiang 2009). These responses take into consideration immediate and short-term acclimation to changes in the environment as well as epigenetic adaptation, whereby gene expression can be up- or down-regulated in response to a particular stressor (Lakeman *et al.* 2009, Sahu *et al.* 2013). In this research, we assessed the optimum hypo- or hyper-osmotic salinity stress to maximize lipid production by selected strains of *Dunaliella*. Cells undergo hypo-osmotic stress when external salinity decreases, and the cells eliminate glycerol to balance the cellular osmotic potential. Therefore, it was expected that *Dunaliella* would not produce neutral lipids during hypo-osmotic shock (Ben-Amotz *et al.* 2009) due to the lack of available glycerol, required for the formation of TAGs.

We also completed a time course experiment with short sampling intervals, with a subset of selected strains, to determine when the highest production of total FAs and glycerol production initially occurs under high salinity stress. The data were helpful in guiding quantitative PCR, which was used in a time course experiment to analyze expression of the glycerol biosynthesis gene, *GPDH*. This gene catalyzes a reversible step in converting dihydroxyacetone phosphate (DHAP) to the intermediate product of glycerol-3-phosphate (G3P). G3P is then converted to the final glycerol product by the enzyme glycerol phosphate isomerase (GPI) (He *et al.* 2007, Chen and Jiang 2009) (Fig. 3.1). We expected that total FAs

would increase rapidly or as a short-term response under high salinity stress; that, as total free glycerol increased, there would be a corresponding expression of *GPDH*; and that a certain proportion of the available glycerol would be incorporated by the cells into TAGs.

3.3 Materials and Methods

3.3.a Culturing and Selection of Strains

Fourteen strains of *Dunaliella* were obtained from the American Type Culture Collection (ATCC), the Culture Collection of Algae and Protozoa (CCAP), the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, formerly the National Center of Marine Phytoplankton, CCMP), and the Culture Collection of Algae at the University of Texas at Austin (UTEX) (Table 3.1).

These strains were labeled by the culture collections within five species of *Dunaliella*. However, in separate work, ITS2 sequence data for each of the 14 strains indicated that the strains actually fall within four distinct species (clades), and that 10 of the 14 strains had been labeled incorrectly (Chapter 2) (Table 3.1). Thus, here we refer to these strains by the species names indicated from the ITS2 sequence data, along with the original strain numbers.

In addition, strain UTEX 1644, which was sent to us 5 yr ago by the culture collection, and was determined from ITS2 sequence data to be a unique strain of *D. viridis* that evidently was a culture contaminant in the collection. It is indeed unique from all of our other strains in major features including cell size, population growth as cell production, sustained growth in continuous light, and high lipid production throughout the cell cycle (see

Chapter 4). Based on that information, the strain was not simply a contaminant from our other *Dunaliella* cultures. UTEX 1644 was ordered recently from the culture collection by colleagues, and was confirmed to be *D. salina* rather than this unique strain. Thus, the unique strain from the UTEX collection evidently was a contaminant that can no longer be obtained under the number, UTEX 1644. Here we refer to it as CONTAM. As it is of special interest for its growth and lipid production, and is not available any longer from a commercial culture, we are depositing it at the NCMA.

All strains were first cultured in the media used by the commercial culture facilities (Table 3.1), except for *D. salina* ATCC 30861 which was provided in L1-Si medium at salinity 105. The planned salinity stress experiments required use of the same medium. Preliminary work indicated that the *Dunaliella* strains grew well in Erdschreiber's (Føyn 1934), Artificial Seawater (ASW, made using f/2-Si nutrients; Guillard 1975, Tompkins *et al.* 1995), and L1-Si media (Guillard and Hargraves 1993), at salinity 30 or 60 depending on the strain. Erdschreiber's and ASW media contained soil water with undefined constituents, and nutrient components of f/2-Si were similar to L1-Si. Therefore, L1-Si was selected for use in this study. Once growth was established for strains in their source media (salinity 30 or 60), each culture was transferred into a series of increasing proportions of L1-Si and the source medium (after Lorenz *et al.* 2005) until the cells finally were acclimated to 100% L1-Si medium. Cell production for each strain was compared in its source medium versus L1-Si medium, and all strains attained comparable or higher maximum cell production in L1-Si (Table 3.2). In a second series of transfers, cultures were adjusted using NaCl (Fisher Scientific, Fairlawn, New Jersey), by salinity ± 5 to 10 for each adjustment (YSI 3200

Conductivity Instrument, Yellow Springs, Ohio), and tested for population growth rates (as cell production) to determine the optimal salinity for each strain (Table 3.1). An analogous approach was used to identify the optimum pH for each strain, using incremental changes of 0.1 pH unit (PerpHecT LogR Meter 350, Boston Massachusetts) from an initial pH of 7.5 to 8.5.

Strains were purified using a combination of differential centrifugation and flow cytometry (Beckman Coulter® EPICS Altra, Hialeah, Florida) to remove bacterial contamination (Guillard 2005, Kawachi and Noël 2005, Chapter 1 and methods therein). Cultures were plated on 1% Agar M (Sigma Aldrich®, St. Louis, Missouri) or 1% Phytoblend (Caisson Laboratories, Logan, Utah) to promote algal growth or bacterial growth, respectively. Subsequent plating was completed aseptically on a monthly basis until each strain was purified, or bacteria-free. Stock culture for each strain (n = 3) was grown under the additional conditions of 23˚C and a light intensity of ~180 μE m^{-2} sec^{-1} (light source, ProLume® compact fluorescence bulbs) under a 12-hr : 12-hr light : dark (L:D) photoperiod.

Population growth rates (as cell production) were assessed by growing each strain in 100 mL volume (n = 3) as unialgal culture until late senescence (~35 days). Subsamples were taken between 0800 and 0900 every other day, preserved in acidic Lugol's solution (1% final concentration; Vollenweider *et al.* 1974), and quantified within 1-2 days using Palmer-Maloney chambers (Wetzel and Likens 2000) under light microscopy at 200x magnification using an Olympus BH-2 light microscope (Germany).

The mean biovolume for each strain was also determined as follows: The cells were immobilized using 1.3% Type V agarose (Sigma Aldrich). Two drops of agarose and two

drops of culture were placed in the center of a warmed slide and gently mixed using a pipette. After the agarose had solidified, at least 30 micrographs of each *Dunaliella* strain were taken under 60X magnification (No. 1.5 25mm^{-2} coverslip, Corning®), using an Olympus AX70 microscope (Japan) connected to an Olympus DP70 camera. Biovolume was calculated using the formula for a prolate spheroid (Hillebrand *et al.* 1999; Table 3.1).

$$V = (\frac{\pi}{6} * d^2 * h)$$

The 14 strains were also screened visually for high neutral lipid content using the fluorescent Nile Red dye (NR, 9-diethylamino-5-benzo[α]phenoxazinone; Sigma Aldrich). NR preferentially stains neutral lipids (triacylglycerides, TAGs), and has been used as a rapid screening technique to qualitatively assess the neutral lipid content of microalgal cells (Cooksey *et al.* 1987, Lee *et al.* 1998, Chen *et al.* 2009). The technique was optimized for use with *Dunaliella* (see Chapter 1): Briefly, 10 µL NR (0.625 µg/mL dissolved in 100% MeOH) was added to slightly heated slides containing 2 drops of culture sample immobilized on 1.3% Type V agarose (Sigma Aldrich). An Olympus AX70 research light microscope and Olympus DP70 camera system, or a Zeiss LSM 710 confocal microscope (excitation at 488 nm, emission at 545-665 nm) was used to photograph NR-stained cells.

We used this rapid screening technique to determine when selected strains (*D. tertiolecta* strains UTEX 999, UTEX 1000, and CCAP 19/9; and *D. viridis* CONTAM) had maximal neutral lipid content, considering both the growth cycle and light period. Samples for these tests were collected every other day at 1000 and 1600 hr for cell counts and

fluorescence microscopy of NR-stained cells, until cultures were senescent. Samples then were taken at 0400, 1000, 1600, and 2200 hr for analysis of neutral lipid content using a rapid screening technique. All tested strains except *D. viridis* CONTAM had the highest neutral lipid content during senescence, and senescent populations had comparable content of neutral lipids throughout the light period. In contrast, rapid screening indicated that the unique strain *D. viridis* CONTAM had high lipid content during active growth as well as senescence. These observations guided sampling for the salinity stress experiments: We sampled the *Dunaliella* populations for total FA content when the populations had reached early onset of senescence, and sampling was conducted between 0800 and 0900 hr.

The data for cell production and rapid screening of neutral lipid content were used to select seven strains for long-term salinity stress experiments (100 mL volume). These strains had high maximal cell production and/or visually high amounts of neutral lipids, ideally both features. Growth curves (as cell production) were generated for each of these strains (Fig. 3.2 A-C).

Of the seven strains, the four strains with highest lipid production in the long-term salinity stress experiment were further tested for their immediate and short-term responses to increased salinity. *D. viridis* CONTAM was also tested for glycerol production in these short-term experiments. In addition, two strains (*D. tertiolecta* UTEX 999 and *D. viridis* CONTAM) were grown in mass culture (~150 to 175 L volume) to check feasibility of scale-up.

3.3.b Long-term salinity stress

Preliminary experiments were completed to determine appropriate intervals for the salinity stress experiments. Increasing or decreasing salinity shifts of 5, 10, 15, 25, 30, and 50 were tested on select strains of *Dunaliella.* The smaller levels of change, by salinity 5 and 10, did not appear to stress the cells based on cell size and shape, and cell production. The upper limit was an increase of 50; when cells were exposed to a salinity of 50 higher than the previous salinity, cell production ceased and after 7 days there was no recovery from the hyperosmotic shock. When exposed to a salinity of 15 or 30 above the previous salinity, there was a slight lag in cell production over several days. Based on these data, in the long-term salinity stress experiments we imposed a series of salinity shifts of 15, with shifts separated by 7-day intervals, in order to allow the populations to slowly acclimate to increasingly higher or lower salinity levels.

Seven selected strains (*Dunaliella tertiolecta* strains CCAP 19/9, CCAP 19/24, CCAP 19/26, UTEX 999, and UTEX 1000; *D. pseudosalina* CCAP 19/18; and *D. viridis* CONTAM; Table 3.1) were separately grown in replicate cultures (n = 3) in 100 mL volumes for 14 days until the onset of early senescence. Small-volume samples (~1 mL) were taken at 3-day intervals and preserved in acidic Lugol's solution for cell counts as described above. After the first week, 5 mL of exponentially-growing culture were inoculated into 100 mL of fresh medium that was higher or lower in salinity (by 15) than the previous medium. This procedure was repeated with increasing or decreasing salinity until the cultures attained one-third to one-half of the maximal cell production in the controls (maintained at the initial, optimal salinity for each strain). The salinity at this point was interpreted as the upper or

lower tolerance limit of the strain to salinity stress. After the onset of early senescence at each salinity, cultures were centrifuged (3,000 RPM, 10 min – CL2 Centrifuge, ThermoScientific, Millford, Massachusetts) and the pellets were immediately frozen at -80˚C until FA analysis was completed (within 7-10 days for all experiments; L. Dean, U.S. Department of Agriculture, Raleigh NC, personal communication).

3.3.c Short-term salinity stress from increasing salinity

Four selected strains (*Dunaliella tertiolecta* strains CCAP 19/9, CCAP 19/24, and UTEX 999, and *D. viridis* CONTAM; n = 3) were each grown in unialgal culture at ~3.5 L volume under optimal salinity and pH, as above. When cultures reached senescence, the culture volume of the three replicate flasks was evenly distributed into seven sterile 50-mL tubes (Corning® Centristar™) by centrifuging (3,000 RPM, 10 min). Each 50-mL aliquot was centrifuged repeatedly until a total of ~500 mL of culture was obtained within each tube. Two salinity stress treatments were imposed by adding L1-Si medium at a salinity +15 or +30, adjusted with NaCl (n = 3 for each strain at each salinity). Control pellets were re-suspended with 50 mL of L1-Si medium at the initial, optimal salinity (30 or 60) for each strain. Treatment pellets were re-suspended with 50 mL of L1-Si at a salinity +15 above the control salinity. A shift of +30 above the control salinity was also tested to assess the effect of more severe short-term salinity stress on cell production and total FA content. After re-suspension, all replicates were subsampled for cell counts and rapid screening of neutral lipid content at 30 sec, 5 min, 30 min, 1 hr, 2 hr, and 24 hr after the salinity adjustments. Re-suspended samples (concentrated from ~500 mL of culture) were centrifuged (3,000 RPM, 10 min) and the pellets immediately frozen at -80˚C until FA analysis was completed.

3.3.d Total FAs

Direct transesterification (DT; Griffiths *et al.* 2010) was performed to convert saponifiable lipids to fatty acid methyl esters, hereafter referred to as total FAs, which were then quantified using gas chromatography (GC) with a flame ionization detector (FID). This technique offers several important advantages over conventional solvent-based extractions. DT combines lipid extraction and purification steps in one tube, thus minimizing sample loss, and can be completed on the small sample sizes needed in this research. The combination of adding a basic and acidic catalyst allows for samples to be processed even if the samples contain small amounts of water (Griffiths *et al.* 2010), which was important because these samples were not dried before processing.

For this technique, we used a combination of acidic and basic transesterification catalysts (see Chapter 1). Briefly, 0.5 M methanolic KOH was added to each sample, homogenized, and heated to 85˚C. Then, BF_3 (14% in methanol) was added and the samples were reheated to 85˚C. Next, equal volumes of water and hexane were added and the samples were allowed to separate into layers by density. The lighter hexane layer containing the neutral lipids was removed and analyzed with a GC HP5890 series II equipped with a FID (Hewlett Packard, refurbished by Primera Scientific LLC, Princeton, New Jersey) and a 7673A autosampler (Alpha Omega Technologies, Inc., New Jersey, USA). Separation was achieved in an Rtx-2330 capillary column (Alpha Omega Technologies, Inc., refurbished by Primera Scientific LLC, Princeton, New Jersey). The temperature was programmed to include an initial 3 min at 60˚C, and then was increased to 230˚C at a rate of 4˚C / min. Both injector and detector temperatures were set at 265˚C. Injections were performed under the

splitless mode (Sparkman *et al.* 2011). Data acquisition and analysis were completed using the GC Chemstation Rev. A.08.03 (847) software. Calculations for total FAs were completed using the relative response factor (RRF) method (Sparkman *et al.* 2011; L. Dean, USDA, Raleigh NC, personal communication) and area ratios were compared to the internal standard (KEL-FIM-FAME-5 Mixture, Matreya, LLC, Pennsylvania, USA).

3.3.e Total free glycerol

A short-term salinity stress experiment was completed as above for *D. viridis* CONTAM. This experiment deviated from the above approach only in that the culture was divided into 15-mL centrifuge tubes, each of which contained a pellet concentrated from ~175 mL of stock culture (n = 3). Each pellet was then re-suspended in 15 mL of fresh medium within a sterile centrifuge tube, either at the control salinity (60, for this strain) or at salinity 90. After each exposure period, a set of tubes was centrifuged and the supernatant was saved in a new, sterile 15-mL centrifuge tube. Both the pellet and its corresponding supernatant were quantified for total free glycerol content. Pelleted samples were each dispersed into 1 mL of internal standard mix consisting of cellobiose in water (Patee *et al.* 2000). From this solution, a 50 µL aliquot was diluted with 2 mL water. Samples of the supernatant (1 mL each) were spiked with 50 µL of the internal standard and diluted to 2 mL with deionized water. Total free glycerol was analyzed as described by Patee *et al.* (2000); the data were reported as mg/g for pellets and mg/mL for supernatants.

3.3.f Time course of glycerol production

A short-term salinity experiment as detailed above (Section 3.3.c) was completed using *D. viridis* CONTAM. Centrifuged pellets re-suspended into 50 mL of culture medium

at the control salinity of 60 or at the stress salinity of 90. After re-suspension, replicates were centrifuged (3,000 RPM, 10 min, 4˚C) and immediately frozen at -80˚C until RNA extractions were completed in 7-10 days. Total RNA was isolated with a RNeasy Plant Mini Kit (Qiagen, Maryland) following manufacturer's instructions. The RNA was visualized by gel electrophoresis (1% agarose) to evaluate its integrity. A NanoDrop^{TM} ND-1000 spectrophotometer was used to measure the total RNA concentration (ng/µL). High-quality RNA (~ 500 ng) was prepared using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, California) to synthesize first-strand cDNA (complementary DNA), following manufacturer's instructions using an Oligo$(dT)_{20}$ primer.

The endogenous gene *ACTIN* was selected as the housekeeping gene for these experiments because it expresses at the same level regardless of physiological stress (He *et al.* 2007, Chen *et al.* 2011). Forward and reverse primer pairs for the two genes (*ACTIN* and *GPDH*) were initially designed following Chen *et al.* (2011 – for *ACTIN*; GenBank accession no. AF163669.2) and He *et al.* (2007) and Chen *et al.* (2011 – for *GPDH*; GenBank accession no. EU624407.1), and following criterion guidelines for primer design (Klatte and Bauer 2009, and methods therein). The primer sequences were confirmed using the online program Primer3 (Rozen and Skaletsky 1998), and then were slightly modified with MacVector (MacVector, Inc. V. 12.6 2013) and Amplify3 (Amplify3 V.3.1.4 2005) to ensure a single 153-bp product would be amplified (Table 3.3). Thermocycler conditions were as follows: an initial heating cycle of 95˚C for 30 sec, 30 cycles of 95˚C for 5 sec, 54˚C for 34 sec, 68˚C for 5 sec, and a final annealing stage of 68˚C for 7 min. A single 153-bp product

was confirmed by gel electrophoresis (1% agarose) and sequencing (GENEWIZ, Inc., South Plainfield, New Jersey).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was run on an Applied Biosystems StepOnePlus^{TM} real-time qPCR system (NCSU) following manufacturer's instructions. A master mix was prepared for each cDNA (control, 30 sec, 5 min, 30 min, 1 hr, 2 hr, 24 hr, and non-template control or NTC). For a 20-µL total volume, 10 µL of SYBR® Select Master Mix (Invitrogen), 6 µL of the primer pair, 3 µL DEPC-treated water, and 1 µL cDNA were prepared per well for a 96-well plate (n = 3)(Invitrogen). Primer pairs with each set of cDNA were run in triplicate. Relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001, Edwards *et al.* 2004) relative to the endogenous gene *ACTIN*.

3.3.g Mass Cultures

The mass culturing facility consisted of a series of growth tubes, each 1.21 m height x 30 cm diameter, made of lightweight 0.1-cm polymer fiberglass, providing ~90% transparency. Each tube held a maximum of ~175 L. Unless otherwise specified, cultures were grown under a 12:12 L:D photoperiod. The light source consisted of light banks (Standard Utilitech 48 ½ in fluorescent light, Lowes, Mooresville, North Carolina) that provided ~160 to 180 µE/m^2/sec for each tube. The temperature for these experiments was maintained at 23 ± 1^{o}C. Tubes were sampled daily between 0800 and 0900 hr for temperature, pH (Oakton Waterproof pH Testr 30 Pocket pH Tester, Oakton Instruments, Vernon Hills, Illinois), and cell counts (Section 2.1).

In preparation for mass culturing, tubes were filled with ~150 L deionized water adjusted to the desired salinity (30 or 60) (Hydrolab MiniSonde 4, Hach Company, Loveland, Colorado) and pH (8.3-8.4) with Coralife® salts (CL; Franklin, Wisconsin) + ~19.35 mL each of parts ProLine® A/B (F/2; Aquatic Eco-Systems, Apopka, Florida) nutrient mixtures (Guillard 1975). Stock culture was grown by inoculating ~3 L volume (salinity 30 or 60, adjusted with [CL], + L1-Si nutrients) with ~300 mL of culture (i.e. a 10% inoculum) under growth conditions described above (Section 2.1). After the stock culture reached late exponential growth (as determined by cell counts – see Section 2.1), it was inoculated to a freshly prepared mass culture tube (~3.5 L stock culture in ~150 L prepared medium). When this inoculum tube reached late exponential growth (typically within 7-10 days), ~15 L were dispensed into each freshly prepared mass culture tube that was filled with ~150 L culture medium as described above. Thus, the maximum volume for any given tube was ~165 L including culture. The total number of tubes prepared varied by experiment. *Dunaliella* cultures were grown to a density of 10^6 (rarely 10^7) cells/mL within 7-10 days depending on the strain and the experimental conditions.

3.3.h Statistics

Data were analyzed by one-way ANOVA with repeated measures (SAS v.9.2). Treatment effects were considered significant at $p \leq 0.05$ (one-sided). Type 3 Tests of Fixed Effects were completed to ensure that controls were not significantly different, and indicated that replicates were similar in all experiments.

3.4 Results

3.4.a FA composition

Total FAs were analyzed for constituent FAs for each of the seven strains tested (Table 3.4). In general, the FA composition of the strains was similar, regardless of their optimal salinity (30 or 60). However, *D. viridis* CONTAM contained a small amount of C 17:0 (0.42% of the total), which was not observed in any of the other six strains (Table 3.4). In addition, *D. pseudosalina* CCAP 19/18 lacked the FAs C18:2, cis9,12 and C18:3, cis6,9,12, which were present in the other strains. Higher proportions of C18:1, cis9 and C18:2, cis9,12 (~7.6 and 7.2%, respectively) were found in *D. viridis* CONTAM and *D. tertiolecta* CCAP 19/24 when compared to the remaining four *D. tertiolecta* strains CCAP 19/9, CCAP 19/26, UTEX 999, and UTEX 1000, which exhibited ~5.6 and 3.7% of these FAs, respectively (Table 3.4). *D. tertiolecta* CCAP 19/24 has an optimal salinity of 60, compared to the other *D. tertiolecta* strains tested that have an optimal salinity of 30. The four strains tested under short-term salinity stress (*D. tertiolecta* strains CCAP 19/9, CCAP 19/24, and UTEX 999, and *D. viridis* CONTAM) all exhibited similar proportions of FAs when compared to their FA composition under optimal salinity conditions (Table 3.4).

The four *D. tertiolecta* strains (CCAP 19/9, CCAP 19/26, UTEX 999, and UTEX 1000) all behaved similarly in response to long-term salinity stress with regard to % FA composition. In general, these three strains declined in C16:0 (~15 to 12%) when exposed to high salinities (salinity 30 to 120). Proportions of C18:1 cis9 also decreased in these strains (from 7 to 1.5%) when exposed to high salinities. The FA C16:3, cis increased from ~12 to 19% under the same high salinity conditions.

Strains *D. viridis* CONTAM and *D. tertiolecta* CCAP 19/24 increased in the FA C18:3, cis9,12,15 when exposed to salinities 60 to 150 (~25 to 30% and ~31 to 38%, respectively by strain). Both strains also exhibited an increase in proportions of the FA C18:2 cis9,12 under high salinity stress of 60 to 150, but *D. viridis* CONTAM yielded higher proportions (~11 to 13%) when compared to *D. tertiolecta* CCAP 19/24 (~3 to 5%).

3.4.b Long-term salinity stress in bench-scale experiments

All seven strains attained highest growth as cell production at the control salinity (30 or 60) that had been tested as optimal for each strain. Maximal population density among strains ranged from 2 to 8 x 10^6 cells/mL (Table 3.1, Fig. 3.2 A-C). Within this range, the strain with the smallest mean cell size, *D. viridis* CONTAM (biovolume 74 ± 5 μm^3), had the highest maximal density, whereas the strain with the largest cells, *D. pseudosalina* CCAP 19/18 (biovolume 215 ±10 μm^3), had the lowest maximal density (see Table 3.1 for strain biovolume and cell production). All strains except *D. pseudosalina* CCAP 19/18 were able to survive in salinities of 90 higher than the control salinity; the exception survived at a salinity of 105, only 45 higher than the control salinity of 60 for that strain. Sample weights for each strain remained constant over the course of the experiment (~ 0.1 g). DT and subsequent analysis was sensitive and accurate at these weights. Thus, while cell production varied among the strains, total biomass remained consistent.

Total FA content significantly increased under high salinity stress in comparison to the total FA content of controls for four of the five *D. tertiolecta* strains (Fig. 3.3), including CCAP 19/9 (Fig. 3.3 A), CCAP 19/26 (Fig. 3.3 B), UTEX 999 (Fig. 3.3 C), and UTEX 1000 (Fig. 3.3 D). In contrast, low salinity stress promoted an increase in total FAs for two strains,

D. tertiolecta CCAP 19/24 (Fig. 3.4) and *D. pseudosalina* CCAP 19/18 (Fig. 3.5). The seventh strain, the unique *D. viridis* CONTAM, had the highest total FA content at its optimum salinity of 60 (Fig. 3.6). For example, total FA production by *D. tertiolecta* CCAP 19/9 was significantly higher under increased salinity than at the optimum salinity for population growth, including salinities of 45 ($p = 0.016$), 75 ($p = 0.014$), and 105 ($p < 0.001$) (Table 3.5). Maximum total FA content (60.6% ± 7.76; percent total FAs ± 1 standard error [SE]) occurred at salinity 105, versus total FA content of only 19.6% ± 1.33 at the optimal salinity (30) for cell production ($p = 0.006$; Table 3.5, Fig. 3.3 A). In contrast, the total FA content of *D. viridis* CONTAM at all salinities was significantly lower than that of control cultures maintained at the optimal salinity for cell production (salinity 60; total FAs 58.2% ± 0.59; $p < 0.0001$ to 0.007; Table 3.5, Fig. 3.6).

D. tertiolecta CCAP 19/24 had significantly lower total FA content at salinity 90 than did control cultures for that strain at the optimum salinity (60) for population growth ($p = 0.007$; Table 3.5). It attained maximal total FA content (35.7% ± 0.86) under low salinity stress (salinity 45), whereas the controls had 30.2% ± 1.07 total FA content at salinity 60 (Table 3.5, Fig. 3.4). *D. tertiolecta* UTEX 999 had significantly lower total FA content at salinity 15 than at the control salinity (optimum for cell production, salinity 30; $p = 0.045$) or at higher salinities of 90, 105, and 120 ($p < 0.001$ to 0.009; Table 3.5). This strain produced comparable total FA content at salinities 30 and 75 (36.4% ± 0.99 – 41.6% ± 6.19) (Table 3.5, Fig. 3.3 C).

3.4.c Growth and long-term salinity stress in mass culture

D. tertiolecta strains UTEX 999 and 1000, and *D. viridis* CONTAM were grown separately in mass culture (~150 to 175 L). All three strains were assessed for population growth at the optimal salinity for cell production in bench-scale experiments (30, for the two *D. tertiolecta* strains; 60 for *D. viridis* CONTAM). *D. viridis* was also retested for its response to long-term, decreased salinity. Maximum cell density of *D. tertiolecta* UTEX 1000, ~2.5 x 10^6 cells/mL (n = 8), occurred on day 5, whereas high lipid content (based on visual observations under fluorescence microscopy) occurred on day 11 at ~1.0 x 10^6 cells/mL. The large cell size and relatively high cell yield of this strain in association with maximal lipid content made *D. tertiolecta* UTEX 1000 a promising candidate for biofuel production. In addition, the low optimum salinity for its growth (30, versus 60 for *D. viridis* CONTAM) would have minimized the cost of culture medium. After several months, however, this strain unfortunately lost its ability to produce high amounts of lipids, and when an additional culture of this strain was obtained, it grew slowly and had poor lipid production. Thus, this strain was not considered for further study.

The second strain tested in mass culture, *D. tertiolecta* UTEX 999, was unable to sustain rapid growth in mass culture and attained a maximum of only ~2.5 x 10^5 cells/mL at salinity 30, ~ten-fold less than its growth in bench-scale cultures under otherwise-similar conditions. The third strain, *D. viridis* CONTAM, grew comparably in bench-scale and mass cultures. At its optimal salinity of 60 for population growth, however, corrosion occurred on overhanging light fixtures and mass culture tubes had to be acid-stripped between experiments due to residue accumulation on the walls. In mass culture, this strain produced

two-fold more cells at salinity 60 than at salinity 30 (~4 x 10^6 cells/mL versus ~2 x 10^6 cells/mL, respectively), but still had relatively high cell production at the sub-optimal salinity. Thus, *D. viridis* CONTAM was successfully mass-cultured at the sub-optimal as well as the optimal salinity for population growth.

3.4.d Short-term salinity stress

All three *D. tertiolecta* strains tested in this experimental series (CCAP 19/9, CCAP 19/24, and UTEX 999) reached senescence and maximum cell production (~5.0 x 10^5 cells/mL) within 7 days of inoculation. The fourth strain assessed in the short-term trials, *D. viridis* CONTAM, attained maximal cell production (~1.0 to 1.5 x 10^6 cells/mL) more slowly, at 10 days. In general, total FAs increased within 5 min to 1 hr after cultures were exposed to higher salinity (15 or 30 above the optimal in controls; Figs. 3.7-3.10).

All three *D. tertiolecta* strains tested exhibited both immediate and short-term increases in total FA production in response to high salinity stress. At salinity 60, *D. tertiolecta* CCAP 19/9 significantly increased its total FAs within 1 hr (34.8% ± 1.30 total FAs ± 1 standard error [SE]; $p = 0.015$) in comparison to the control cultures at salinity 30 (22.9% ± 1.80; Table 3.6, Fig. 3.7). At a salinity of 45, this strain significantly increased its total FA content at 24 hr ($p = 0.036$). Total FA content was comparable to that of controls at all other time intervals.

D. tertiolecta CCAP 19/24 significantly increased in total FA content, relative to the control cultures maintained at salinity 60 at time points of 5 min (36.4% ± 1.69) and 30 min (33.3% ± 1.76) after exposure to salinity 75 ($p = 0.005$ to 0.034) (Table 3.6, Fig. 3.8). At salinity 90, this strain significantly increased in total FAs at 5 min, 30 min, and 24 hr ($p =$

0.012 to 0.050) in comparison to the control cultures (24.4% ± 0.1 to 25.9% ± 0.92; Table 3.6, Fig. 3.8). As with *D. tertiolecta* CCAP 19/9, there was an increase in total FAs at each time point although not significantly different from the controls.

The third *D. tertiolecta* strain, UTEX 999, increased in in total FA content at a salinity of 45 or 60 relative to the control cultures as an immediate response (30 sec to 5 min; Fig. 3.10). At salinity 45, its total FAs increased at 30 sec, 5 min and 1 hr ($p = 0.009$ to 0.032) relative to the control cultures (Table 3.6, Fig. 3.9). At 30 sec, 5 min, and 30 min after exposure to salinity of 60, *D. tertiolecta* UTEX 999 exhibited an increase in total FAs when compared to the controls ($p < 0.0001$ to 0.0444). This strain exhibited an increase in total FAs when compared to the controls at each time interval, albeit not at a statistically significant level. Highest total FAs were found at 30 sec after exposure to either a salinity of 45 or 60 (37.8% ± 2.74 and 35.8% ± 2.22, respectively, Fig. 3.9). There was a significant difference in total FAs at 1 hr between the salinity stress treatments (30 to 45 and 30 to 60; $p = 0.017$).

Finally, *D. viridis* CONTAM significantly increased total FA content 30 sec and 5 min after being exposed to salinity 75 ($p < 0.0001$ to 0.0066) and to salinity 90 ($p < 0.0001$ to 0.0014) when compared to salinity 60 (Fig. 3.10). There was a significant increase in total FAs at a salinity of 90 at 30 min and 24 hr after exposure ($p = 0.030$ to 0.041). At 30 sec after exposure there was a significant difference among treatments (60 to 75 and 60 to 90; $p = 0.007$). Total FAs increased at each time period regardless of salinity stress when compared to the controls (Table 3.6, Fig. 3.10).

3.4.e Total free glycerol

Although the total FA content of *D. viridis* CONTAM was comparable at the optimal salinity (60) versus higher salinities as above, the total glycerol content of pelleted *D. viridis* CONTAM increased significantly relative to that of control cultures in response to salinity 90 at 1 hr ($p = 0.03435$) and 2 hr ($p = 0.00665$). Maximum total free glycerol was measured at 24 hr after exposure to salinity 90 (17.8 mg/g ± 3.31, total free glycerol ± 1 SE; $p < 0.0001$) (Fig. 3.11 A). In the supernatant from the centrifuged *D. viridis* CONTAM cultures, total free glycerol significantly increased from 30 min up to 24 hr after exposure to salinity 90 ($p < 0.0001$ to 0.0264), with maximal glycerol produced at 24 hr (0.012 mg/mL ± 0.0006) (Fig. 3.11 B).

3.4.f Time course with real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR indicated that *ACTIN* was constitutively expressed by *D. viridis* CONTAM regardless of salinity stress. Stable expression confirmed that *ACTIN* acted as a housekeeping gene throughout the experiment. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of *GPDH* to the endogenous gene *ACTIN* and to the calibrator sample (Before cDNA). *GPDH* was significantly expressed, when compared to the baseline *ACTIN* expression, at 30 min ($p = 0.0107$), 1 hr ($p = 0.0102$), and 2 hr ($p = 0.0035$) after exposure to hyperosmotic stress. *GPDH* expression continued for 24 hr, but was not statistically significant at this time point (Fig. 3.12). Expression was evaluated as significant if the n-fold expression was ≥ 2. The corresponding melting curve confirmed a single peak in all dissociation curves, indicating that there was no non-specific amplification of products during the RT- qPCR procedure.

3.5 Discussion

Microalgae have high surface area-to-volume ratios, which facilitate rapid responses to changing environmental conditions (Becker 1994, Rodolfi *et al.* 2009), and they can quickly alter lipid metabolism in response to environmental stressors (Roessler 1990) such as salinity stress. This research built from the short-term and long-term experimental approach of Takagi *et al.* (2006), and examined *rapid*, *short-term*, and *long-term* responses (after Chen and Jiang 2009) of selected *Dunaliella* strains to environmental stressors. During the short-term experiments, a reversible physiological change was expected, which would have allowed the population to survive during the altered conditions. The length of the long-term salinity stress experiments (2 weeks) would have allowed epigenetic adaptation resulting in the synthesis of salinity-induced proteins and expression of salinity stress genes (Chen and Jiang 2009, Lakeman *et al.* 2009). Plants have developed a wide range of responses to environmental stressors, such as DNA methylation, chromatin remodeling, and small RNA-based mechanisms that are involved in regulating gene expression especially under abiotic stress (Sahu *et al.* 2013).

As we expected based on previous research with *Dunaliella* (Hanaa *et al.* 2004 – with *D. salina*, strain number unavailable, from the Botany Department of Texas University; Takagi *et al*. 2006 – with *D. tertiolecta* strain ATCC 30929 from the American Type Culture Collection), high salinity stress generally promoted FA production, but we also found very high intraspecific variation. *Dunaliella tertiolecta* strains CCAP 19/9, CCAP 19/26, and UTEX 999 increased in total FA content as percent dry weight when exposed to long-term hyperosmotic stress, albeit at different salinity levels (Fig. 3.3 A-C). In contrast, the percent

total FA content of *D. viridis* CONTAM *decreased* under high salinity stress, relative to a maximum of nearly 60% (dry weight basis) in the controls (optimal salinity, 60) (Fig. 3.6). And, *D. tertiolecta* CCAP 19/24 increased in total FA content under *low* salinity stress (Fig. 3.4).

In the experiments testing long-term response salinity stress, four of the seven strains increased total FA content under high salinity stress, whereas two strains showed an increase in total FAs under low salinity stress. *D. viridis* CONTAM had the highest amounts of total FAs at its optimal salinity of 60. This small-celled strain grew most rapidly, as expected, in comparison to the other strains tested in this study. It was also unique in two other features: Unlike any of the other 13 strains, it produced neutral lipids during active growth as well as during senescence, and it grew well under continuous light (see Chapter 4).

Previous research by Takagi *et al.* (2006) showed that total lipid content of *D. tertiolecta* ATCC 30929 increased to ~70% whether cultures were grown initially at higher salinity or sustained hyperosmotic stress at the middle or end of the logarithmic growth phase. Concomitantly, however, cell production significantly decreased in response to high salinity stress. Takagi *et al.* (2006) suggested that this strain of *D. tertiolecta* may be able to recover normal cell production if allowed to acclimate to higher salinity through *gradual* increase of NaCl concentrations. The present study supports that premise: These *Dunaliella* populations grew normally if allowed to acclimate, to a point. Six of the seven strains tested (exception, *D. pseudosalina* CCAP 19/18) were able to maintain similar growth patterns under increasing salinity, until the salinity was 90 higher than the control (optimum) salinity. That point marked the limit of tolerance to high salinity stress for the six strains and cell

production significantly declined. In addition, *D. pseudosalina* CCAP 19/18 lacked two FAs that were present in all other strains tested. These two FAs, C18:2, cis9,12 and C18:3, cis6,9,12, are unsaturated, meaning that they contain at least one double carbon-carbon bond. The degree of unsaturation is important in cells such as *Dunaliella* because they assist in adapting to salinity stress (Xu and Beardall 1997, Vanitha *et al.* 2007). The data suggest that, lacking these unsaturated FAs, *D. pseudosalina* CCAP 19/18 may not have been able to successfully acclimate to higher salinities.

In response to fluctuating salinities, *Dunaliella* generally osmoregulates by producing or releasing glycerol (in hyperosmotic or hypo-osmotic conditions, respectively) as a mechanism to return the cells to their original volume (Chitlaru and Pick 1991, Ginzburg *et al.* 1995, Ben-Amotz *et al.* 2009, Chen and Jiang 2009). Based on research with various *Dunaliella* strains, cell size and shape are known to decrease immediately after exposure to hyperosmotic stress (Avron and Ben-Amotz 1992, Ben-Amotz *et al* 2009, Chen and Jiang 2009). As shown in this study, total FA content significantly increased in tested strains within sec to hr after exposure to high salinity. These responses were strain-specific. However, lipid composition was similar throughout the short-term study. Like various other algae (Graham *et al.* 2010), *Dunaliella* lacks a polysaccharide cell wall; instead, the outer covering is a thin membrane (Avron and Ben-Amotz 1992, Ben-Amotz *et al.* 2009). The lack of a rigid cell wall facilitates rapid changes in volume and shape in response to changes in salinity (Chitlaru and Pick 1991, Ben-Amotz *et al.* 2009, Chen and Jiang 2009). Cells typically remain a constant volume after the immediate exposure, independent of external salinity (Avron 1986, Azachi *et al.* 2002). Our glycerol experiment narrowed the window of

time during which glycerol significantly increases in response to hyper-osmotic shock (sudden salinity increase from 60 to 90) for up to ~1 hr after exposure, earlier than proposed by Chen and Jiang (2009). If confronted by hyper-osmotic stress cells increase their total FA content as an immediate or short-term response. This increase may occur because the cells incorporate a proportion of the available glycerol as TAGs, thus explaining the low amounts of total free glycerol. As a long-term response (i.e. 24 hr), intracellular glycerol significantly increased (Fig. 3.11). In addition, the cells likely began dividing at or after 24 hr and the available glycerol would then be metabolized into other products such as starch, sugars, or pyruvate required for active growth. Future experiments would be needed to assess when cell division occurs after cells are exposed to salinity stress.

Glycerol and glycerol biosynthesis genes have been extensively studied in *Dunaliella* because of their importance in osmoregulation. The amount excreted or produced under hypo-osmotic and hyperosmotic conditions, respectively, depends mostly on the concentration of NaCl in the surrounding medium (Borowitzka and Brown 1974, Chitlaru and Pick 1991, Ginzburg *et al.* 1995, Ben-Amotz *et al.* 2009), and can be as high as > 6 M in media with salinity greater than 230 (Oren 2005). In agreement with other studies on *Dunaliella*, glycerol began increasing within 30 min of exposure to hyperosmotic stress (Ben-Amotz 1973, Belmans and Van Laere 1987, Goyal 2007) and continued to accumulate up to 24 hr. A steady-state condition was not observed here; similarly, work by other researchers has shown that glycerol may continue to be produced for 1 to 1.5 hr when the cells attain an internal glycerol concentration similar to that of fully adapted cells (Ben-Amotz and Avron 1973, Belmans and Van Laere 1987, Goyal 2007). In this study at 24 hr

after exposure to high salinity stress, the internal glycerol concentration had increased to double that of control populations. Similar findings were reported by He *et al.* (2007).

Many previous studies investigating glycerol accumulation have examined only one time point at the end of the experiment. Our time course experiment was similar to that of Belmans and Van Laere (1987); both studies analyzed intracellular glycerol accumulation within sec to min up to 2 hr after hyperosmotic shock, but our study additionally extended to 24 hr. Both Belmans and Van Laere (1987) and our work detected an increase in glycerol immediately after hyperosmotic shock, and a steady-state condition at 2 hr. We additionally were able to establish that glycerol continued to accumulate after 24 hr before the cells fully adapted, well beyond the apparently temporary steady-state condition detected at 2 hr. Had sampling ended at 2 hr, the accumulation of glycerol mistakenly would have appeared to reach steady-state, when in reality the population required considerably longer to fully adapt to hyperosmotic stress.

In the pathway of glycerol biosynthesis, *GPDH* is an important enzyme converting dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G-3-P), which is then converted to the end product glycerol by the enzyme glycerol-3-phosphate isomerase (*GPI*) (Haus and Wegmann 1984, Chen and Jiang 2009). This enzyme is also referred to DHAP reductase because at physiological conditions (i.e. pH and substrate) it is inactive as a dehydrogenase (Gee *et al.* 1998). The RT-qPCR data from this research showed that the relative expression of *GPDH* increased 30 min, 1 hr, and 2 hr after high salinity stress. These data supported the findings for total free glycerol (Fig. 3.12). At 24 hr, maximal total glycerol was found; however, this timing did not correlate with a significant expression of

GPDH. It is likely that because glycerol was produced in large quantities, *GPDH* was no longer up-regulated.

This gene expression experiment was only completed for the strain *D. viridis* CONTAM. In further research, gene expression could be investigated in other strains to assess whether *GPDH* expression correlates with glycerol production and/or total FA production. Other researchers have conducted similar measurements on *Dunaliella* (identified by the culture collection as *D. salina* UTEX 200) after 2 hr of high salinity stress (e.g. Chen *et al.* 2011). Our study with *D. viridis* CONTAM used shorter time periods (sec to min) in order to capture more rapid changes, and showed that *GPDH* begins to be expressed (at activities at least 2-fold higher than unstressed control populations) within 30 min after exposure to high salinity stress, continuing to 2 hr. In general, when total free glycerol was low, the four strains that we tested increased in total FAs, but there were considerable differences among the strains in their response. Our study builds from Belmans and Van Laere (1987), who showed increased activity of *GPDH* due to the cellular increase of G-3-P content during the period of rapid glycerol synthesis. Triose phosphates and fructose-1,6-bisphosphate remained constant during the same period, suggesting *GPDH* activity (Belmans and Van Laere 1987). Our RT-qPCR data provide evidence supporting the premise that *GPDH* is up-regulated in response to hyperosmotic stress. As indicated above, glycerol is involved in many metabolic pathways. Future work should include quantification of total starch or sugars to further validate the glycerol results.

3.6 Conclusion

Among 14 strains within four *Dunaliella* species that were screened for cell production and lipid content, seven strains were selected for tests of enhanced lipid production under both low and high salinity stress in long-term (≥ 24 hr) experiments, and four of these strains additionally were tested for their short-term response (sec, min, to less than 24 hr). Under long-term high salinity stress, *Dunaliella tertiolecta* CCAP 19/9 produced the highest proportion of total FAs per cell mass (up to 65% by dry weight) relative to controls (~10-25% total FAs). In the short-term tests, FA production significantly increased within sec to hr *of exposure to high salinity stress. The most promising strain, *D. viridis* CONTAM, was shown to produce glycerol within 30 min to 24 hr. Total free glycerol was low inside the cell when total FAs were increasing as an immediate or short-term response to hyperosmotic stress, suggesting that the cells are incorporating a portion of the free glycerol as TAGs. *D. viridis* CONTAM was successfully mass cultured (~150 to 175 L) with high cell production in salinities of 60 or 30.

3.7 References

Avron, M., 1986. The osmotic components of halotolerant algae. Trends Biochem. Sci. 11, 5-6.

Avron, M., Ben-Amotz, A. (Eds.), 1992. *Dunaliella*: Physiology, Biochemistry, and Biotechnology. CRC Press, Boca Raton (Florida). 256 pp.

Azachi, M., Sadka, A., Fisher, M., Goldshlag, P., Gokhman, I., Zamir, A., 2002. Salt induction of fatty acid elongase and membrane lipid modifications in the extreme halotolerant alga *Dunaliella salina*. Plant Physiol. 129, 1320-1329.

Becker, E.W., 1994. Microalgae: Biotechnology and Microbiology. Cambridge University Press, Cambridge (New York). 293 pp.

Belmans, D., Van Laere, A., 1987. Glycerol cycle enzymes and intermediates during adaptation of *Dunaliella tertiolecta* cells to hyperosmotic stress. Plant Cell Environ. 10, 185-190.

Ben-Amotz, A., Avron, M., 1973. The role of glycerol in the osmotic regulation of the halophilic alga *Dunaliella parva*. Plant Physiol. 51, 875-878.

Ben-Amotz, A., Polle, J.E.W., Rao, D.V.S. (Eds.), 2009. The Alga *Dunaliella*: Biodiversity, Physiology, Genomics and Biotechnology. Science Publishers, Enfield (New Hampshire). 555 pp.

Borowitzka, L.J., Brown, A.D., 1974. The salt relations of marine and halophilic species of the unicellular green alga, *Dunaliella*. Arch. Microbiol. 96, 37-52.

Chen, H., Jiang, J.G., 2009. Osmotic responses of *Dunaliella* to the changes of salinity. J. Cell Physiol. 219, 251-258.

Chen, H., Lao, Y.-M., Jiang, J.-G., 2011. Effects of salinities on the gene expression of a (NAD^+)-dependent glycerol-3-phosphate dehydrogenase in *Dunaliella salina*. Sci. Total Environ. 409, 1291-1297.

Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.Y.S., Salley, S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour. Technol. 102, 1649-1655.

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126-131.

Chitlaru, E., Pick, U., 1991. Regulation of glycerol synthesis in response to osmotic changes in *Dunaliella*. Plant Physiol. 96, 50-60.

Edwards, K., Logan, J., Saunders, N. (Eds.), 2004. Real-Time PCR: An Essential Guide. Horizon Bioscience, Norfolk (United Kingdom). 300 pp.

Føyn, B. 1934. Lebenszyklus, Cytologie und Sexualiät der Chlorophycee *Cladophora suhriana* Küzing. Arch. Protistenk. 83, 1-56.

Gardner, R.D., Cooksey, K.E., Mus, F., Macur, R., Moll, K., Eustance, E., Carlson, R.P., Gerlach, R., Fields, M.W., Peyton, B.M., 2012. Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte *Scenedesmus* sp. and the diatom *Phaeodactylum tricornutum*. J. Appl. Phycol. 24, 1311-1320.

Gee, R., Goyal, A., Gerber, D., Byerrum, R.U., Tolbert, N.E., 1988. Isolation of dihydroxyacetone phosphate reductase from *Dunaliella* chloroplasts and comparison with isozymes from spinach leaves. Plant Physiol. 88, 896-903.

Ginzburg, M., 1987. *Dunaliella*: a green alga adapted to salt. Adv. Bot. Res. 14, 93-183.

Ginzburg, B.Z., Ginzburg, M., Cohen, M., 1995. A salt-sensitive *Dunaliella* mutant I. Growth and osmoregulatory responses to increases in salt concentration. J. Exp. Bot. 46, 1505-1513.

Goyal, A., 2007. Osmoregulation in *Dunaliella*, part II: photosynthesis and starch contribute carbon for glycerol synthesis during a salt stress in *Dunaliella tertiolecta.* Plant Physiol. Biochem. 45, 705-710.

Graham, L.E., Graham, J.M., Wilcox, L.W., 2009. Algae. 2nd Edition. Pearson Education, Inc., San Francisco (California). 616 pp.

Griffiths, M.J., van Hille, R.P., Harrison, S.T.L., 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 45, 1053-1060.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York (New York). pp. 29-60.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234-236.

Guillard, R.R.L., 2005. Purification Methods for Microalgae. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts), pp. 117-132.

Hanaa, H., El-Baky, A., El-Baz, F.K., El-Baroty, G.S., 2004. Production of lipids rich in omega 3 fatty acids from the halotolerant alga *Dunaliella salina*. Biotechnology 3, 102-108.

Haus, M., Wegmann, K., 1984. Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) from *Dunaliella tertiolecta.* I. Purification and kinetic properties. Physiol. Plant. 60, 283-288.

He, Q., Qiao, D., Bai, L., Zhang, Q., Yang, W., Li, Q., Cao, Y., 2007. Cloning and characterization of a plastidic glycerol 3-phosphate dehydrogenase cDNA from *Dunaliella salina*. J. Plant Physiol. 164, 214-220.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621-639.

Kawachi, M., Noël, M.-H., 2007. Sterilization and Sterile Technique. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts). pp. 65-81.

Klatte, M., Bauer, P., 2009. Accurate real-time reverse transcription quantitative PCR. Plant Sig. Transduct. 479, 61-77.

Lakeman, M.B., von Dassow, P., Cattolico, R.A. 2009. The strain concept in phytoplankton ecology. Harmful Algae. 8, 746-758.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402-408.

Lorenz, M., Friedl, T., Day, J.G., 2005. Perpetual Maintenance of Actively Metabolizing Microalgal Cultures. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts). pp. 145-156.

Miner, C.S., Dalton, N.N. (Eds.), 1953. Glycerol. Reinhold Publishing Corporation, New York. 460 pp.

Oren, A., 2005. A hundred years of *Dunaliella* research: 1905-2005. Aquat. Biosyst. 1, 1-14.

Patee, H. E., Isleib, T.G., Giesbrecht, F.G, McFeeters, R.F., 2000. Investigations into genotypic variations of peanut carbohydrates. J. Agric. Food Chem. 48, 750-756.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100-112.

Roessler, P.G., 1990. Environmental control of glycolipid metabolism in microalgae: commercial implications and future research directions. J. Phycol. 26, 393-399.

Rozen, S. Skaletsky, H.J., 1998. *Primer3*. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

Sahu, P.P., Pandey, G., Sharma, N., Puranik, S., Muthamilarasan, M., Prasad, M. 2013. Epigenetic mechanisms of plant stress responses and adaptation. Plant Cell. Rep. 32, 1151-1159.

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B., 2008. Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioeng. Res. 1, 20-43.

Shariati, M., Hadi, M.R. 2011. Microalgal biotechnology and bioenergy in *Dunaliella*. Prog. Molec. Environ. Bioeng. 22, 483-506.

Sparkman, O.D., Penton, Z.E., Kitson, F.G., 2011. Gas Chromatography and Mass Spectrometry: A Practical Guide, 2nd Edn. Elsevier Inc., Burlington (Massachusetts). 611 pp.

Taiz, L., Zeiger, E., 2006. Plant Physiology. 4th Edition. Sinauer Associates, Inc., Sunderland (Massachusetts). 764 pp.

Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J. Biosci. Bioeng. 101, 223-226.

Tompkins, J., DeVille, M.M., Day, J.G., Turner, M.F., 1995. Culture Collection of Algae and Protozoa. Catalog of Strains. Ambleside (United Kingdom). 204 pp.

Vanitha, A., Narayan, S., Murthy, K.N.C., Ravishankar, G.A. 2007. Comparative study of lipid composition of two halotolerant alga, *Dunaliella bardawil* and *Dunaliella salina*. Int. J. Food Sci. Nutr. 58: 373-382.

Vollenweider, R.A., Talling, J.F., Westlake, D.F. 1974. A Manual on Methods for Measuring Primary Production in Aquatic Environments, 2nd Edition. Blackwell Scientific, Oxford. 225 pp.

Wetzel, R.G., Likens, G.E., 2001. Limnological Analyses, 2nd Edition. W.B. Saunders, New York (New York). 429 pp.

Xu, X.-Q., Beardall, J., 1997. Effect of salinity on fatty acid composition of a green microalga from an Antarctic hypersaline lake. Phytochemistry 45, 655-658.

Table 3.1 – Species, strain source and identification number, and characteristics (mean biovolume, maximum population growth as cell production, source culture medium, optimum salinity in L1-Si medium) for the 14 strains of *Dunaliella* examined in this study, also indicating the strains that were used in the long-term and/or short-term salinity stress experiments.

Species Name	Commercial Source and Strain Number	Biovolume (μm^3; means ± 1 SE; n = 30 cells)	Maximum Cell Production (100 mL volume; 10^6 cells/mL; means ± 1 SE (n = 3)	Source Culture Medium	Optimal Salinity (L1-Si)	Use in Salinity Stress Experiment
D. pseudosalina	CCAP 19/18	215 ± 10	1.9 ± 0.2	2ASW	60	Long-term
D. pseudosalina	UTEX 1983	240 ± 11	3.4 ± 0.1	Erdschreiber's	30	N/A
D. pseudosalina	UTEX 200	183 ± 8	3.3 ± 0.4	2X Erdschreiber's	60	N/A
D. salina	ATCC 30861	646 ± 218	0.3 ± 0.04	L1-Si	60	N/A
D. tertiolecta	CCAP 19/9	120 ± 6	6.2 ± 0.9	f/2-Si	30	Long-term, short-term
D. tertiolecta	CCAP 19/24	132 ± 6	3.4 ± 0.4	f/2-Si	60	Long-term, short-term
D. tertiolecta	CCAP 19/26	120 ± 5	3.2 ± 0.3	f/2-Si	30	Long-term
D. tertiolecta	CCMP 364	135 ± 8	3.6 ± 0.3	L1-Si	30	N/A
D. tertiolecta	CCMP 1320	136 ± 6	3.9 ± 0.4	L1-Si	30	N/A
D. tertiolecta	UTEX 999	163 ± 9	4.4 ± 0.8	ASW	30	Long-term, short-term
D. tertiolecta	UTEX 1000	168 ± 8	6.6 ± 0.8	Erdschreiber's	30	Long-term
D. viridis	CCAP 19/10	46 ± 2	1.9 ± 0.1	2ASW	60	N/A
D. viridis	CCAP 19/3	183 ± 8	3.3 ± 0.4	2ASW	60	N/A
D. viridis	CONTAM	74 ± 5	10.0 ± 0.6	2X Erdschreiber's	60	Long-term, short-term

Table 3.2 – Comparison of the maximum cell production (mean cell number x 10^6/mL) for each *Dunaliella* strain in its source medium (commercial culture facility) versus in L1-Si medium. For both media, we used a volume of 50 mL that initially contained 10^6 cells/mL.

Clade Determined from ITS Sequence Data (Chapter 2)[3]	Species and Strain Number Given by Commercial Source	Source Medium (n = 1)[4]	Li-Si (means ± 1 SE; n = 3)
Dunaliella pseudosalina	(*D. salina* CCAP 19/18)	1.6	1.9 ± 0.2
Dunaliella pseudosalina	(*D. salina* UTEX 200)	1.1	3.3 ± 0.4
Dunaliella pseudosalina	(*D. parva* UTEX 1983)	0.2	3.4 ± 0.1
Dunaliella salina	(*D. bardawil* ATCC 30861)	0.1	0.3 ± 0.04
Dunaliella tertiolecta	(*D. parva* CCAP 19/9)	0.1	6.2 ± 0.9
Dunaliella tertiolecta	(*D. tertiolecta* CCAP 19/24)	1.0	3.4 ± 0.4
Dunaliella tertiolecta	(*D. parva* CCAP 19/26)	0.2	3.2 ± 0.3
Dunaliella tertiolecta	(*D. tertiolecta* CCMP 364)	3.8	3.6 ± 0.3
Dunaliella tertiolecta	(*D. tertiolecta* CCMP 1320)	2.5	3.9 ± 0.4
Dunaliella tertiolecta	(*D. tertiolecta* UTEX 999)	0.9	4.4 ± 0.8
Dunaliella tertiolecta	(*D. primolecta* UTEX 1000)	3.7	6.6 ± 0.8
Dunaliella viridis	(*D. salina* CCAP 19/3)	6.9	7.7 ± 0.8
Dunaliella viridis	(*D. parva* CCAP 19/10)	0.3	1.9 ± 0.1
Dunaliella viridis	(contaminant of *D. salina* UTEX 1644)	5.2	10.0 ± 0.6

[3]ITS2 sequence data for each of the 14 strains indicate that the strains fall into four distinct species (clades) as indicated, and that many of the strains were incorrectly designated by the commercial culture facilities (Chapter 2). Note that strain UTEX 1644, sent to us ~5 yr ago, evidently was a culture contaminant in the strain received. It is no longer available from the culture collection under that strain number, but we have deposited it at the NCMA (see text).

[4]Experiment not replicated because preliminary trials showed that higher growth was obtained in L1-Si.

Table 3.3 – Primer pairs for each gene used in real-time PCR from 5’ to 3’.

Primer	Sequence (5’ to 3’)
ACTIN – Forward	TAGCTGTTTGCGTGTGTGTGCT
ACTIN – Reverse	CATCCTGCATTCCTTCCATT
GPDH – Forward	GGCAATGGCATGTGTAGTTG
GPDH - Reverse	TACAGGTCTCCCTGCTCTCG

Table 3.4 – Breakdown of FA composition as percent (%) of total FAs for strains grown at optimal salinity 30 (*D. tertiolecta* strains CCAP 19/9, CCAP 19/26, UTEX 999, and UTEX 1000) compared to strains grown at optimal salinity 60 (*D. pseudosalina* CCAP 19/18, *D. tertiolecta* CCAP 19/24, and *D. viridis* CONTAM). *D. viridis* UTEX CONTAM exhibited a small amount of C 17:0, not observed in the other strains tested. *D. pseudosalina* CCAP 19/18 lacked certain FAs as indicated (N/A). Data are presented as mean % total FAs ± 1 standard error [SE].

	CCAP 19/9	CCAP 19/26	UTEX 999	UTEX 1000	CCAP 19/18	CCAP 19/24	UTEX CONTAM
C16:0	17.1 ± 1.30	18.19 ± 0.73	15.66 ± 0.52	16.06 ± 0.98	16.51 ± 0.33	14.65 ± 0.94	13.48 ± 0.18
C 16:1, cis9	5.30 ± 0.11	5.03 ± 0.22	4.66 ± 0.04	4.68 ± 0.19	5.79 ± 0.20	4.91 ± 0.01	4.74 ± 0.01
C 16:3, cis	14.03 ± 0.11	12.85 ± 0.61	12.15 ± 0.11	13.04 ± 0.72	13.62 ± 0.32	13.63 ± 0.18	11.84 ± 0.47
C 17:0	N/A	N/A	N/A	N/A	N/A	N/A	0.42 ± 0.42
C 18:1, cis9	5.58 ± 0.71	8.70 ± 1.63	8.85 ± 0.64	5.48 ± 1.55	N/A	5.32 ± 1.37	10.03 ± 0.63
C18:2, cis9,12	3.84 ± 0.09	5.40 ± 0.87	5.05 ± 0.44	4.11 ± 0.40	6.62 ± 0.25	3.58 ± 0.45	11.45 ± 0.26
C 18:3, cis6,9,12	3.74 ± 0.07	3.60 ± 0.12	3.72 ± 0.02	3.21 ± 0.24	N/A	3.59 ± 0.11	2.47 ± 0.16
C 18:3, cis9,12,15	30.72 ± 0.28	22.25 ± 7.07	27.24 ±0.46	29.82 ± 1.16	26.99 ± 0.37	30.57 ± 0.74	25.32 ± 0.74
Unknowns	13.44 ± 0.15	9.37 ± 1.25	11.63 ± 0.83	15.01 ± 1.27	21.37 ± 1.40	14.85 ± 1.66	12.46 ± 0.59

Table 3.5 – Total percent FAs for each of the seven strains under long-term salinity stress, with corresponding p-values if statistically significant from percent total FA content produced at the control (optimal) salinity. LS Mean Diff = the least squared means difference from the control salinity (30 or 60); NS = not significant.

Strain	Final Salinity	Mean FA	± 1 SE	LS Mean Diff	P-value
D. tertiolecta CCAP 19/9	15	23.01	1.57	3.39	NS
	30	19.61	2.67	N/A	N/A
	45	35.88	4.32	16.27	0.016
	60	23.25	3.57	3.64	NS
	75	36.44	2.62	16.83	0.014
	90	23.56	4.07	3.95	NS
	105	60.64	15.53	41.03	<0.001
	120	27.40	5.70	7.79	NS
D. tertiolecta CCAP 19/24	45	35.72	1.71	5.53	NS
	60	30.19	2.14	N/A	N/A
	75	28.75	10.92	-1.45	NS
	90	15.87	2.12	-14.3	0.007
	105	22.08	3.05	-8.11	NS
	120	32.36	3.84	2.17	NS
	135	26.07	0.95	-4.13	NS
	150	22.90	7.08	-7.29	NS
D. tertiolecta CCAP 19/26	15	30.29	9.22	0.61	NS
	30	29.69	6.61	N/A	N/A
	45	23.44	1.97	-6.25	NS
	60	33.38	7.62	3.69	NS
	75	32.99	5.10	3.30	NS
	90	31.47	5.88	1.78	NS
	105	29.87	1.67	0.19	NS
	120	28.02	4.66	-1.67	NS

Table 3.5 Continued

Strain	Final Salinity	Mean FA	± SE	LS Mean Diff	P-value
D. tertiolecta UTEX 999	15	26.30	5.41	-10.1	0.045
	30	36.38	1.98	N/A	N/A
	45	35.73	1.45	-0.65	NS
	60	28.33	4.95	-8.05	NS
	75	41.66	12.38	5.28	NS
	90	21.56	5.58	-14.8	0.009
	105	10.71	0.68	-25.7	<0.001
	120	20.78	1.95	-7.29	0.007
D. tertiolecta UTEX 1000	15	31.69	8.15	-2.08	NS
	30	33.77	1.78	N/A	N/A
	45	35.26	5.15	1.49	NS
	60	31.97	1.42	-1.79	NS
	75	39.08	9.84	5.31	NS
	90	32.63	5.87	-1.13	NS
	105	18.29	2.87	-15.5	0.006
	120	26.06	2.24	-7.71	NS
D. pseudosalina CCAP 19/18	45	21.53	7.51	5.10	NS
	60	16.43	1.82	N/A	N/A
	75	20.19	2.14	3.75	NS
	90	6.07	2.21	-10.4	0.015
	105	12.64	3.77	-3.80	NS
D. viridis CONTAM	45	40.34	7.79	-17.8	<0.001
	60	58.18	1.18	N/A	N/A
	75	44.18	4.24	-14.0	0.003
	90	17.69	4.23	-40.5	<0.001
	105	31.27	5.82	-26.9	<0.001
	120	36.85	3.33	-21.3	<0.001
	135	22.47	2.18	-35.7	<0.001
	150	18.45	1.07	-39.7	<0.001
	120	20.78	1.95	-15.6	0.007

Table 3.6 – Total percent FAs for each of the four strains under short-term salinity stress, with corresponding p-values if statistically significant from percent total FA content produced at the control (optimal) salinity. LS Mean Diff = the least squared means difference from the control salinity (30 or 60); NS = not significant.

Strain	Salinity	Time Point	FA Mean	± 1 SE	LS Mean Diff	P-value
D. tertiolecta CCAP 19/9	Control	30 sec	27.42	0.64	N/A	N/A
		5 min	25.23	6.34	N/A	N/A
		30 min	28.27	0.58	N/A	N/A
		1 hr	22.89	1.80	N/A	N/A
		2 hr	22.29	1.66	N/A	N/A
		24 hr	19.14	1.22	N/A	N/A
	30 to 45	30 sec	32.83	3.55	5.41	NS
		5 min	26.20	2.94	0.97	NS
		30 min	27.79	3.51	-0.48	NS
		1 hr	25.75	2.36	2.86	NS
		2 hr	25.40	2.87	3.20	NS
		24 hr	31.69	2.18	12.82	0.0355
	30 to 60	Before	29.06	1.61	N/A	N/A
		30 sec	35.94	1.74	8.52	NS
		5 min	37.83	1.13	12.6	NS
		30 min	31.95	2.42	3.68	NS
		1 hr	34.87	1.12	11.98	0.0145
		2 hr	30.54	1.37	8.31	NS
		24 hr	26.55	3.14	7.68	NS
D. tertiolecta CCAP 19/24	Control	30 sec	26.18	0.92	N/A	N/A
		5 min	24.41	0.10	N/A	N/A
		30 min	25.87	0.92	N/A	N/A
		1 hr	24.52	1.80	N/A	N/A
		2 hr	26.20	2.40	N/A	N/A
		24 hr	24.15	1.04	N/A	N/A
	60 to 75	30 sec	32.96	2.54	6.78	NS
		5 min	36.36	1.46	11.95	0.005
		30 min	33.31	1.53	7.44	0.034
		1 hr	34.34	2.28	9.82	NS
		2 hr	31.61	0.97	5.41	NS
		24 hr	26.98	0.54	2.83	NS
	60 to 90	30 sec	33.05	0.68	6.87	NS
		5 min	31.02	1.03	6.62	0.050
		30 min	35.52	0.67	9.65	0.012
		1 hr	32.82	2.04	8.30	NS
		2 hr	32.53	1.18	6.33	NS
		24 hr	28.98	0.98	4.83	0.043

Table 3.6 Continued

D. tertiolecta UTEX 999	Control	30 sec	23.95	4.31	N/A	N/A
		5 min	21.66	1.27	N/A	N/A
		30 min	25.63	3.64	N/A	N/A
		1 hr	21.80	1.47	N/A	N/A
		2 hr	25.50	4.38	N/A	N/A
		24 hr	25.43	0.78	N/A	N/A
	30 to 45	30 sec	37.84	2.38	13.89	0.0261
		5 min	33.68	2.67	12.02	0.0324
		30 min	35.86	1.20	10.23	NS
		1 hr	37.23	1.56	14.71	0.0087
		2 hr	33.61	2.80	8.07	NS
		24 hr	28.35	1.57	2.92	NS
	30 to 60	30 sec	24.98	1.92	11.80	0.0444
		5 min	37.84	2.18	10.89	0.0444
		30 min	33.68	2.82	15.30	<0.0001
		1 hr	35.86	1.62	5.33	NS
		2 hr	37.23	0.67	3.67	NS
		24 hr	33.61	1.20	2.87	NS
D. viridis CONTAM	Control	30 sec	18.93	2.38	N/A	N/A
		5 min	21.36	0.29	N/A	N/A
		30 min	23.60	4.41	N/A	N/A
		1 hr	23.69	5.13	N/A	N/A
		2 hr	24.59	4.34	N/A	N/A
		24 hr	21.45	2.26	N/A	N/A
	60 to 75	30 sec	34.20	1.31	15.27	<0.0001
		5 min	31.96	0.67	10.60	0.0066
		30 min	30.98	1.94	7.38	NS
		1 hr	30.95	1.77	7.26	NS
		2 hr	31.45	0.58	6.86	NS
		24 hr	25.50	4.32	2.92	NS
	60 to 90	30 sec	30.89	0.40	21.95	<0.0001
		5 min	40.88	1.96	15.26	0.0014
		30 min	35.54	2.00	11.15	0.030
		1 hr	34.75	1.75	8.95	NS
		2 hr	32.64	1.63	7.85	NS
		24 hr	32.44	2.80	12.70	0.0408

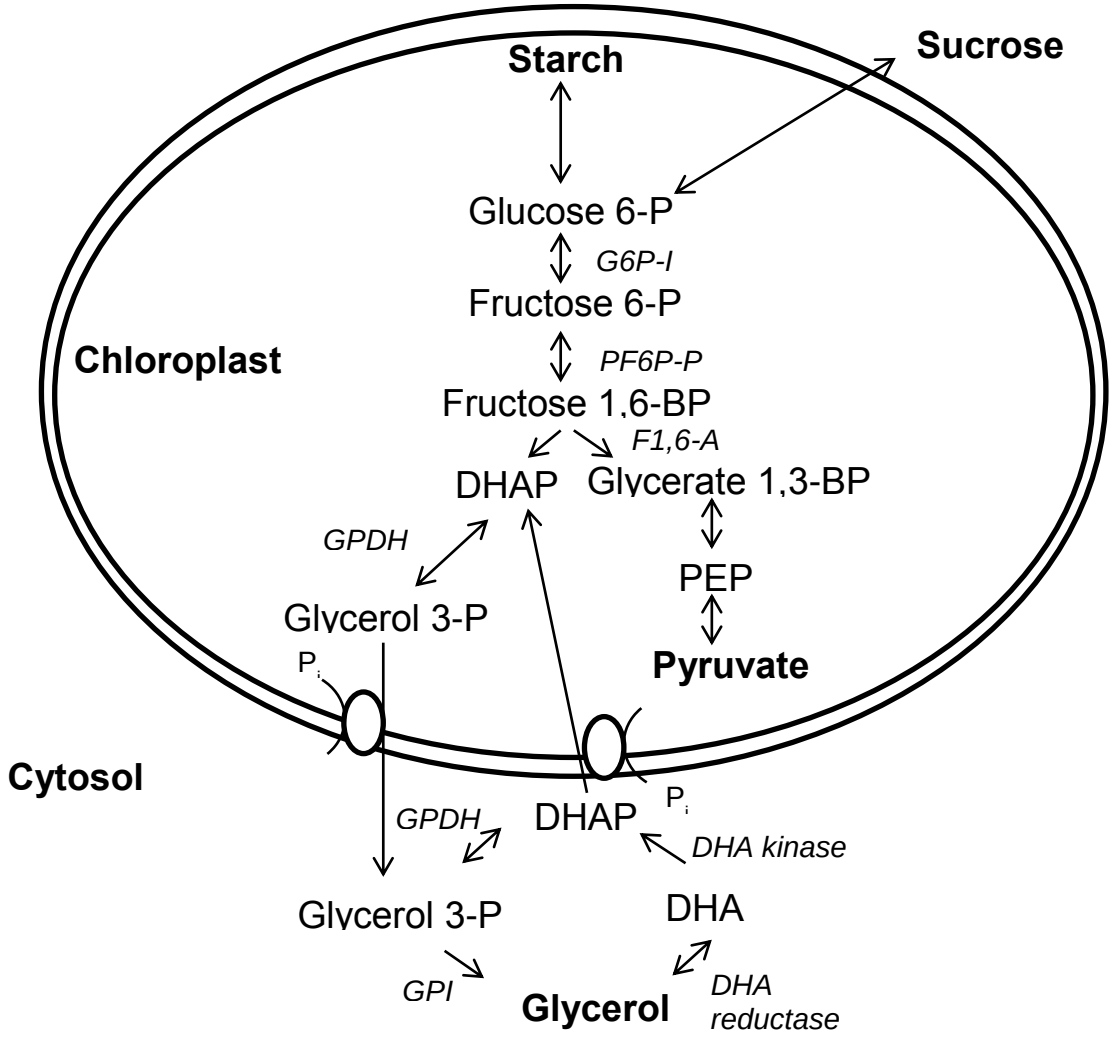

Figure 3.1 – Simplified schematic of glycerol metabolism in *Dunaliella*. Reversible reactions are indicated by the double arrow and important enzymes are indicated by italicized abbreviations (*G6P-I*, glucose-6-phosphate isomerase; *PF6P-P*, pyrophosphate fructose-6-phosphate phosphotransferase; *F1,6-A*, fructose 1,6-bisphosphate aldolase; *GPDH*, glycerol phosphate dehydrogenase; *GPI*, glycerol phosphate isomerase; and the intermediate products of *DHA*, dihydroxyacetone; *DHAP*, dihydroxyacetone phosphate; *PEP*, phosphoenolpyruvate).

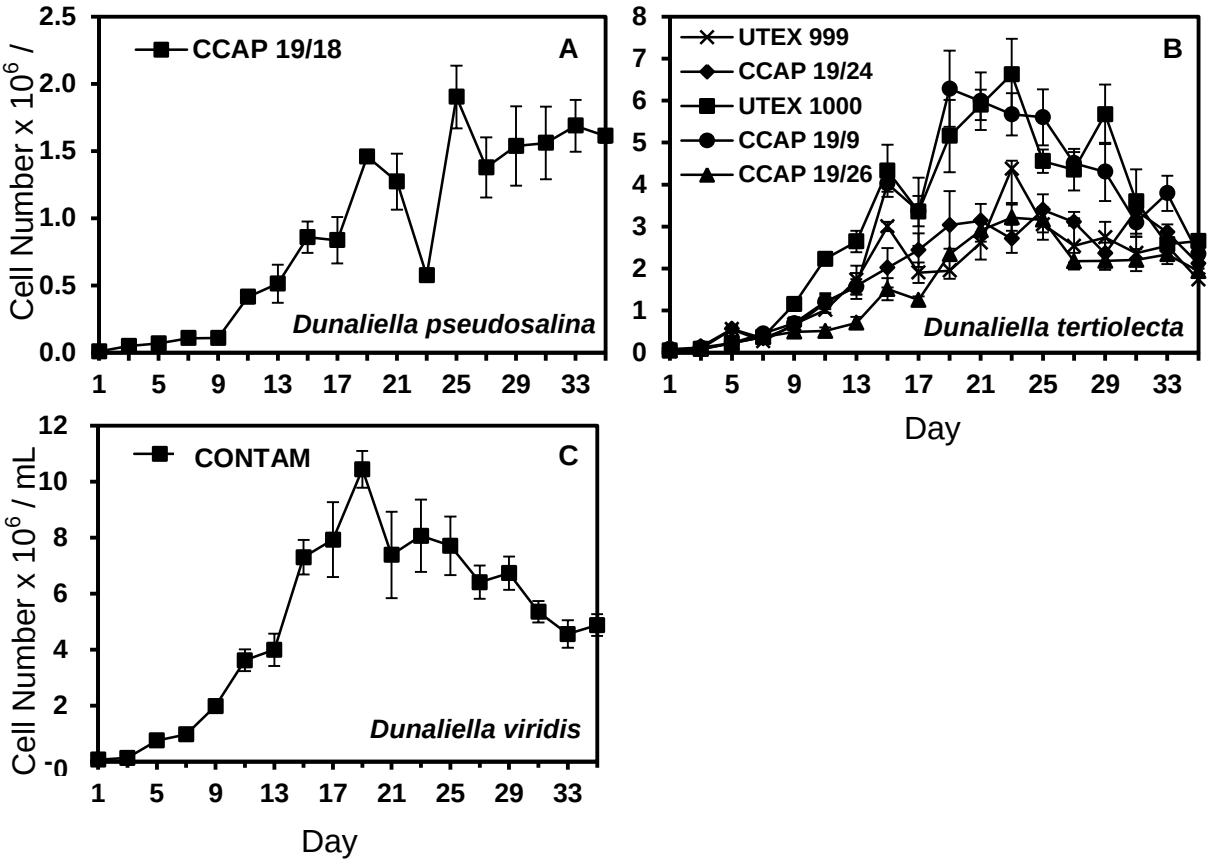

Figure 3.2 – Population growth curves as cell production for the 7 strains of *Dunaliella* selected for long-term salinity stress (see text for culture conditions) including (A) *D. pseudosalina* CCAP 19/18, (B) *D. tertiolecta* strains CCAP 19/9, CCAP 19/24, CCAP 19/26, UTEX 999, UTEX 1000; and (C) *D. viridis* CONTAM. Data are given as means ± 1 SE (n = 3).

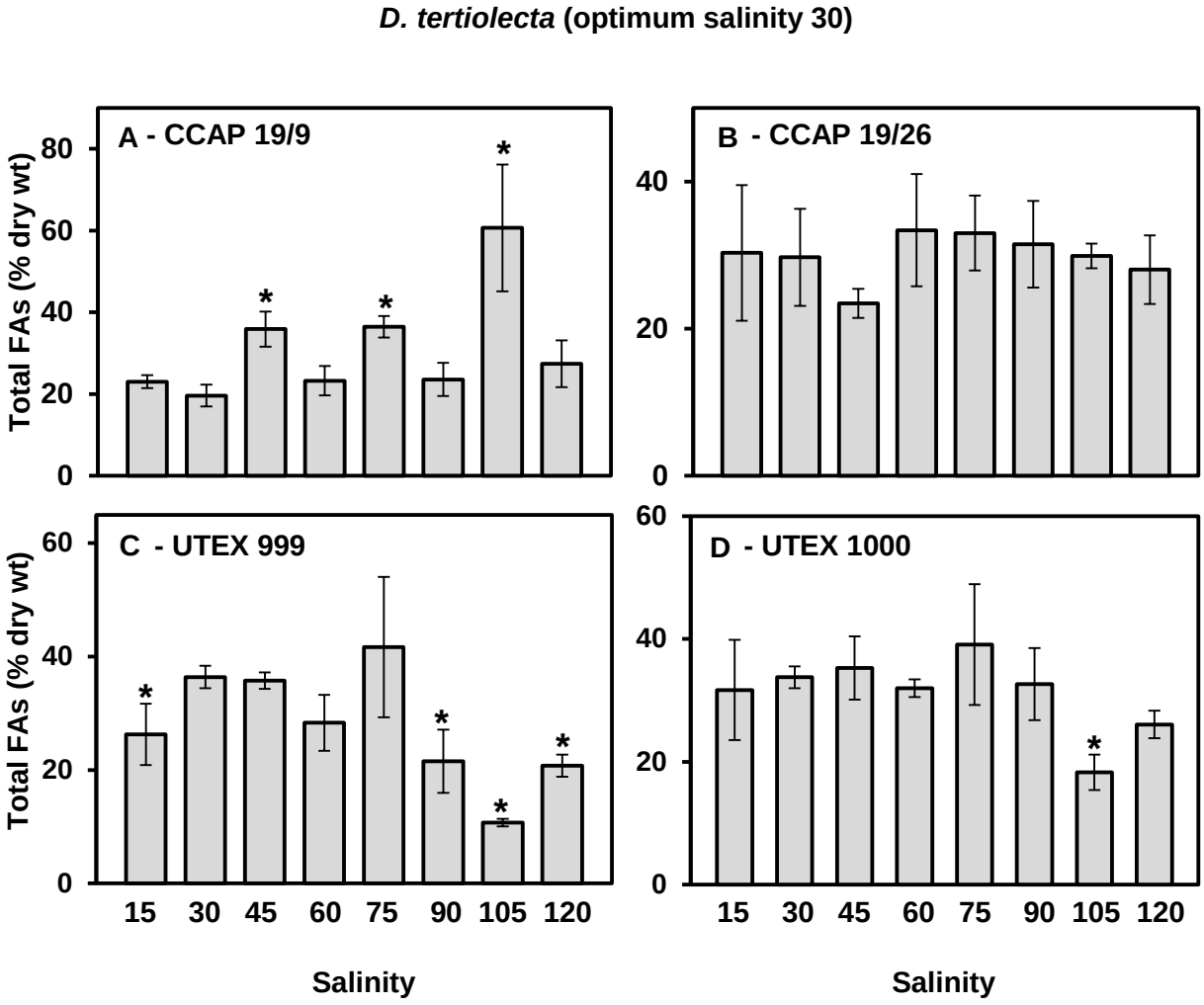

Figure 3.3 – Total FAs (as percent dry wt) for four strains of *D. tertiolecta* under long-term salinity stress, including (A) strain CCAP 19/9, (B) strain CCAP 19/26, (C) UTEX 999; and (D) UTEX 1000. These strains had an optimal salinity of 30. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

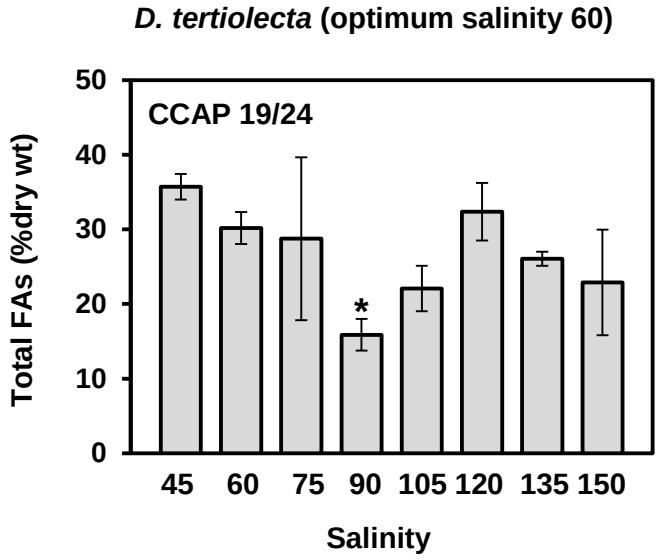

Figure 3.4 – Total FAs (as percent dry wt) for *D. tertiolecta* CCAP 19/24 under long-term salinity stress. This strain was different from the other *D. tertiolecta* strains tested because it had an optimum salinity of 60. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

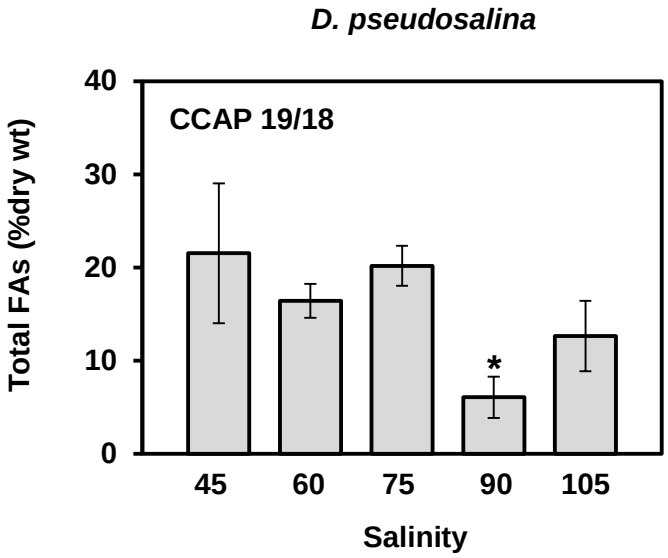

Figure 3.5 – Total FAs (as percent dry wt) for *D. pseudosalina* CCAP 19/18 under long-term salinity stress. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

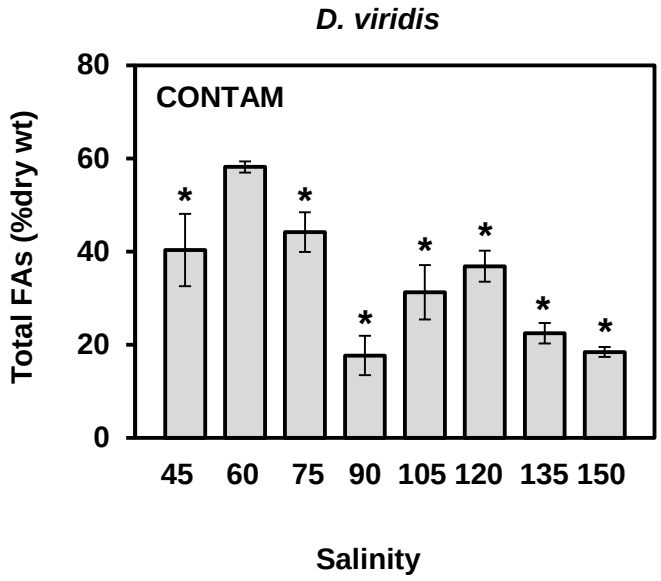

Figure 3.6 – Total FAs (as percent dry wt) for *D. viridis* CONTAM under long-term salinity stress. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

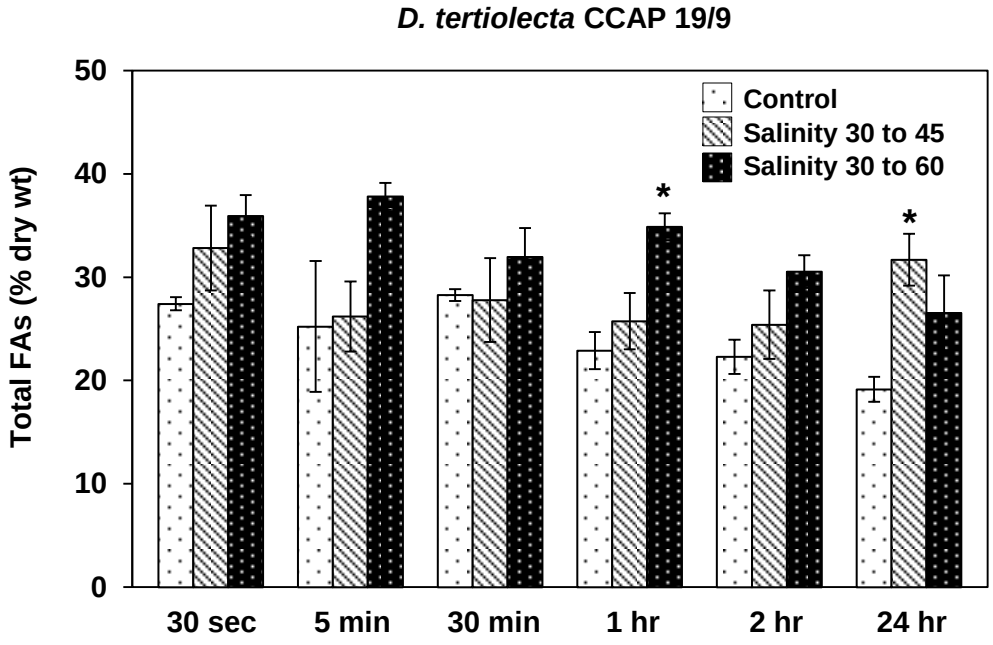

Figure 3.7 – Total FAs (as percent dry wt) for *D. tertiolecta* CCAP 19/9 under short-term high-salinity stress, when the control salinity of 30 was increased to 45 or 60. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

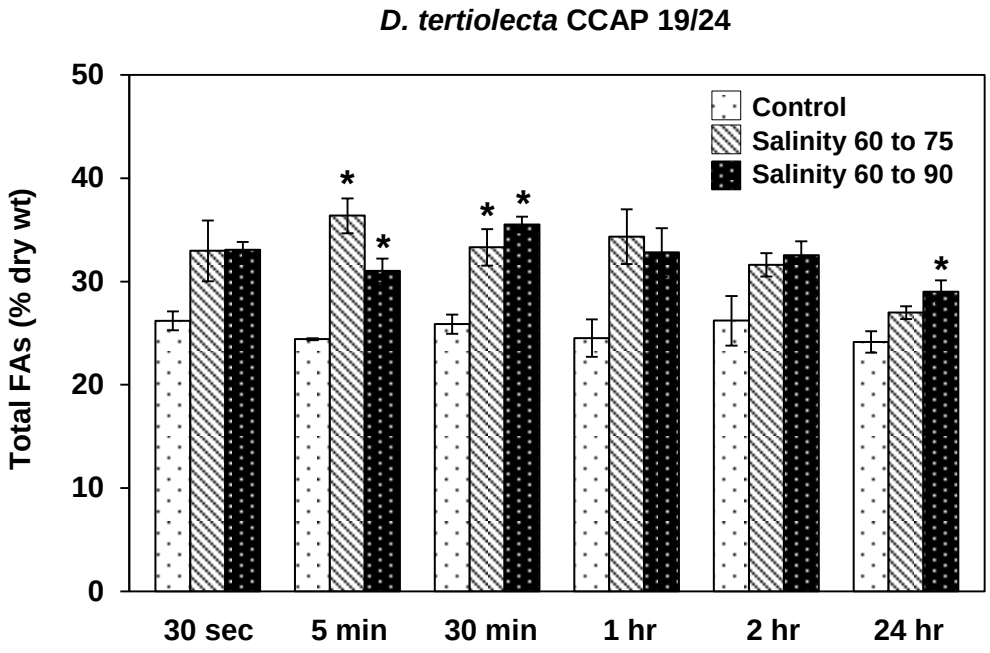

Figure 3.8 – Total FAs (as percent dry wt) for *D. tertiolecta* CCAP 19/24 under short-term high salinity stress, when the control salinity of 60 was increased to 75 or 90. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

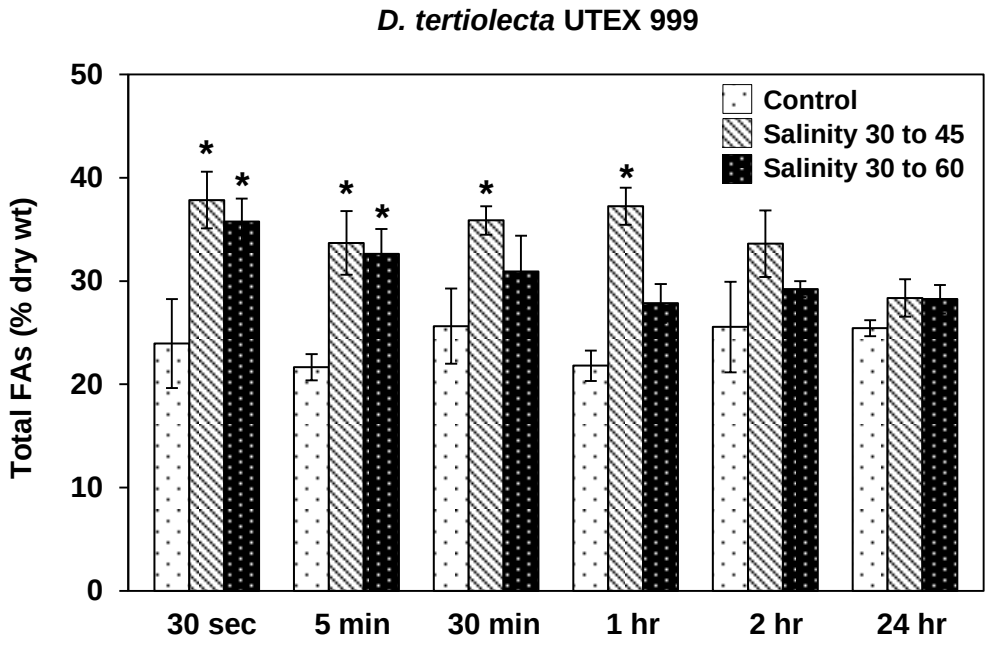

Figure 3.9 – Total FAs (as percent dry wt) for *D. tertiolecta* UTEX 999 under short-term high salinity stress, when the control salinity of 30 was increased to 45 or 60. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

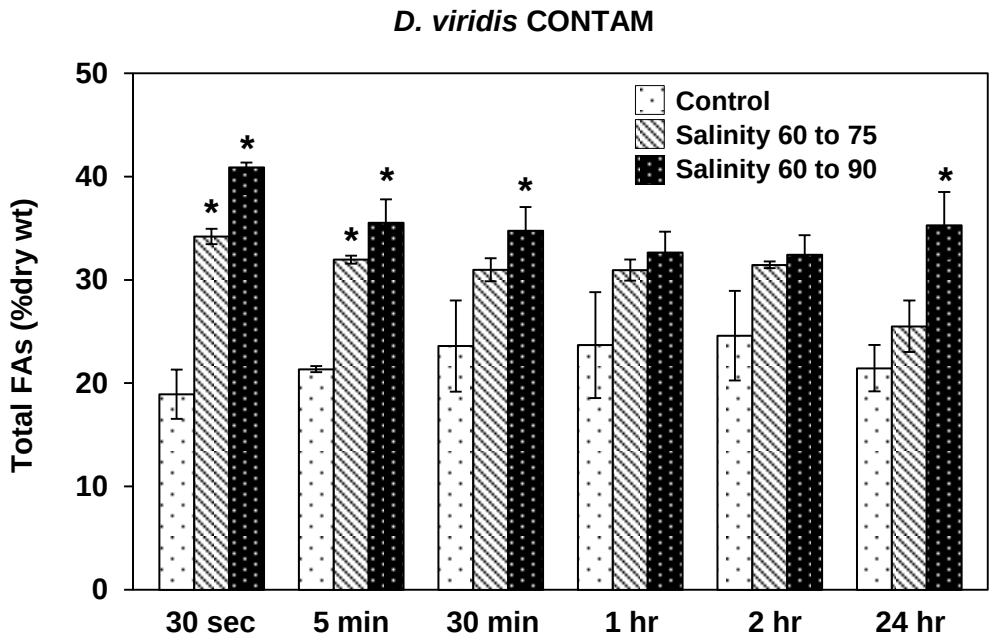

Figure 3.10 – Total FAs (as percent dry wt) for *D. viridis* CONTAM under short-term high salinity stress, when the control salinity of 60 was increased to 75 or 90. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

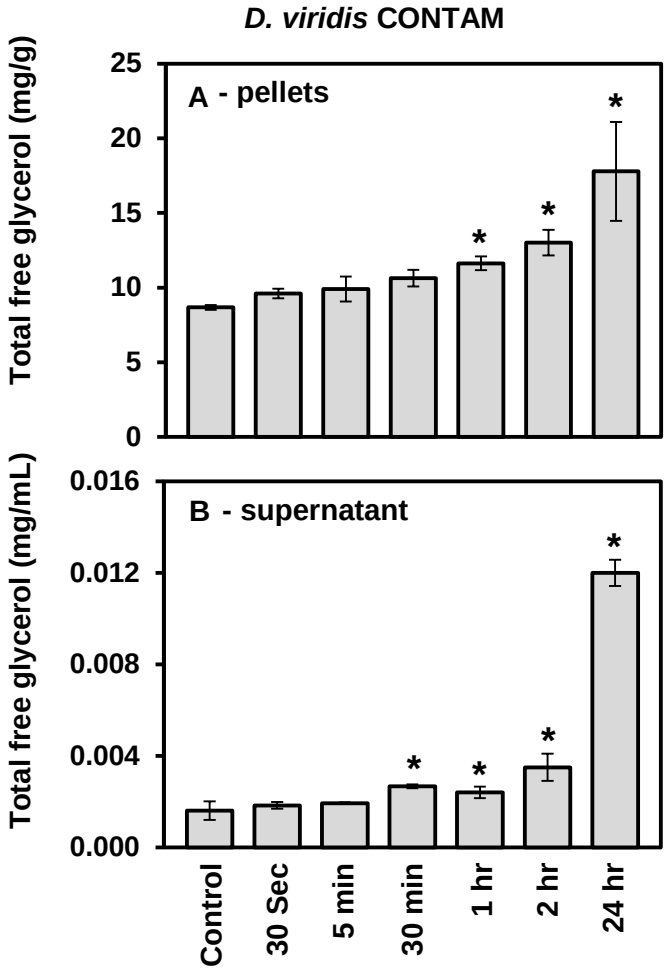

Figure 3.11 – Total free glycerol content in *D. viridis* CONTAM under short-term high salinity stress when the control salinity of 60 was increased to 90 - following centrifugation, total free glycerol A) In the pellets (mg/g); and B) In the supernatant (mg/mL). Note the change in scale for these two graphs. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE ($n = 3$).

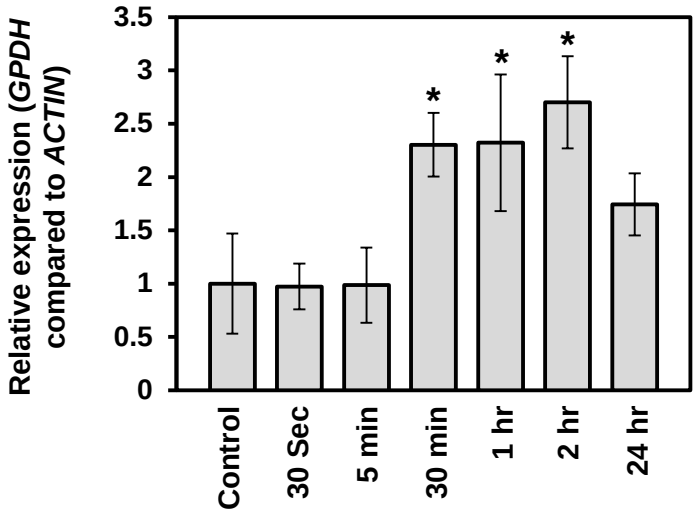

Figure 3.12 – Expression of *GPDH* in *D. viridis* CONTAM under short-term high salinity stress when the control salinity was increased to 90. Expression values are given as ratios relative to the values of *ACTIN*. Data are given as means ± 1 SD (n = 3).

4. EFFECTS OF ENVIRONMENTAL STRESSORS ON TOTAL FATTY ACID PRODUCTION IN *DUNALIELLA* SPECIES

Submitted to Journal of Experimental Marine Biology and Ecology

4.1 Abstract

Fourteen strains within four species of the halophytic microalga *Dunaliella* were rapidly screened for the presence of cellular lipid content during senescence using an optimized Nile Red technique. The five most promising strains were assessed for enhanced lipid production as total fatty acids (FAs) in bench-scale cultures (0.5 to 3.5L) under environmental stressors including nutrient limitation or deprivation (of inorganic (N) nitrogen and/or phosphorus (P), pH stress (control pH of early senescent cultures versus pH 10 or pH 7), a 12-hr : 12-hr light:dark photoperiod versus continuous light, and un-enriched versus enriched carbon dioxide (± 4-hr pulse of bubbling with CO_2). Strains capable of both rapid population growth and enhanced lipid production were also tested in mass culture (~150 to 175 L) to test feasibility of scale-up. All but one strain had minimal lipid production during active growth, and increased lipid production under senescence. The unique strain produced high amounts of total FAs during active growth as well as senescence, whether in a 12:12 L:D photoperiod or under continuous light. High pH x low salinity, pH 7, and continuous light x CO_2 yielded the maximum total FA content of 56%, 43%, and 42% (dry weight basis), respectively, in comparison to controls (~10 to 25% total FAs). Under continuous light ± CO_2 bubbling, cells likely incorporated the excess carbon (from CO_2 bubbling) as carbon-containing products such as FAs. Low N and/or P under 12:12 L:D photoperiod or continuous light, as well as

enriched CO_2 with a 12:12 L:D photoperiod, did not significantly enhance total FA content. High intraspecific and interspecific variation in population growth and lipid production was found in *Dunaliella* in response to each environmental stressor, suggesting fundamental metabolic differences. One *D. viridis* strain, was unique among the strains tested because it produced high amounts of neutral lipids during active growth as well as senescence, whether in a 12:12 L:D photoperiod or under continuous light.

4.2 Introduction

Microalgae have high surface area-to-volume ratios, facilitating rapid responses to environmental stressors (Becker 1994, Rodolfi *et al.* 2009). They can rapidly alter lipid metabolism in both total content and composition (Roessler 1990) in response to factors such as temperature (Lynch and Thompson 1982, Thompson *et al.* 1992), light (Peeler and Thompson 1990, Gordillo *et al.* 1998), pH (Gardner *et al.* 2012), and salinity (Xu and Beardall 1996, Azachi *et al.* 2002, Takagi *et al.* 2006). *Dunaliella* spp. (Chlorophyceae), as promising candidates for sustainable biofuel production, have been studied in great detail with regard to salinity stress and its effect on increasing total fatty acids (FAs) (Azachi *et al.* 2002, Takagi *et al.* 2006, Ben-Amotz *et al.* 2009, see Chapter 3). Here we investigated the effects of various abiotic stressors including low nutrients or deprivation, pH stress, and CO_2 x light under optimal or low salinity.

Inorganic nitrogen (N) limitation has been found to promote an increase in neutral lipids in several chlorophytes (Shifrin and Chisholm 1981, Roessler 1990, Rodolfi *et al.* 2009). Lipid accumulation has been expected to occur in the presence of N deficiency partly

because N is not a component of storage and membrane lipids (Roessler 1990). Shifrin and Chisholm (1981) completed an in-depth study on the effects of N depletion on total lipids within species of chlorophytes; however, found that *Dunaliella* spp. did not increase neutral lipids. In general, when phosphorus (P) becomes deficient, protein, chlorophyll *a*, RNA, and DNA tend to decrease while carbohydrate storage products increase (Kaplan *et al.* 1986). Little research has been completed to examine the effects of low P on lipid production in *Dunaliella*, or the effects of combined low N+P.

As microalgae consume carbon, cell division occurs and the extracellular pH in the medium increases (Sager and Granick 1953, Azov 1982, Guckert and Cooksey 1990). Species respond differently physiologically and biologically to external pH, and there can also be high intraspecific variation. In a recent study of the interaction between high pH and N limitation on triacylglyceride (TAG) production, two chlorophytes accumulated TAGs in response to high pH (> 9) + N deprivation, as indicated by Nile Red fluorescence (Gardner *et al.* 2011). Our pH experiments eliminated the nutrient limitation variable indicated by Gardner *et al.* (2011), so that any increase in total FAs was in response to pH stress alone. These studies additionally considered *rapid*, *short-term*, and *long-term* responses, briefly defined here as the time frames 30 sec to ≤ 5 min, up to 2-3 hr, and ≥ 24 hr, respectively (also see Chapter 3).

Carbon (C) accounts for almost 50% of the overall dry weight of microalgal biomass (Becker 1994). Phototrophic microalgae use dissolved inorganic carbon (DIC) to synthesize intracellular carbon compounds (Kaplan *et al.* 1986, Carvalho and Malcata 2004) in the presence of light. Algae commonly are cultured in a 12-hr :12-hr (light, 12-14 hr; dark, 10-12

hr) light:dark (L:D) photoperiod. Although many algae grow poorly or cease growth in continuous light (Graham *et al.* 2001, Carvalho and Malcata 2004), continuous light has promoted higher biomass production than L:D photoperiods for algae such as the rhodophyte *Porphyridium* (Porphyridiophyceae; Yongmanitchai and Ward 1992) and the chlorophyte *Dunaliella bardawil* (Ben-Amotz and Avron 1983). We extended this information by examining the response of two other *Dunaliella* strains to increased pulses of CO_2 x continuous light. In addition, environmental stressors that appeared to be especially promising in enhancing *Dunaliella* lipid production in preliminary trials were tested in both bench scale (~0.5 to 3.5 L) and mass culture experiments (~150 to 175 L). The difference in experimental scale was imposed because of the "container effects" shown for various algae (Ben-Amotz and Avron 1989, García-González *et al.* 2003), and in recognition of the importance of scale-up feasibility in harnessing algae for biofuel production.

4.3 Materials and Methods

4.3.a Culturing of Strains

Fourteen strains of *Dunaliella* were visually screened for high neutral lipid content with a fluorescent Nile Red dye technique optimized for use with *Dunaliella* spp. (see Chapter 1). Of these, five were selected for the environmental stressor experiments. These five strains were obtained from the Culture Collection of Algae and Protozoa (CCAP) and the Culture Collection of Algae at the University of Texas at Austin (UTEX) (Table 4.1).

In a companion study (Chapter 2), ITS2 sequence data for each of the 5 strains indicated that the strains fall within two distinct species (clades) instead of the expected

three. Three of the five strains tested in these experiments additionally had been labeled incorrectly (Chapter 2) (Table 4.1). Additionally, strain UTEX 1644, which was obtained ~5 yr ago from the culture collection, was determined to be a highly unique strain of *D. viridis*. This strain is indeed unique in many features when compared to other strains of *Dunaliella*, including cell size, population growth as cell production, lipid production throughout the cell cycle, and sustained growth in continuous light (below). Thus, the strain was not contaminated by our other cultures. When UTEX 1644 was ordered recently from the culture collection by colleagues, it was confirmed to be *D. salina* and not this unique strain. Here, we refer to the strain as CONTAM, because the culture can no longer be obtained under the number UTEX 1644 from the culture facility. We are depositing this strain at the NCMA so that it is available to other researchers. We refer to the other strains by the species names indicated from the ITS2 sequence data, along with the original strain numbers (Chapter 2). They included *D. tertiolecta* strains CCAP 19/9 (120 ± 6 μm^3, mean ± 1 SD, n = 30 cells), CCAP 19/24 (132 ± 6 μm^3), UTEX 999 (163 ± 9 μm^3), and UTEX 1000 (168 ± 8 μm^3), and *D. viridis* CONTAM (74 ± 5 μm^3) (Table 4.1).

Culture cell densities and cell production were assessed from subsamples preserved in acidic Lugol's solution (1% final concentration; Vollenweider *et al.* 1974), and quantified within 1-2 days using Palmer-Maloney chambers (Wetzel and Likens 2000) under light microscopy at 200x magnification using an Olympus BH-2 light microscope (Germany).

Preliminary work indicated that the *Dunaliella* strains grew well in Erdschreiber's (Føyn 1934), Artificial Seawater (ASW, made using f/2-Si nutrients; Guillard 1975, Tompkins *et al.* 1995), and L1-Si medium (Guillard and Hargraves 1993), at salinity 30 or 60

depending on the strain. Erdschreiber's and ASW media contained soil water with undefined constituents, and nutrient components of f/2-Si were similar to L1-Si. Therefore, L1-Si was selected for use in this study. Once growth was established for strains in their source media (Table 4.1; salinity 30 or 60), each culture was transferred into a series of increasing proportions of L1-Si and the source medium (after Lorenz *et al.* 2005) until the cells finally were acclimated to 100% L1-Si medium. Cell production for each strain was compared in its source medium versus L1-Si medium, and all strains attained comparable or higher maximum cell production in L1-Si (see Chapter 3). In a second series of transfers, cultures were adjusted using NaCl (Fisher Scientific, Fair Lawn, New Jersey), by salinity ± 5 to 10 for each adjustment (YSI 3200 Conductivity Instrument, Yellow Springs, Ohio), and tested for population growth rates (as cell production) to determine the optimal salinity for each strain (See Chapter 3). An analogous approach was used to identify the optimum pH for each strain, using incremental changes of 0.1 pH unit (PerpHecT LogR Meter 350, Boston Massachusetts) from an initial pH of 7.5 to 8.5. Accordingly, three strains (*D. tertiolecta* strains CCAP 19/9, UTEX 999, and UTEX 1000) were grown at an optimal salinity of 30 and an optimal initial pH of 8.0-8.4 for cell production; the other two strains (*D. tertiolecta* CCAP 19/24 and *D. viridis* CONTAM) were grown at their optimum salinity of 60 and an optimal initial pH of 8.0-8.4. Stock cultures for each strain was grown under the additional conditions of 23˚C and a light intensity of ~180 $\mu Em^{-2}sec^{-1}$ (light source, ProLume® compact fluorescent bulbs) under a 12-hr : 12 hr light : dark (L:D) photoperiod.

Strains were purified using a combination of differential centrifugation and flow cytometry (Beckman Coulter® EPICS Altra, Hialeah, Florida) to remove bacterial

contamination (Guillard 2005, Kawachi and Noël 2005, Chapter 1 methods therein). Cultures were plated on 1% Agar M (Sigma Aldrich®, St. Louis, Missouri) or 1% Phytoblend (Caisson Laboratories, Logan, Utah) to promote algal growth or bacterial growth, respectively. Subsequent plating was completed aseptically on a monthly basis until each strain was purified.

4.3.b Mass Cultures

The mass culturing facility consisted of a series of growth tubes, each 1.21 m height x 30 cm diameter, made of lightweight 0.1-cm polymer fiberglass, providing ~90% transparency. Each tube held a maximum of ~175 L. Unless otherwise specified, cultures were grown under a 12:12 L:D photoperiod. The light source consisted of light banks (Standard Utilitech 48 ½ in fluorescent light, Lowes, Mooresville, North Carolina) that provided ~160 to 180 $\mu E/m^2/sec$ for each tube. The temperature for these experiments was maintained at 23 ± 1 ^oC. Tubes were sampled daily between 0800 and 0900 hr for temperature, pH (Oakton Waterproof pH Testr 30 Pocket pH Tester, Oakton Instruments, Vernon Hills, Illinois), and cell counts (Culturing of strains Section).

In preparation for mass culturing, tubes were filled with ~150 L deionized water adjusted to the desired salinity (30 or 60) (Hydrolab MiniSonde 4, Hach Company, Loveland, Colorado) and pH (8.3-8.4) with Coralife® salts (CL; Franklin, Wisconsin) + ~19.35 mL each of parts ProLine® A/B (F/2; Aquatic Eco-Systems, Apopka, Florida) nutrient mixtures (Guillard 1975). Stock culture was grown by inoculating ~3 L volume (salinity 30 or 60, adjusted with CL + L1-Si nutrients) with ~300 mL of culture (i.e. a 10% inoculum) under growth conditions described above. Once the stock reached late-exponential growth, as

determined by cell counts, it was then added to a freshly prepared mass culture tube (~3.5 L stock culture in ~150 L prepared medium) and grown as inoculum for mass culture experiments. When this inoculum tube reached late exponential growth (typically within 7-10 days), ~15 L was dispensed into each freshly prepared mass culture tube that was filled with ~150 L culture medium, described above. Therefore, the maximum volume for any given tube was ~165 L including culture. The total number of tubes prepared varied by experiment. *Dunaliella* cultures were grown to a density of 10^6 (rarely 10^7) cells/mL within 7-10 days depending on the strain and the experimental conditions.

4.3.c Low Nitrogen and/or Phosphorus

In bench-scale preliminary trials, *D. viridis* CONTAM and *D. tertiolecta* UTEX 1000 were grown under low N or N deprivation (N as nitrate, NO_3^- N, added as 10% or 0% of replete conditions, respectively) and/or low P or deprivation (P as PO_4^{-3}P, added as 10% or 0% of replete conditions). N deprivation was included to compare these results to other studies that used N-free medium in an attempt to enhance total lipids in *Dunaliella* (Shifrin and Chisholm 1981, Davidi *et al.* 2012). Use of NH_4^+ as the N source resulted in poor growth, so the N experiments focused on NO_3^-N.

The two strains attained maximal lipid content of 40-60% as total fatty acids (FAs). In preliminary trials, *D. tertiolecta* UTEX 1000 attained maximal biomass of 73.5 grams fresh weight (g fr wt) per mass culture tube on day 5 (at an optimal salinity of 30), and maximal lipid production of 29.4 g fr wt per tube on day 11. By comparison, *D. viridis* CONTAM had maximal biomass of 42.7 g fr wt per tube occurred on day 7 (at an optimal salinity of 60), and maximal lipid production of 36.4 g fr wt per tube on day 9. Shortly after

the preliminary trials, however, *D. tertiolecta* UTEX 1000 inexplicably lost its ability to produce significant amounts of neutral lipids. Thus, it had to be eliminated from further consideration in the environmental stressor experiments.

Low nutrient experiments included controls (N- and P-replete; 8.82×10^{-4} M and 3.62×10^{-5} M, respectively) versus treatments as N and P deprivation (no N or P added; negligible measured concentrations); 10% limitation (10% of the control, replete media concentrations(s) of N and/or P added); and a low N and/or P condition (20% of control, replete media concentration(s) of N and/or P added). Replicates (bench scale only, ~500 mL volume, n = 4) were grown for one week under the growth conditions described. Two replicates were then moved to continuous light (CL) for an additional week. The remaining two replicates per treatment were maintained under 12:12 L:D photoperiod for the additional week. Subsamples were taken at 0800 hr daily until the onset of senescence, and cell production was quantified as above. At the end of the experiment, samples (~ 450 mL) were centrifuged (3,000 RPM, 10 min - CL2 Centrifuge, ThermoScientific, Milford, Massachusetts) and immediately frozen at -80˚C until analysis of total FAs (for this and all other experiments, within 7-10 days; L. Dean, U.S. Department of Agriculture, Raleigh, NC, personal communication).

4.3.d Long-Term pH Stress

D. viridis CONTAM was grown in mass culture as described above. Replicate controls (n = 3) were transferred from salinity 60 to 60 or from salinity 60 to 30 (n = 3 for each salinity) and were maintained at pH 8.3-8.4 after addition of the nutrient mixture and the *Dunaliella* culture inoculum. Likewise, replicate treatment cultures in salinity 60 or

salinity 30 (n = 3 for each salinity) were adjusted to pH 10 using 6N NaOH. Replicates were grown until senescence (~21 days) as determined by cell counts (Section 2.1) and pH readings (Section 2.2). Sub-samples (~500 mL) were centrifuged at ~3 day intervals and immediately frozen at -80˚C until total FA analysis. Controls were compared to treated (high pH) at each salinity level, allowing for the determination of the effect of pH alone on FA production. Note: hypo-osmotic stress, alone, was previously reported (see Chapter 3).

4.3.e Short-Term pH Stress

In preliminary work, *Dunaliella* growth as cell production in response to pH was tested across a pH range of 2 to 12 with four strains including *D. tertiolecta* strains CCAP 19/9, CCAP 19/24, and UTEX 999, and *D. viridis* CONTAM. Low or no growth as cell production was observed for cultures grown in an initial pH of 2, 4, 6, or 12. From those observations, we selected low and high pH treatments at pH 7 and pH 10, respectively. Each strain was grown in replicate cultures (n = 3) at ~3.5 L volume under the conditions described above. Cells were quantified from preserved subsamples (Section 2.1) until cultures reached early senescence.

Each replicate culture was evenly distributed into seven sterile 50-mL centrifuge tubes (Corning® Centristar™) by centrifuging (3,000 RPM, 10 min) ~500 mL culture into each tube. Thus, the final pellet in each tube was concentrated from ~500 mL of culture. Pellets were re-suspended into 50 mL of medium at either low pH (7) or high pH (10). After re-suspension, replicates were sampled for cell counts and semi-quantitative assessment of lipid content via fluorescence microscopy after Nile Red (NR) staining (Chapter 1) before (at the control pH) and at 30 sec, 5 min, 30 min, 1 hr, 2 hr, and 24 hr after exposure to the

altered pH. Briefly, 10 µL of NR (0.625 µg/mL dissolved in 100% MeOH) was added to slides (slightly heated by placing slides on a hotplate set to low) containing 2 drops of culture with cells that had been immobilized on 1.3% Type V agarose (SigmaAldrich). An Olympus AX70 microscope (excitation, 488 nm; emission, 545-655 nm) and an Olympus DP70 camera were used to photograph each strain at 600x. Samples were centrifuged (3,000 RPM, 10 min) and immediately frozen at -80˚C until FA analysis.

4.3.f CO_2 x Light Regime

D. viridis CONTAM was tested in bench-scale preliminary trials (~500 mL cultures) and was shown to produce neutral lipids under both 12:12 L:D photoperiod and CL based upon NR fluorescence microscopy, described above . We also tested effects of continuous versus pulsed bubbling of CO_2. Continuous bubbling resulted in pH readings below neutral (~6.5-7.0), which was detrimental to cell growth. Therefore, CO_2 was bubbled for a daily pulse of 4 hr between 1000 and 1400 hr when highest photosynthetic activity was expected.

Stock culture of *D. viridis* CONTAM was grown at salinity 30 and other growth conditions described above at ~3.5 L volume and was added to a freshly prepared mass culture tube ("mass cultures stock A"; Mass culture Section). After one week, the culture was in exponential growth phase (~8.50 x 10^5 cells/mL). Eight freshly prepared tubes were each inoculated with 15 L of mass culture stock A. All tubes were sampled daily between 0800 and 0900 for temperature, pH, and cell counts. When the mass cultures had reached mid-exponential growth phase (usually on day 6), CO_2 was bubbled for 4 hr (at 1000 – 1400 hr) daily for the remainder of the experiment in 4 of the 8 tubes (12:12 L:D) The remaining 4 tubes were also sampled and maintained as controls.

A second experiment in a separate set of mass culture tubes was set up similarly; then continuous light (CL) was imposed to all eight tubes on day 6 and for the remainder of the experiment. Four tubes also received 4 hr of bubbling CO_2 (at 1000 – 1400 hr) daily beginning on day 6 for the remainder of the experiment.

Control tubes did not receive CO_2 enrichment in either experiment. For both experiments (12:12 L:D ± CO_2, and CL ± CO_2), a ~500 mL subsample was taken between 0800 and 0900 hr, and centrifuged (3,000 RPM, 10 min) on days 6, 9, and 12 representing mid-exponential, late exponential, and early senescence growth phases, respectively. The pellets were stored at -80˚C until FA analysis.

4.3.g Total FAs

Direct transesterification (DT; Griffiths *et al.* 2010, Chapter 1) was performed to convert saponifiable lipids to FA methyl esters, hereafter referred to as total FAs, which were then directly quantified using gas chromatography (GC) with a flame ionization detector (FID). In this process, a combination of acidic and basic transesterification catalysts was used. Briefly, 0.5 M methanolic KOH was added to each sample, homogenized, and heated to 85˚C. Then, BF_3 (14% in methanol) was added and the samples were re-heated again to 85˚C. Equal volumes of water and hexane were then added and the samples were allowed to separate into layers. The hexane layer, containing the neutral lipids, was removed and analyzed with a GC HP5890 series II equipped with a FID (Hewlett Packard, refurbished by Primera Scientific LLC, Princeton, New Jersey) and a 7673A autosampler (Alpha Omega Technologies, Inc., refurbished by Primera Scientific LLC, Princeton, New Jersey). Separation was achieved in an Rtx-2330 capillary column. The temperature was programmed

for an initial 3 min at 60˚C increasing at a rate of 4˚C min^{-1} to a final temperature of 230˚C. Both injector and detector temperatures were set at 265˚C. Injections were performed under the splitless mode. Data acquisition and analysis was completed using the GC Chemstation Rev. A.08.03 (847) software. Calculations for total FAs were completed using the relative response factor (RRF) method (Sparkman *et al.* 2011; L. Dean, USDA, Raleigh NC, personal communication) and area ratios were compared to the internal standard (KEL-FIM-FAME-5 Mixture, Matreya, LLC, Pennsylvania, USA).

4.3.h Statistics

Data were analyzed by either a one-way or two-way ANOVA with repeated measures (SAS v.9.2 – SAS Institute, Inc. 2010). Treatment effects were considered significant at $p \leq 0.05$ (one-sided). Type 3 Tests of Fixed Effects were completed to ensure that controls were not significantly different, and indicated that replicates were similar.

4.4 Results

4.4.a Low Nitrogen and/or Phosphorus

Cell production by *D. viridis* CONTAM under N or P deprivation or limitation (cultures from enriched media transferred to 0% or 10% of nutrient-replete conditions) was so low that there was not enough material for FA analysis at the end of the 14-day duration. The cells were yellowish-whitish in color in comparison to the bright kelly green cells of control cultures.

Maximum cell production for the nutrient-replete cultures was 2.8 x 10^6 cells/mL under 12:12 L:D and 2.3 x 10^6 cells/mL under CL (Fig. 4.1). Replicates grown in the lower

nutrient regime (20% of nutrient-replete conditions) peaked at 1.8 x 10^6 cells/mL regardless of the light regime. Cultures in the lower nutrient regime (20% N, 20% P, or 20% N & P) under 12:12 L:D consistently had lower pH measurements than cultures grown under CL (Table 4.2). Regardless of light treatment, nutrient-replete controls were similar in pH to one another throughout the culture's growth phase (Table 4.2). The overall findings from this set of experiments was that low-nutrient stress as N and/or P (20% of replete conditions) did not increase total FAs under the 12:12 L:D regime or under CL (Fig. 4.2). *D. viridis* CONTAM attained higher total FA content under 12:12 L:D than in CL, but this was not statistically significant (Fig. 4.2).

4.4.b Long-Term pH Stress

Adjusting the pH on exponentially growing culture revealed a potentially valuable harvesting mechanism (Chapter 5). Addition of 2N or 6N sodium hydroxide (NaOH) to the culture medium resulted in the *Dunaliella* cells flocculating to the bottom of the flasks or mass culture tubes (Fig. 4.3). At salinity 30, mass cultures that had been adjusted to pH 10 recovered and the suspended algal populations began to increase cell production at 9 days post-inoculation (Fig. 4.4). In contrast, mass cultures at salinity 60 that had been adjusted to pH 10 did not recover from the apparent adverse effect of high pH. These high pH-treated cultures attained maximum mean cell production (~1.25 x 10^6 cells/mL) at ~day 18, whereas control mass cultures peaked in mean cell production (~2 x 10^6 cells/mL) by ~day 12. Comparison of total FAs for the control mass cultures grown in salinity 30 and an initial pH 8.3-8.4 versus mass cultures grown at salinity 30 and adjusted to pH 10 showed that the high-pH mass cultures recovered within 9 days (Fig. 4.4).

In the control mass cultures (salinity taken from 60 to 30; no pH adjustment), total FAs were highest on day 12 (41.7% ± 6.63, % total FAs ± 1 standard error [SE]) in comparison to day 3 (9.6% ± 1.66) ($p < 0.001$) (Table 4.3). The treatment mass cultures (salinity taken from 60 to 30; pH adjusted to 10) behaved similarly, with total FA content increasing significantly on day 9 in comparison to day 3 ($p = 0.014$) (Table 4.3). At day 9, tubes at salinity 60 taken to salinity 30 and adjusted to pH 10 yielded the maximal total FA content (55.6% ± 3.05) when compared to other days in that treatment or to the control mass cultures (Fig. 4.5).

To ensure that hypo-osmotic stress did not affect total FAs, control mass cultures (salinity taken from 60 to 60, or salinity taken from 60 to 30; no pH adjustment) were compared. A similar trend in production of total FAs was observed for control tubes transferred from a salinity of 60 to 60 wherein total FAs increased throughout the growth phase (Fig. 4.6). For control tubes transferred from a salinity of 60 to 60, total FAs increased significantly on days 9, 12, and 14 when compared to day 3 ($p <0.001$ to 0.019) (Table 4.3). Within the tubes transferred from a salinity of 60 to 30, total FAs increased significantly on all days in comparison to FA content on day 3 ($p < 0.001$ to 0.003) (Table 4.3). There was no significant difference in total FAs between control tubes transferred from salinity 60 to 60 or from salinity 60 to salinity 30 on each sampling date (Fig. 4.6). Therefore, hypo-osmotic stress did not affect total FA content.

4.4.c Short-Term pH Stress

As expected, responses to short-term pH stress were strain-specific. *Dunaliella tertiolecta* CCAP 19/9 significantly increased in total FAs when exposed to pH 7 (Fig. 4.7 A), but only after 24 hr exposure to pH 10 (Fig. 4.7 B). After re-suspension in pH 7 medium, there was a significant increase in total FAs at all time periods from 30 sec to 24 hr ($p = 0.001$ to 0.015) (Table 4.4). However, the maximum total FA content was only 19.9% ± 0.78 when compared to the control FA content (15.3% ± 1.54). At pH 10, there was a significant increase by 24 hr after re-suspension ($p = 0.046$) (Table 4.4). *D. tertiolecta* CCAP 19/24 did show an increased total FA content when exposed to pH 7 or pH 10 medium (Fig. 4.8). Maximum total FA content (35.6% ± 1.12) was measured 30 sec after exposure to pH 7, and was higher than the total FA content of controls (30.8% ± 0.61) (Fig. 4.8 A). After re-suspension in pH 10 medium, *D. tertiolecta* CCAP 19/24 yielded a significant decrease in total FA content at 30 sec, 1 hr, and 24 hr ($p = 0.001$ to 0.049) (Table 4.4).

There was no significant difference in total FA production when *D. tertiolecta* UTEX 999 was exposed to pH 7 medium (Fig. 4.9 A). In response to pH 10, total FA content significantly increased at 30 sec, 5 min, and 24 hr after exposure ($p = 0.006$ to 0.035) for *D. tertiolecta* UTEX 999 (Table 4.4, Fig. 4.9 B). The maximum total FA content was 23.9% ± 1.24 at 30 sec when re-suspended in pH 10 compared to the control total FA content (18.6% ± 1.52). Lastly, there was a significant *decrease* in total FA content for *D. viridis* CONTAM when exposed to either pH 7 (except at 5 min, when there was a significant increase) or pH 10 medium (Fig. 4.10 A-B). When re-suspended in pH 10, this strain yielded significantly decreased total FA content at every time period from 30 sec to 24 hr ($p < 0.001$ to 0.003)

(Table 4.4). *D. viridis* CONTAM yielded maximal total FA content of 43.3% ± 5.53 at 5 min after exposure to pH 7, which was statistically significant ($p = 0.004$) (Fig. 4.10 A). At every other time point, this strain yielded a significant decrease in total FA content ($p = 0.005$ to 0.021) (Table 4.4).

4.4.d CO_2 x Light Regime

Maximum cell production for *D. viridis* CONTAM under 12:12 L:D (Fig. 4.11) or continuous light (CL) in mass culture occurred on day 11 (Fig. 4.11). Tubes under 12:12 L:D ± CO_2 or CL without CO_2 reached a maximum cell production of 1.4×10^6 cells/mL compared to tubes under CL with CO_2 that reached 1.9×10^6 cells/mL by day 11 (Fig. 4.11 A-B). The pH decreased from ~9.3 to ~8.0 (12:12 L:D) (Fig. 4.11 A) or from ~9.8 to ~8.4 (CL) (Fig. 4.11 B) when cultures received pulsed CO_2 enrichment. Culture tubes that received both CL and 4hr bubbling of CO_2 were much darker green in color (Fig. 4.12) than cultures that were under 12:12 L:D ± CO_2 or CL without CO_2 (Fig. 4.12 A,B).

Total FAs increased throughout growth to senescence of mass-cultured *D. viridis* CONTAM (Fig. 4.13). The total FA content significantly increased when comparing day 6 (baseline) to days 9 and 12 in mass cultures grown under 12:12 L:D ± CO_2 ($p < 0.001$ to 0.003) (Table 4.5) and in cultures grown under CL ± CO_2 ($p < 0.001$ to 0.002) (Table 4.5). Mass cultures grown under CL and enriched with pulses of CO_2 yielded maximal total FA content of 42.8% ± 8.22 when compared cultures grown under CL without enriched CO_2 (22.6% ± 0.90) by day 12 (Fig. 4.13 B). This difference in total FA content was statistically significant ($p = 0.026$) (Table 4.6). An overall comparison of cultures ± CO_2 enrichment was compared and was statistically significant ($p = 0.00855$) (Table 4.6). Therefore, adding the

variable of CO_2 enrichment significantly impacted total FA content. By day 12, cultures under 12:12 L:D with CO_2 pulsing reached a maximum total FA content of 31.5% ± 1.78 (Fig. 4.13 A), which was significantly different from cultures under CL with CO_2 pulsing (42.8% ± 8.22) ($p = 0.0486$) (Table 4.6, Fig. 4.13 B).

4.5 Discussion

4.5.a Low Nitrogen and/or Phosphorus

N accounts for up to 10% of the total dry weight in exponentially growing non-siliceous algae (Kaplan *et al.* 1986, Wetzel 2001). Some algae have been shown to prefer NH_4^+ over other N forms, with NO_3^- taken up only after NH_4^+ is depleted (Kaplan *et al.* 1986). Yet, NH_4^+ can be inhibitory or toxic to cells when used as the sole form of N (Kaplan *et al.* 1986) and this inorganic N form has been termed a paradoxical nutrient (Britto and Kronzucker 2002). The threshold of toxicity responses to NH_4^+ is species-specific. In this study, NH_4^+ appeared to inhibit both *Dunaliella* strains tested even at the lowest concentrations used, and this N substrate resulted in poor growth.

N deficiency would have been expected to increase lipid accumulation partly because storage and membrane lipids do not contain N (Roessler 1990). These lipids could continue to be synthesized while N-containing compounds such as proteins and nucleic acids are slowed or stopped as N becomes limiting (Roessler 1990). Inorganic N limitation has been found to promote an increase in neutral lipids in various chlorophytes, such as *Chlorella* spp. (Ben-Amotz *et al.* 1985, Roessler 1990), *Nannochloropsis* spp. (Suen *et al.* 1987, Rodolfi *et al.* 2009), and *Chlamydomonas reinhardtii* (Wang *et al.* 2009, Moellering and Benning

2010). However, studies with strains of *Dunaliella* have yielded varying results. Some strains within the species *D. bardawil, D. primolecta, D. tertiolecta,* and *D. viridis* have not increased total lipid content in response to N deficiency (Shifrin and Chisholm 1981, Ben-Amotz *et al.* 1985, Uriarte *et al.* 1993, Adam 1997, Gordillo *et al.* 1998, present study), while others (within strains of *D. salina* and of *D. tertiolecta*) have produced more lipids (Weldy and Huesemann 2007, Chen *et al.* 2011, Davidi *et al.* 2012). High variation among species and strains of *Dunaliella* response to N was reflected by the fact that, unlike the findings from previous studies, in this research *Dunaliella* CONTAM did not increase neutral lipids in response to N limitation. Interspecific variation was expected, as shown for other microalgae (e.g. Shifrin and Chisholm 1981, Lombardi and Wangersky 1995). Here, certain strains of *D. viridis* and *D. tertiolecta* did not increase total FA content in response to N stress.

Luxury consumption of P by microalgae is a well-known phenomenon (Beardall *et al.* 2001, Wetzel 2001). Under P-limited conditions algae exhibit reduced photosynthetic capacity, increased P uptake, and P-dependent transients in oxygen evolution (Beardall *et al.* 2001). Few studies have examined the potential role of P limitation or deprivation in enhancing lipid production in microalgae. The diatom *P. tricornutum* has been reported to significantly increase in total FAs in P-deprived medium when compared to P-replete medium, but this response has not been found in *D. tertiolecta* (Siron *et al.* 1989) or in *D. viridis* CONTAM (present study).

4.5.b Long-term pH Stress

An interesting potential harvesting mechanism was found when cultures were adjusted from pH 8.3-8.4 to pH 10. Spontaneous or auto-flocculation occurred due to the

sharp increase in pH (Richmond and Becker 1986). The *Dunaliella* cells excreted sufficient glycerol to enhance flocculation and removal from the water column (Chapter 5). The combination of high salinity (60) and pH adjustment to10 resulted in no recovery of the culture from flocculation and settlement. In contrast, in media with salinity 30 x pH 10, the *Dunaliella* cultures were able to recover from flocculation/settlement. Although the treated cultures maintained lower maximal cell densities than the controls, total FAs increased by the end of the experiment. The only significant difference between the control and high pH-treated cultures occurred on day 9, when cultures in the high pH treatment increased total FAs to ~56%, whereas the total FA content of controls was only ~29%. Gardner *et al.* (2011) found that the chlorophyte *Scenedesmus* sp. produced a significant amount of lipids in alkaline medium (> 9); however, this observation also correlated with nitrogen depletion. By contrast, in this study *D. viridis* CONTAM did not increase total FAs in response to low N, thus suggesting that high pH alone increased total FAs for this strain of *Dunaliella* in mass culture.

4.5.c Short-term pH Stress

Responses to pH stress were strain-specific and, in general, increases in total FAs were not observed. After exposure to pH 7, *Dunaliella tertiolecta* CCAP 19/9 significantly increased in total FAs at every time point (30 sec to 24 hr) and *D. viridis* CONTAM also increased total FA production, but only 5 min after exposure. However, at every other time point, *D. viridis* CONTAM had a significant *decrease* in total FAs in response to pH 7. Exposing *Dunaliella* cells to low or high pH led to variation among strains with regard to total FA production as a short-term response. With the exception of *D. tertiolecta* CCAP 19/9

(pH 10) and *D. tertiolecta* UTEX 999 (pH 7), the imposed short-term pH stress led to a *decrease* in total FAs in comparison to the FA content of controls. It was expected that pH 10 would stimulate FA production, as reported for other chlorophyte microalgae (*Scenedesmus* spp.) by Gardner *et al.* (2012). However, as indicated above, *Dunaliella* spp. have differed in responses to environmental stressors relative to other chlorophytes. In addition, Gardner *et al.* (2012) did not quantify lipid production but, rather, observed an increase in Nile Red fluorescence that was calibrated to TAG concentrations.

4.5.d CO_2 x Light

In this experiment total FAs accumulated throughout the growth cycle for *D. viridis* CONTAM cultures, with the highest production by early senescence. Most microalgae have been found to accumulate lipids only during senescence, not in active growth (Graham *et al.* 2010). Additionally, most algae tested have not grown well in continuous light (Carvalho and Malcata 2004, Sforza *et al.* 2012).

D. viridis CONTAM cultures grown under CL and bubbled with 4 hr of CO_2 had maximal total FAs by day 12 (42.8%, Fig. 4.12). In CL these cultures continually maintained photosynthesis absent dark cycle respiration. Typically, during the dark cycle plant cells use FAs that accumulated in the light period for energy (Guckert and Cooksey 1990, Gardner *et al.* 2012). Without a dark cycle to break down carbon products, the excess C as CO_2 apparently was converted by *D. viridis* CONTAM cells into FAs. Gardner *et al.* (2012) investigated adding sodium bicarbonate ($NaHCO_3$) to cultures that were also N-depleted. Adding bicarbonate triggered FA production in *Scenedesmus* spp., but the cells were grown under 14:10 L:D in addition to being N-deprived. Here, *D. viridis* CONTAM did not respond

to N and/or P limitation. In the enriched CO_2 x light regime study, more C became available for cell use when cultures were bubbled for 4 hr with CO_2. Thus, the combination of CL and pulsed bubbling with CO_2 significantly increased total FA production by *D. viridis* CONTAM when compared to cultures grown under CL alone, or in a 12:12 L:D period ± enriched CO_2. In addition, cultures that received both CL and enriched CO_2 had much more green pigment under 12:12 ± enriched CO_2 or CL alone. It is hypothesized that the additional available CO_2 resulted in enhanced chlorophyll *a* production, as has been found in other work. For example, Tripathi *et al.* 2001 found that certain strains of the chlorophytes *Chlorella vulgaris*, *Scenedesmus obliquus* and cyanobacteria *Spirulina*, *Nostoc*, and *Stigonema* exhibited a 2-3 fold increase in both chlorophyll and carotenoid contents when cells were exposed to 2% CO_2 after 21 days and 10 days, respectively. Another experiment investigated the effects of CO_2 deprivation on chlorophyll content in *Synechococcus lividus* (Miller and Hold 1977). Within 100 hr of CO_2 depletion, the chlorophyll *a* content of the cells significantly declined below controls. When cells were then given additional CO_2 for 48 hr, the chlorophyll *a* content of the cells significantly declined below that of controls. When the cells were then given additional CO_2 for 48 hr, the chlorophyll *a* levels increased back to control levels.

4.6 Conclusion

The environmental stressors of high pH x low salinity, low pH (short-term), and CL x CO_2 significantly enhanced lipid production on average by 56%, 43%, and 42%, respectively, in comparison to controls (23%). In contrast to reports for other chlorophytes, low N and/or P,

high pH alone, and 12:12 L:D x CO_2 in the strains of *Dunaliella* investigated did not enhance total FAs. The response of *Dunaliella* cells to pH stress resulted in an increase of total FAs as both a short-term (sec to hr) and a long-term ($\geq$ 24 hr) response. Enriched CO_2 under CL resulted in higher total FA production by the unusual strain *D. viridis* CONTAM during active growth, rather than only during senescence as for most microalgae, in the absence of dark cycle respiration. Furthermore, high intraspecific variation in both growth and neutral lipid production was observed within each environmental stressor. Future research can build upon this study to further elucidate the complex relationships among environmental stressors and total FA production in efforts to harness *Dunaliella* as a source of sustainable biofuel. Of the strains tested here, the unique strain *D. viridis* CONTAM can sustain both high maximal cell production and high total FAs content under continuous light or L:D periods, in both active growth and senescence.

4.7 References

Adam, M.S., 1997. Metabolic response of the halotolerant green alga *Dunaliella bardawil* to nitrogen : phosphorus ratios in batch culture. Folia Microbiol. 42, 357-360.

Azachi, M., Sadka, A., Fisher, M., Goldshlag, P., Gokhman, I., Zamir, A., 2002. Salt induction of fatty acid elongase and membrane lipid modifications in the extreme halotolerant alga *Dunaliella salina*. Plant Physiol. 129, 1320-1329.

Azov, Y., 1982. Effect of pH on inorganic carbon uptake in algal cultures. Appl. Environ. Microbiol. 43, 1300-1306.

Beardall, J., Berman, T., Heraud, P., Kadiri, M.O., Light, B.R., Patterson, G., Roberts, S., Sulzberger, B., Sahan, E., Uehlinger, U., Wood, B., 2001. A comparison of methods for detection of phosphate limitation in microalgae. Aquat. Sci. 63, 107-121.

Becker, E.W., 1994. Microalgae: Biotechnology and Microbiology. Cambridge University Press, Cambridge (New York). 293 pp.

Ben-Amotz, A., Avron, M., 1983. On the factors which determine massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil.* Plant Physiol. 72, 593-597.

Ben-Amotz, A., and Avron, M., 1989. The Biotechnology of Mass Culturing *Dunaliella* for Products of Commercial Interest. In: Creeswell, R.C., Rees, T.A.V, Shah, N. (Eds.), Algal and Cyanobacterial Biotechnology, Longman Scientific and Technical Press, New York, (New York). pp. 90-114.

Ben-Amotz, A., Tornabene, T.G., Thomas, W.H., 1985. Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol. 21, 72-81.

Ben-Amotz A, Polle JEW, Rao DVS (Eds), 2009. The Alga *Dunaliella*: Biodiversity, Physiology, Genomics and Biotechnology. Science Publishers, Enfield (New Hampshire). 555 pp

Britto, D.T., Kronzucker, H.J., 2002. NH_4^+ toxicity in higher plants: a critical review. J. Plant Physiol. 159, 567-584.

Carvalho, A., Malcata, F.X., 2004. Optimization of ω-3 fatty acid production by microalgae: crossover effects of CO_2 and light intensity under batch and continuous cultivation modes. Mar. Biotechnol. 7, 381-388.

Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.Y.S., Salley, S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour. Technol. 102, 1649-1655.

Davidi, L., Katz, A., Pick, U., 2012. Characterization of major lipid droplet proteins from *Dunaliella.* Planta 236, 19-33.

Føyn, B. 1934. Lebenszyklus, Cytologie und Sexualiät der Chlorophycee *Cladophora suhriana* Küzing. Arch. Protistenk. 83, 1-56.

Gardner, R., Peters, P., Peyton, B., Cooksey, K.E., 2011. Medium pH and nitrate concentration effects on accumulation of triglycerol in two members of the chlorophytes. J. Appl. Phycol. 23, 1005-1016.

Gardner, R.D., Cooksey, K.E., Mus, F., Macur, R., Moll, K., Eustance, E., Carlson, R.P., Gerlach, R., Fields, M.W., Peyton, B.M., 2012. Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte *Scenedesmus* sp. and the diatom *Phaeodactylum tricornutum*. J. Appl. Phycol. 24, 1311-1320.

García-González, M., Moreno, J., Cañavate, J.P., Anguis, V., Prieto, A., Manzano, C., Florencio, F.J., Guerrero, M.G., 2003. Conditions for open-air outdoor culture of *Dunaliella salina* in southern Spain. J. Appl. Phycol. 15, 177-184.

Gordillo, F.J.L., Goutx, M., Figueroa, F.L., Niell, F.X., 1998. Effects of light intensity, CO_2 and nitrogen supply on lipid class composition of *Dunaliella viridis*. J. Appl. Phycol. 10, 135-144.

Graham, L.E., Graham, J.M., Wilcox, L.W., 2009. Algae. 2nd Edition. Pearson Education, Inc., San Francisco (California). 616 pp.

Griffiths, M.J., van Hille, R.P., Harrison, S.T.L., 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 45, 1053-1060.

Guckert, J.B., Cooksey, K.E., 1990. Triglyceride accumulation and fatty acid profile changes in *Chlorella* (Chlorophyta) during high pH-induced cell cycle inhibition. J. Phycol. 26, 72-79.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York (New York). pp. 29-60.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234-236.

Guillard, R.R.L., 2005. Purification Methods for Microalgae. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts) pp. 117-132.

Kaplan, D., Richmond, A.E., Dubinsky, Z., Aaronson, S., 1986. Algal Nutrition. In: Richmond, A. (Ed.), CRC Handbook of Microalgal Mass Culture. CRC Press, Boca Raton, Florida. pp. 147-198.

Kawachi, M. & Noël, M.-H., 2007. Sterilization and Sterile Technique. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts) pp. 65-81.

Lombardi, A.T., Wangersky, P.J., 1995. Particulate lipid class composition of three marine phytoplankters *Chaetoceros gracilis*, *Isochrysis galbana* (Tahiti), and *Dunaliella tertiolecta* grown in batch culture. Hydrobiologia 306, 1-6.

Lorenz, M., Friedl, T., Day, J.G., 2005. Perpetual Maintenance of Actively Metabolizing Microalgal Cultures. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts) pp. 145-156.

Lynch, D.V. Thompson, Jr., G.A., 1982. Low temperature-induced alterations in the chloroplast and microsomal membranes of *Dunaliella salina*. Plant Physiol. 69, 1369-1375.

Miller, L.S., Holt, S.C. 1977. Effect of carbon dioxide on pigment and membrane content in *Synechococcus lividus*. Arch. Microbiol. 115, 185-198.

Moellering, E.R., Benning, C., 2010. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryot. Cell. 9, 97-106.

Peeler, T.C., Thompson, Jr., G.A., 1990. Effects of light on phospholipid metabolism in *Dunaliella salina*. Physiol. Plant. 78, 324-330.

Richmond, A., E.W. Becker., 1986. Technological Aspects of Mass Cultivation – A General Outline. In: Richmond, A. (Ed.), CRC Handbook of Microalgal Mass Culture. CRC Press, Inc., Boca Raton (Florida). pp. 245-264.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Gonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100-112.

Roessler, P.G., 1990. Environmental control of glycolipid metabolism in microalgae: commercial implications and future research directions. J. Phycol. 26, 393-399.

Sager, R., Granick, S., 1953. Nutritional studies with *Chlamydomonas reinhardtii*. Ann. NY. Acad. Sci. 56, 831-838.

Sforza, E., Simionato, D., Giacometti, G.M., Bertucco, A., Morosinotto, T., 2012. Adjusted light and dark cycles can optimize photosynthetic efficiency in algae growing in photobioreactors. PLoS ONE 7:e38975.

Shifrin, N.S., Chisholm, S.W., 1981. Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. J. Phycol. 17, 374-384.

Siron, R., Giusti, G., Berland, B., 1989. Changes in the fatty acid composition of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* during growth and under phosphorus deficiency. Mar. Ecol. Prog. Ser. 55, 95-100.

Sparkman, O.D., Penton, Z.E., Kitson, F.G., 2011. Gas Chromatography and Mass Spectrometry: A Practical Guide, 2nd Edition. Elsevier Inc., Burlington (Massachusetts). 611 pp.

Suen, Y., Hubbard, J.S., Holzer, G., Tornabene, T.G., 1987. Total lipid production of the green alga *Nannochloropsis* sp. QII under different nitrogen regimes. J. Phycol. 23, 289-296.

Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J. Biosci. Eng. 101, 223-226.

Thompson, P.A., Guo, M.-X., Harrison, P.J., Whyte, J.N.C., 1992. Effects of variation in temperature. II. On the fatty acid composition of eight species of marine phytoplankton. J. Phycol. 28, 488-497.

Tompkins, J., DeVille, M.M., Day, J.G., Turner, M.F., 1995. Culture Collection of Algae and Protozoa. Catalog of Strains. Ambleside (United Kingdom). 204 pp.

Tripathi, U., Sarada, R., Ravishankar, G.A. 2001. A culture method for microalgal forms using two-tier vessel providing carbon-dioxide environment: studies on growth and carotenoid production. World J. Microbiol. Biotechnol. 17, 325-329.

Uriarte, I., Farías, A., Hawkins, A.J.S., Bayne, B.L., 1993. Cell characteristics and biochemical composition of *Dunaliella primolecta* Butcher conditioned at different concentrations of dissolved nitrogen. J. Appl. Phycol. 5, 447-453.

Vollenweider, R.A., Talling, J.F., Westlake, D.F. 1974. A Manual on Methods for Measuring Primary Production in Aquatic Environments, 2nd Edition. Blackwell Scientific, Oxford. 225 pp.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S., Goodenough, U., 2009. Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. Eukaryot. Cell. 8, 1856-1868.

Weldy, C.S., Huesemann, M., 2007. Lipid production by *Dunaliella salina* in batch culture: effects of nitrogen limitation and light intensity. DOE J. Undergrad. Research V, 115-122.

Wetzel, R.G., Likens, G.E., 2001. Limnological Analyses, 2nd Edition. W.B. Saunders, New York (New York). 429 pp.

Wetzel, R.G., 2001. Limnology: Lake and River Ecosystems, 3rd Edition. Academic Press, San Diego (California). 1006 pp.

Xu, X.-Q., Beardall, J., 1997. Effect of salinity on fatty acid composition of a green microalga from an Antarctic hypersaline lake. Phytochemistry 45, 655-658.

Yongmanitchai, W., Ward, O.P., 1992. Growth and eicosapentaenoic acid production by *Phaeodactylum tricornutum* in batch and continuous culture systems. J. Am. Oil Chem. Soc. 69, 584-590.

Table 4.1 – Species and strain names for 5 strains of *Dunaliella* used for environmental stressor experiments. Mean biovolume, maximum cell production, the original culture medium, and optimal salinity in L1-Si medium for each strain are indicated.

Species Name	Commercial Source and Strain Number	Biovolume (mean μm^3 ± 1 SE; n = 30)	Maximum cell production (10^6 cells/mL ± 1 SE; n = 3) in L1-Si	Original Medium	Optimal Salinity (L1-Si Medium)
D. tertiolecta	CCAP 19/9	120 ± 6	6.2 ± 0.9	f/2-Si	30
D. tertiolecta	CCAP 19/24	132 ± 6	3.4 ± 0.4	f/2-Si	60
D. tertiolecta	UTEX 999	4.4 ± 0.8	4.4 ± 0.8	ASW	30
D. tertiolecta	UTEX 1000	168 ± 8	6.6 ± 0.8	Erdschreiber's	30
D. viridis	CONTAM	10.0 ± 0.6	10.0 ± 0.6	2X Erdschreiber's	60

Table 4.2 – The pH values for bench-scale cultures of *D. viridis* CONTAM under a 12:12 L:D photoperiod or continuous light (CL). Controls were under nutrient-replete conditions and N, P, or N+P were in a lower nutrient regime (20% of the nutrient-replete concentration(s)). After 7 days of growth, flasks either remained under 12:12 L:D or were moved to CL; thus, day 10 and day 14 were 3 and 7 days after flasks were moved, respectively.

Treatment	pH after 10 days under 12:12 L:D	pH after 3 days in CL
Control	9.3925	9.4295
N+P	9.198	9.452
P	9.383	9.5505
N	9.4145	9.6445
	pH after 14 days under 12:12 L:D	**pH after 7 days in CL**
Control	9.2525	9.433
N+P	8.4255	8.985
P	8.7025	9.236
N	8.7985	9.2715

Table 4.3 – Total percent FAs for *D. viridis* CONTAM under long-term pH stress, with corresponding p-values if statistically significant from percent total FA content produced from adjusted or from unadjusted cultures. LS Mean Diff = the least squared means difference from the baseline (day 3); NS = not significant.

Method	Day	Mean	SE	LS Mean Diff	P-value
Salinity 60 to 30 (Unadjusted)	Day 3	9.61	1.66		N/A
	Day 6	29.80	6.95	20.19	0.003
	Day 9	28.56	8.02	18.95	0.004
	Day 12	41.70	6.63	32.09	<0.001
	Day 14	39.84	4.61	30.23	<0.001
Salinity 60 to 30 (Adjusted)	Day 3	19.20	2.51		N/A
	Day 6	33.80	7.55	14.59	NS
	Day 9	55.64	3.05	36.44	0.014
	Day 12	46.06	15.60	26.86	0.031
Salinity 60 to 60 (Unadjusted)	Day 3	13.04	4.61		N/A
	Day 6	18.42	5.89	5.38	NS
	Day 9	23.68	3.63	10.64	0.019
	Day 12	26.53	4.42	13.49	0.007
	Day 14	37.70	1.20	24.66	<0.001

Table 4.4 – Total percent FAs for each of the strains under short-term pH stress, with corresponding p-values if statistically significant from percent total FA content produced at the control pH (before adjustment). LS Mean Diff = the least squared means difference from the control salinity (30 or 60); NS = not significant.

Strain	pH	Time Point (hours)	Mean	SE	LS Mean Diff	P-value
D. tertiolecta CCAP 19/9	pH 10	Control	14.46	1.68		N/A
		30 sec	18.90	1.75	4.44	NS
		5 min	18.86	0.79	4.40	NS
		30 min	17.55	1.20	3.09	NS
		1 hr	19.07	5.24	4.61	NS
		2 hr	17.83	2.91	2.86	NS
		24 hr	19.71	5.96	5.25	0.046
	pH 7	Control	15.26	2.67		N/A
		30 sec	19.63	3.33	4.37	0.004
		5 min	18.67	1.72	3.41	0.015
		30 min	19.63	2.44	4.37	0.004
		1 hr	19.50	3.05	4.23	0.005
		2 hr	20.51	2.27	5.25	0.001
		24 hr	19.61	1.34	4.35	0.004
D. viridis CONTAM	pH 10	Control	40.10	5.19		N/A
		30 sec	32.95	1.88	-7.15	0.001
		5 min	33.05	2.02	-7.05	0.001
		30 min	31.75	4.09	-8.36	<0.001
		1 hr	34.22	3.90	-5.88	0.003
		2 hr	32.26	2.07	-7.84	<0.001
		24 hr	32.39	2.45	-7.71	<0.001
	pH 7	Control	37.24	3.03		N/A
		30 sec	32.83	3.09	-4.41	0.021
		5 min	43.33	9.58	6.09	0.004
		30 min	32.18	3.47	-5.06	0.011
		1 hr	31.52	0.31	-5.72	0.006
		2 hr	32.70	1.10	-4.54	0.019
		24 hr	31.30	1.37	-5.94	0.005

Table 4.4 Continued

Strain	pH	Time Point (hours)	Mean	SE	LS Mean Diff	P-value
D. tertiolecta CCAP 19/24	pH 10	Control	30.18	3.56		N/A
		30 sec	28.01	1.35	-2.18	0.049
		5 min	30.30	0.70	0.11	NS
		30 min	28.43	3.76	-1.76	NS
		1 hr	24.96	0.90	-5.23	0.001
		2 hr	29.50	1.65	-0.24	NS
		24 hr	26.94	1.16	-3.25	0.010
	pH 7	Control	30.76	1.06		N/A
		30 sec	35.58	1.94	4.82	0.081
		5 min	31.95	2.47	1.19	NS
		30 min	32.45	12.81	1.69	NS
		1 hr	30.78	4.25	0.02	NS
		2 hr	29.57	0.63	-1.18	NS
		24 hr	29.85	4.78	-0.90	NS
D. tertiolecta UTEX 999	pH 10	Control	18.65	2.62		N/A
		30 sec	23.86	2.14	5.22	0.006
		5 min	22.47	4.22	3.83	0.025
		30 min	19.90	2.94	1.26	NS
		1 hr	20.05	4.33	1.40	NS
		2 hr	21.72	3.55	3.08	NS
		24 hr	22.15	1.57	3.50	0.035
	pH 7	Control	21.26	5.02	0.00	N/A
		30 sec	21.45	5.93	0.19	NS
		5 min	20.68	5.03	-0.58	NS
		30 min	17.82	4.29	-3.44	NS
		1 hr	18.87	2.52	-2.39	NS
		2 hr	20.08	0.83	-1.18	NS
		24 hr	17.89	0.60	-3.37	NS

Table 4.5 – Total percent FAs for *D. viridis* CONTAM under 12:12 L:D ± CO_2 or continuous light (CL) ± CO_2 and corresponding p-values if statistically significant. LS Mean Diff = the least squared means difference from the baseline (day 6); NS = not significant.

Method	Day	Mean	SE	LS Mean Diff	P-value
12:12 No CO_2	Day 6	15.79	2.34		N/A
	Day 9	22.80	1.33	7.01	0.003
	Day 12	27.15	1.83	11.36	<0.001
12:12 With CO_2	Day 6	14.26	2.91		N/A
	Day 9	27.85	2.47	13.59	<0.001
	Day 12	31.46	1.78	17.20	<0.001
CL No CO_2	Day 6	13.04	1.61		N/A
	Day 9	19.56	2.20	6.52	0.001
	Day 12	22.62	1.15	9.58	<0.001
CL With CO_2	Day 6	14.18	1.52		N/A
	Day 9	33.24	5.92	19.07	0.011
	Day 12	42.84	8.22	28.67	0.002

Table 4.6 – Total percent FAs for *D. viridis* CONTAM under 12:12 L:D ± CO_2 or continuous light (CL) ± CO_2 and corresponding p-values if statistically significant among methods. LS Mean Diff = the least squared means difference at either day 9 or 12; NS = not significant.

Day	Comparison	LS Mean Diff	P-value
Day 9	12:12 No CO_2 vs 12:12 With CO_2	-6.58	NS
	CL No CO_2 vs CL With CO_2	-12.5	0.026
	12:12 No CO_2 vs CL No CO_2	0.49	NS
	12:12 With CO_2 vs CL With CO_2	-5.48	NS
Day 12	12:12 No CO_2 vs 12:12 With CO_2	-5.84	0.189
	CL No CO_2 vs CL With CO_2	-19.1	0.006
	12:12 No CO_2 vs CL No CO_2	1.79	NS
	12:12 With CO_2 vs CL With CO_2	-11.5	0.049
Day 12	12:12 vs CL	-9.67	NS
	No CO_2 vs With CO_2	-24.9	0.009

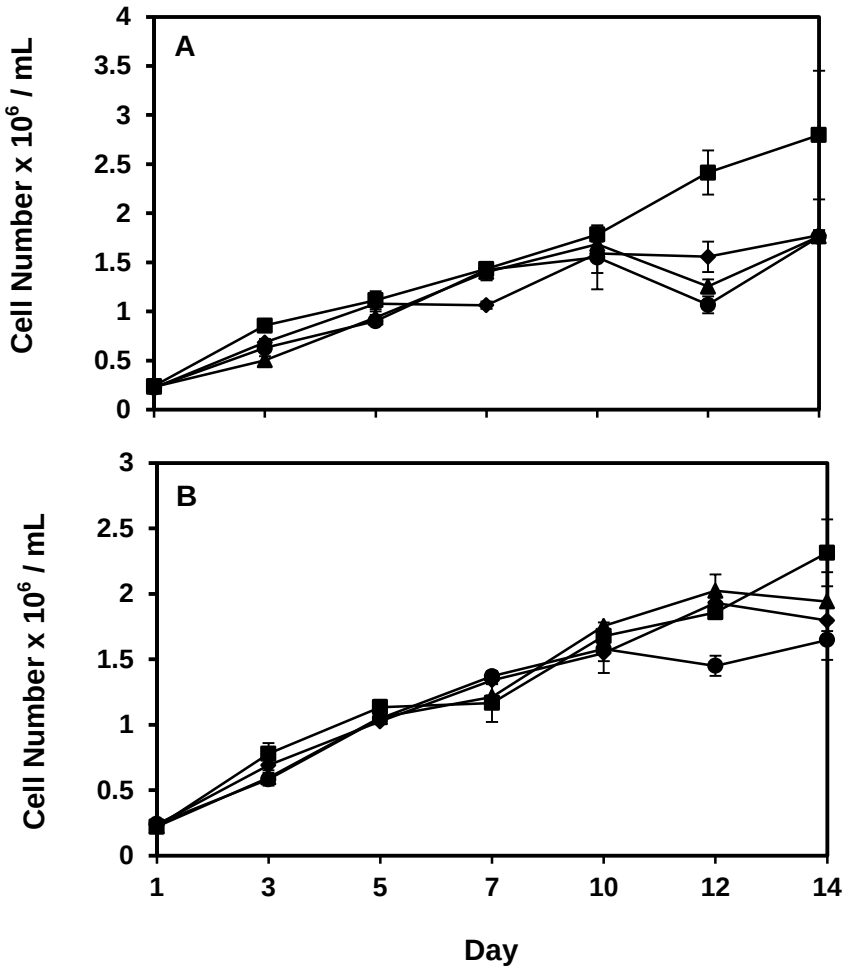

Figure 4.1 – Mean cell production for *Dunaliella viridis* CONTAM comparing low N and/or P (20% of control, replete media concentration(s)). Cell production under replete nutrient concentrations (■), low N (▲), low P (♦), and low N&P (●) under A) 12:12 L:D photoperiod and B) continuous light (CL) (means ± 1 SE; n = 2).

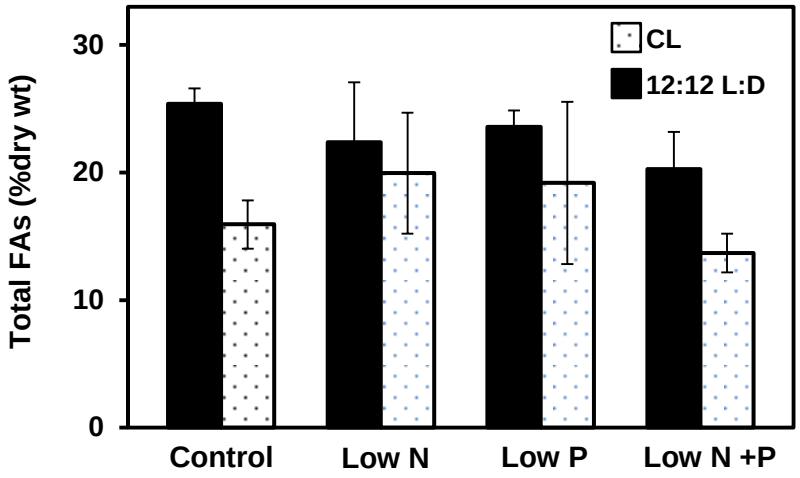

Figure 4.2 – Total FAs as percent dry weight for *Dunaliella viridis* CONTAM under N and/or P-replete conditions (control) versus under low N and/or low P conditions. Each grouping includes 12:12 L:D and continuous light for the control (replete conditions) versus low N and/or P conditions. Data are given as means ± 1 SE; n = 2.

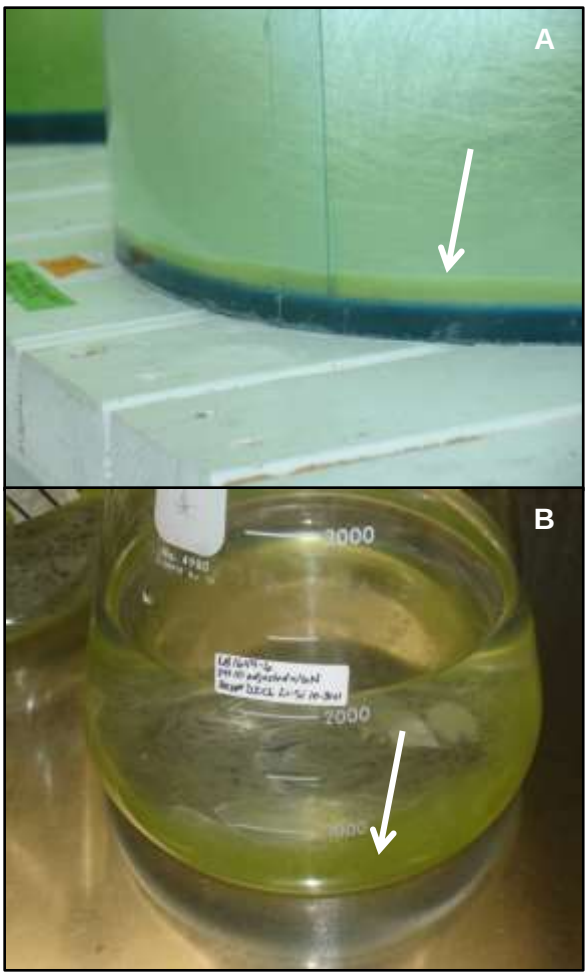

Figure 4.3 – Photographs depicting cell flocculation at either A) mass culture or B) bench-scale in response to high pH. White arrows indicate the layer of *Dunaliella* cells at the bottom of each container.

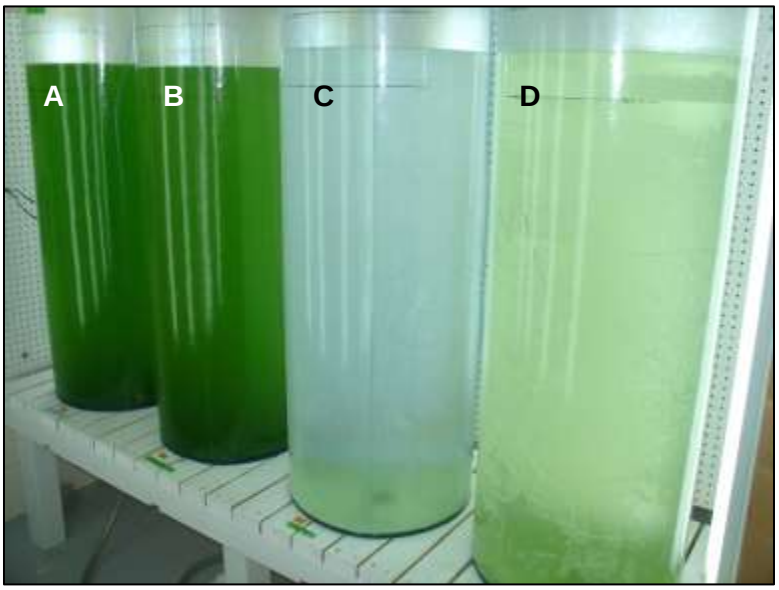

Figure 4.4 – Photograph of mass culture tubes under long-term pH stress experiment after 9 days: A and B) control cultures, both grown in salinity 60; C) culture at salinity 60 x high pH; D) culture at 30 x high pH. C) *Dunaliella* cells flocculated to the bottom of the tube and did not recover. D) *Dunaliella* cells flocculated after high pH adjustment, cells recovered and increased cell production by day 9.

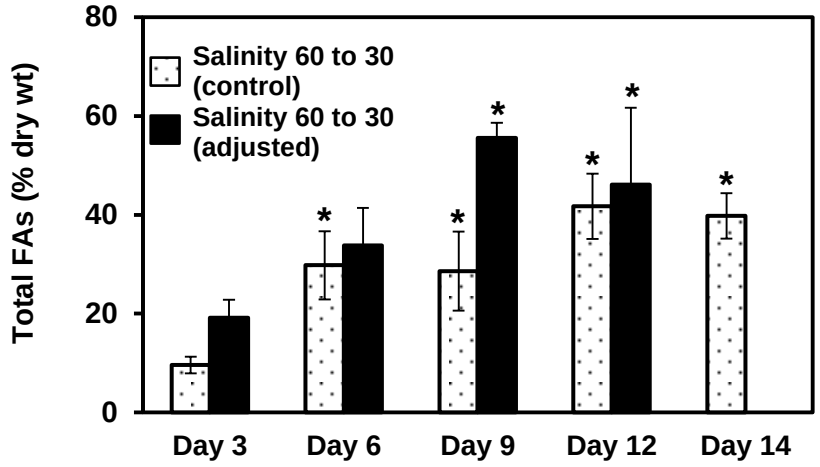

Figure 4.5 – Total FAs as percent by dry weight for *Dunaliella viridis* CONTAM in the long-term pH stress experiment, with controls (no pH adjustment) compared to the treatment (pH 10 adjustment). In response to the high-pH adjustment, the algal populations flocculated and settled out to the bottom of culture vessels, then recovered after 9 days. Thus, samples were taken 3, 6, 9, and 12 days post-recovery. Controls were subsampled at 3-day intervals and at the end of the experiment on day 14. Results were compared every 3 days, using a "baseline" reading at day 3 for controls or day 3 post-recovery for the treatment. Note that because mass cultures adjusted to pH 10 at salinity 60 did not grow, data could not be collected from those cultures for total FAs. Data were collected at early senescence (day 14) for the control cultures, but not for treatments due to incomplete FA analysis. Significant differences from day 3 are indicated by an asterisk ($p \leq 0.05$) Data are given as means ± 1 SE; n = 3.

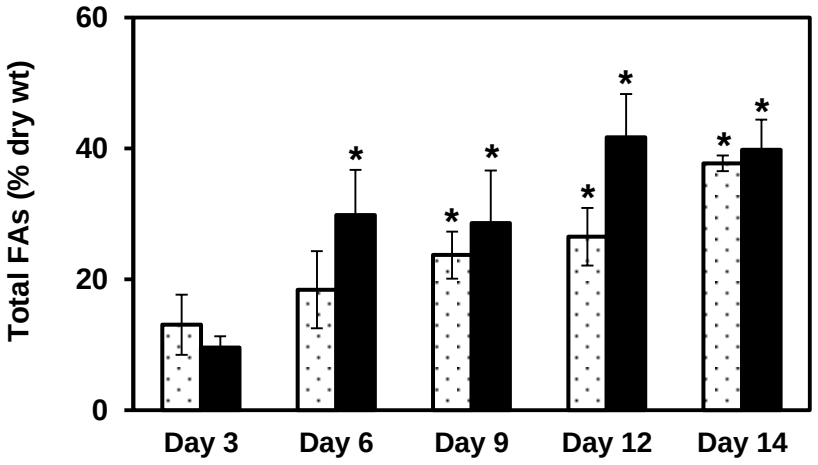

Figure 4.6 – Total FAs as percent dry weight for *Dunaliella viridis* CONTAM under long-term pH stress x hypo-osmotic stress, comparing controls (no salinity adjustment) to the treatment (salinity adjusted from 60 to 30). Samples were taken at early exponential (day 6), mid-exponential (day 9), and late exponential (day 12) growth phases, and in early senescence (day 14). Significant differences from day 3 are indicated by an asterisk ($p \leq 0.05$). Data are presented as means ± 1 SE (n = 3).

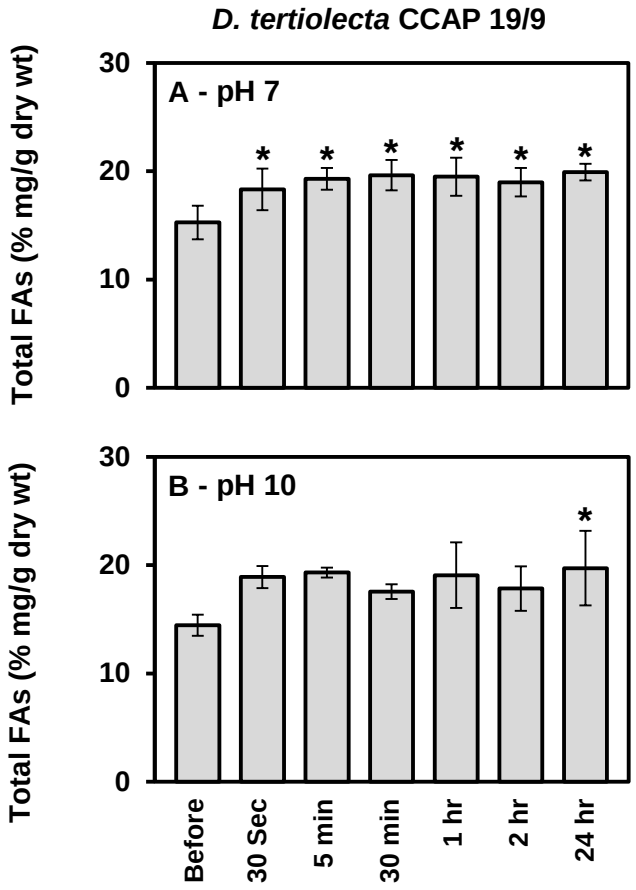

Figure 4.7 – Total FAs as percent by dry weight (mg/g dry wt) for *Dunaliella tertiolecta* CCAP 19/9 under short-term pH stress: A) *D. tertiolecta* CCAP 19/9 under low pH stress (initial pH taken from 9 to 7); and B) *D. tertiolecta* CCAP 19/9 under high pH stress (initial pH taken from 9 to 10). Significant differences from controls (= Before) are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

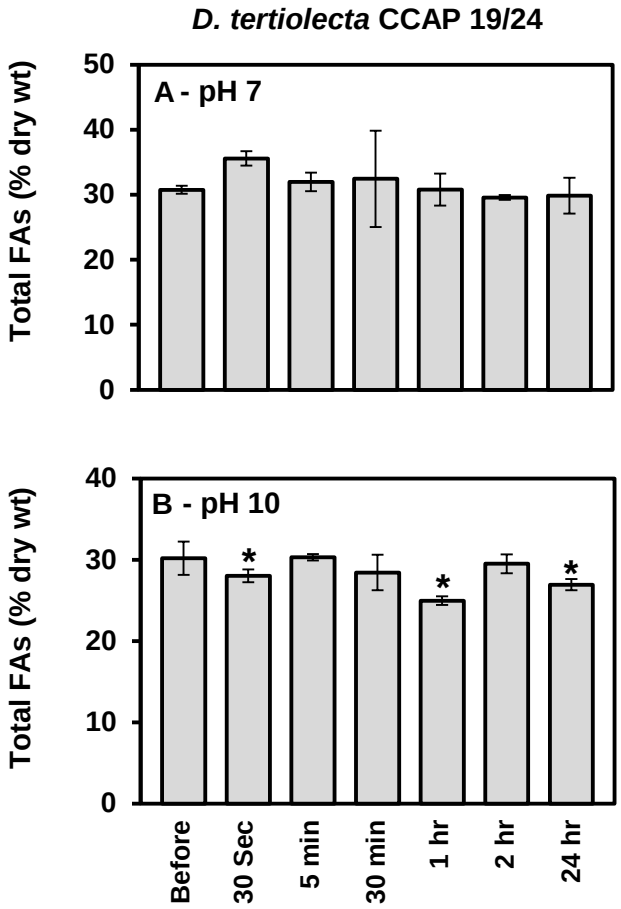

Figure 4.8 – Total percent FAs by dry weight for *Dunaliella tertiolecta* CCAP 19/24 under short-term pH stress: A) *D. tertiolecta* CCAP 19/24 under low pH stress (initial pH taken from 9 to 7); B) *D. tertiolecta* CCAP 19/24 under high pH stress (initial pH taken from 9 to 10). Significant differences from controls (= Before) are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

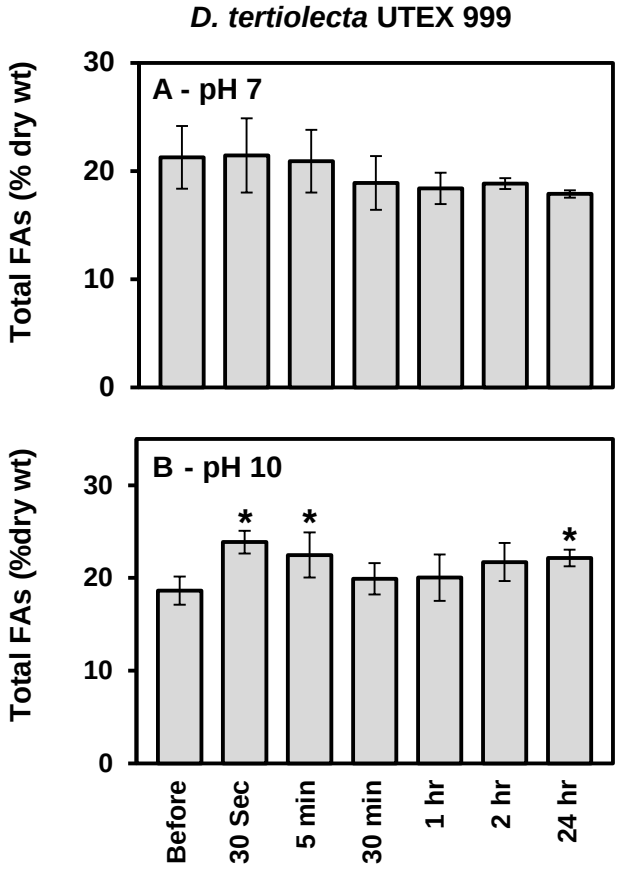

Figure 4.9 – Total FAs as percent by dry weight for *Dunaliella tertiolecta* UTEX 999 under short-term pH stress: A) *D. tertiolecta* UTEX 999 under low pH stress (initial pH taken from 9 to 7); B) *D. tertiolecta* UTEX 999 under high pH stress (initial pH taken from 9 to 10). Significant differences from controls (= Before) are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

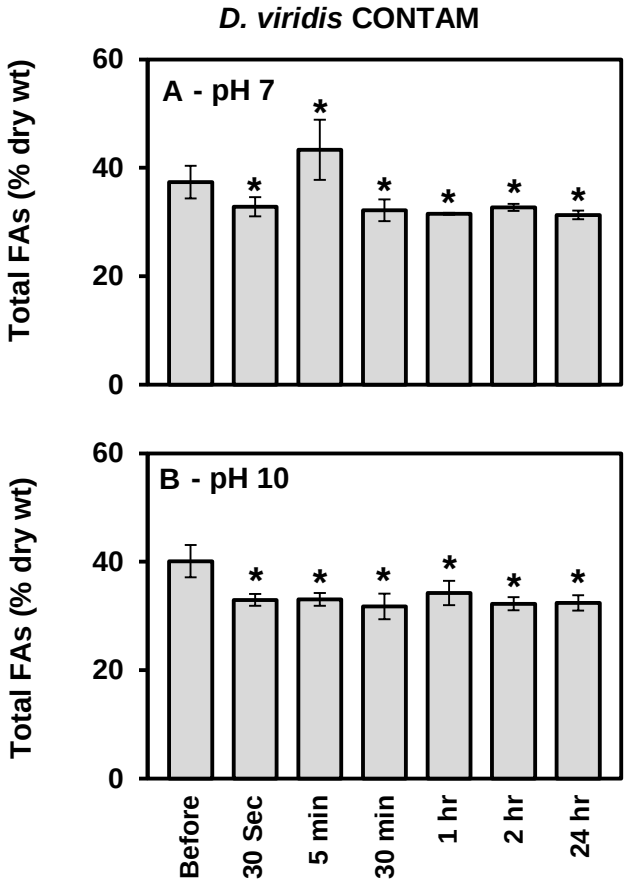

Figure 4.10 – Total FAs as percent by dry weight for *Dunaliella viridis* CONTAM under short-term pH stress: A) *D. viridis* CONTAM under low pH stress (initial pH taken from 9 to 7); B) *D. viridis* CONTAM under high pH stress (initial pH taken from 9 to 10). Significant differences from controls (= Before) are indicated by an asterisk ($p \leq 0.05$) Data are given as means ± 1 SE (n = 3).

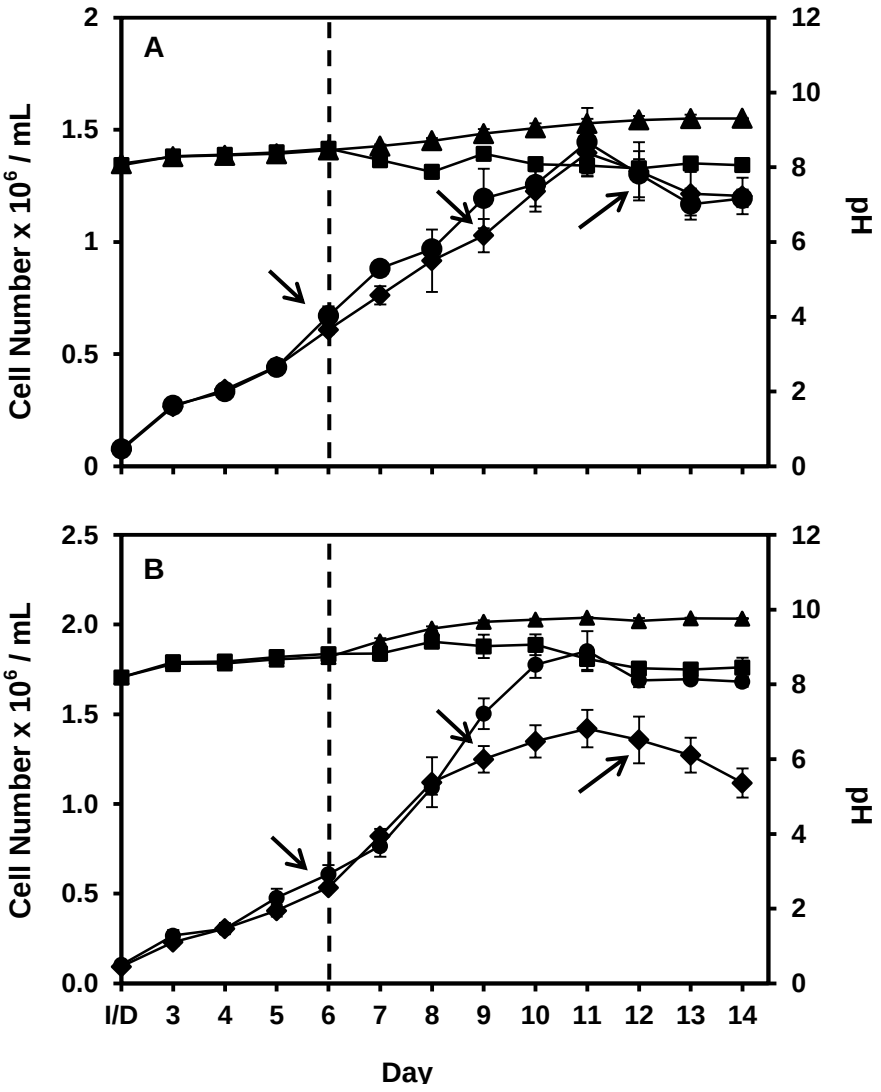

Figure 4.11 – Mean cell production and pH values for mass culture of *D. viridis* CONTAM comparing ± enriched CO_2. Cell production with (●) and without (♦) enriched CO_2, and pH values with (▲) and without (■) enriched CO_2 under A) 12:12 L:D photoperiod and B) continuous light (CL). Arrows indicate where sub-samples were taken for FA analysis. The dashed vertical line indicates initiation of pulsed enrichment with 4 hr of CO_2 bubbling daily

for A) and B), and also the initiation of CL for all cultures in B). Data are given as means ± 1 SE (n = 4).

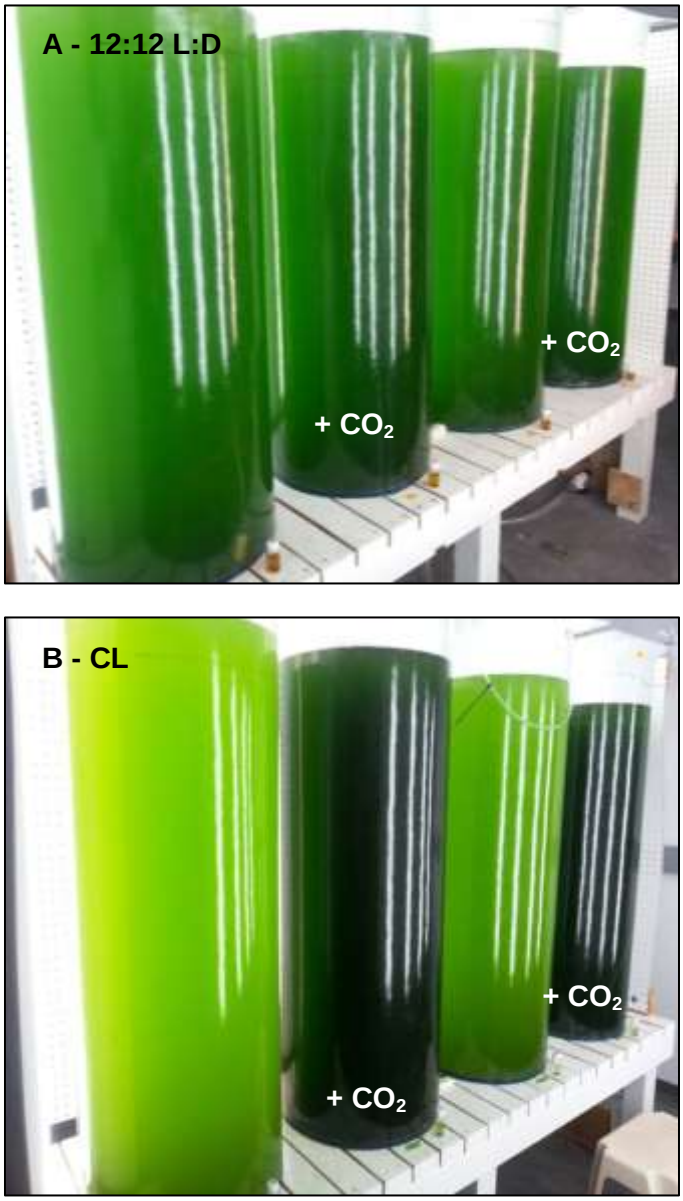

Figure 4.12 – Examples of mass cultures in the CO_2 x light regime experiment: A) 12:12 L:D photoperiod or B) continuous light (CL) and tubes that received 4 hr bubbling of CO_2 are indicated.

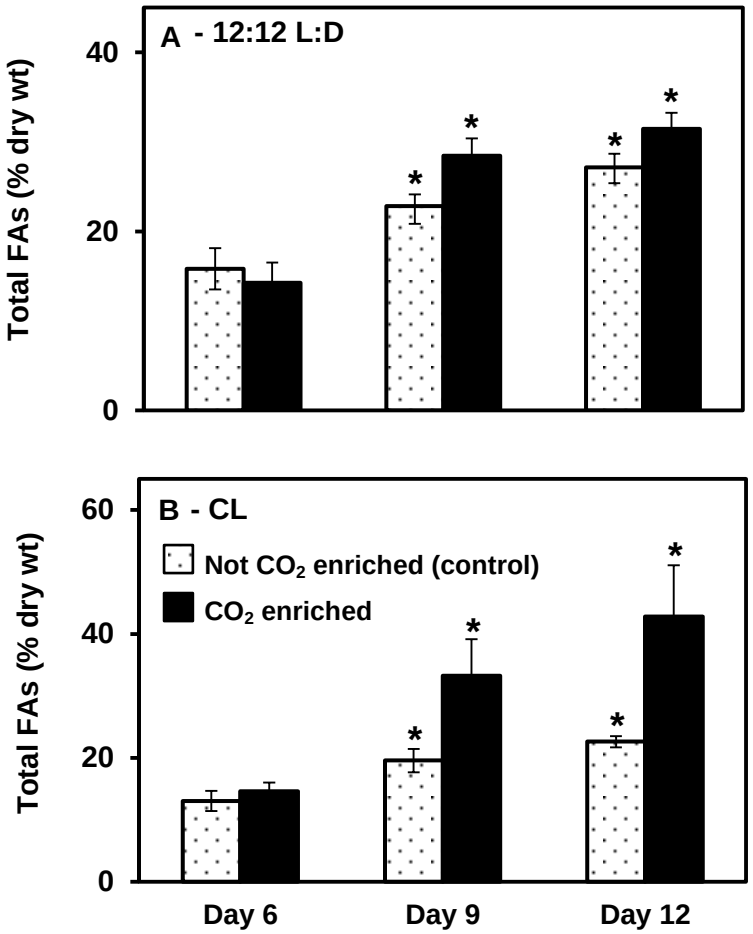

Figure 4.13 – Total FAs as percent by dry weight for the mass culture of *D. viridis* CONTAM ± enriched CO_2: A) All cultures were grown under 12:12 L:D and B) all cultures were grown under continuous light after 6 days of growth. Samples were taken at mid-exponential (day 6), late-exponential (day 9) growth phases, and early senescence (day 12). Cultures were compared ± CO_2. Significant differences from day 6 are indicated by an asterisk ($p \leq 0.05$). Data are presented as means ± 1 SE (n = 4).

5. AUTO-FLOCCULATION, ELECTRO-FLOCCULATION, AND HOLLOW-FIBER FILTRATION TECHNIQUES FOR HARVESTING THE SALTWATER MICROALGA *DUNALIELLA*

Submitted to *Journal of Applied Phycology*

5.1 Abstract

This research assessed the efficiency of several harvesting methods on *Dunaliella* spp. saltwater microalgae, several strains of which have been successfully mass cultured. While it is possible to use lipids from microalgae as a feedstock for biofuels to replace petroleum-based fuel, at this time the commercialization of harvesting has not been proven economically feasible. Auto-flocculation (by adjusting the pH to exponentially growing cells), indirect electro-flocculation (by applying aluminum hydroxide flocculant to culture), and hollow-fiber filtration (by separating biomass from medium using tangential flow) were compared as potential harvesting mechanisms for bench-scale (3 to 10 L) and mass culture scale (30 to 150 L) volumes of *D. viridis* CONTAM. Both auto-flocculation and electro-flocculation had significant biomass recovery (> 95%). The flocculation methods required removal of added chemicals and/or flocculant before the medium could be reused. However, hollow-fiber filtration yielded almost complete biomass recovery (> 99%). In addition, the filtrate from the hollow-fiber filtration experiment was reused as culture medium, with no observed detrimental effects (cell size, shape, or cell production) to the cells. Imposing high salinity stress experiment on the concentrate produced from the filtration method did not increase total fatty acids (FAs). However, total FAs increased after hollow-fiber filtration

(49%) in comparison to total FAs in samples before filtration (36%). This research indicates that hollow-fiber filtration as a commercial harvesting mechanism offers attractive advantages when compared to auto-flocculation and indirect electro-flocculation.

5.2 Introduction

The halotolerant, saltwater microalgae *Dunaliella* spp. accumulate neutral lipids in response to environmental stressors (Roessler 1990, Ben-Amotz *et al.* 2009). These organisms have high population growth rates as cell production, and are successful competitors in scale-up experiments due to their tolerance to high salinities (Avron and Ben-Amotz 1992), making them ideal candidates for mass culturing (Hu *et al.* 2008, Rodolfi *et al.* 2009). Mass culture of *Dunaliella* has been accomplished in an array of open versus closed systems: For example, vertical mass culture tubes provide a high surface-to-volume ratio allowing for efficient gas exchange and mixing of culture (Miyamoto *et al.* 1988). In comparison to open pond or raceway systems, closed photobioreactors allow for better control over the physical, chemical, and biological environment of cultures (Moulton *et al.* 1987, Miyamoto *et al.* 1988).

Once growth has been established, the next step for successful commercialization is the efficient harvesting of the biomass. The biomass must be dewatered so that the products of interest can be extracted; thus, harvesting biomass is a significant cost component for mass culture (Borowitzka 1992, Mallick 2002). Removal of water and the concentration of biomass has been accomplished via centrifugation, electro-flocculation, chemical flocculation, auto-flocculation, or filtration. Centrifugation, while the most direct method, is

an energy-intensive process (Vandamme *et al.* 2010) and is only practical as the sole harvesting technique if there is a highly valuable end product (Grima *et al.* 2003, Şirin *et al.* 2012). The reuse of growth medium after harvesting is imperative to reducing production costs (Borowitzka 1992, Mallick 2002).

Spontaneous or auto-flocculation occurs in response to a sharp increase in pH (Richmond and Becker 1986). Flocculation techniques are used to harvest microalgal biomass because they effectively aggregate microalgal cells to a larger size, thus enhancing biomass recovery (Granados *et al.* 2012). Increasing the pH to 8.0 – 10.5 resulted in ~90% biomass recovery for the cyanobacterium *Anabaena marina* (González-López *et al.* 2009), the diatom *Phaeodactylum tricornutum* (Şirin *et al.* 2012), and the chlorophyte *Dunaliella tertiolecta* (Horiuchi *et al.* 2003).

Electro-flocculation is a common method for the removal of suspended solids (Xu *et al.* 2010). In this method, the oxidation of an electrode by an electrical current and the metal ions released form positively charged hydroxides that attach to negatively charged microalgae and effectively disrupt particle suspension (Grima *et al.* 2003, Granados *et al.* 2012). Xu *et al.* (2010) determined this method to be cost-effective with low energy requirements (0.3 kWh m^{-3}) and ~95% biomass recovery.

Hollow-fiber filtration separates cells in solution using tangential flow of the filtering solution along the surface of a porous fiber membrane (Shimizu *et al.* 1996, Mallick 2002). Because there is flow parallel to the filtration surfaces, particles do not accumulate at the filter surface, as is common with other filtration techniques. The concentrated particles in solution scour the filtration surface, effectively preventing the filter surface and pores from

becoming clogged. This technique can utilize very small pore sizes for filtering solutions with high particle counts without the issues associated with clogging that plague many other filtration techniques.

This research compared the biomass recovery efficiencies of large-scale volumes (defined here as ~10 to 150 L) of *Dunaliella* strain UTEX 1644[5] (10^6 cells/mL) from three harvesting techniques including auto-flocculation, indirect electro-flocculation, and hollow-fiber filtration. This strain was selected for harvesting experiments because it has been shown to be a promising candidate for sustainable biofuel production. This strain is capable of sustaining both high cell production and high total fatty acid (FA) content in mass culture (~150 to 175 L; see Chapter 4). First, we investigated the effectiveness of auto-flocculation as a harvesting method at both bench-scale (3 L) and mass culture scale (150 L), by increasing the pH to 10 using sodium hydroxide (NaOH). Second, we tested an indirect electro-flocculation technique, wherein flocculant was produced in a controlled environment and then was applied to culture (3 L) separately. We used this approach because the flocculant dosing rate and effectiveness were better controlled by separating the production and application portions of electro-flocculation. Third, we used hollow-fiber filtration to concentrate *Dunaliella* cultures consisting of 10^6 cells/mL in 10 L or 30 L volume. We also exposed the dewatered concentrate to high salinity.

[5] ITS2 sequence data and morphology features indicate that this strain falls within the *D. viridis* clade (Chapter 2). Note that strain UTEX 1644, as sent to us ~5 yr ago by the culture collection, was not a strain of *D. salina* but, rather a unique strain of *D. viridis* that evidently was a culture contaminant in the collection. Clearly, it was not contaminated by other cultures in our laboratory because all of them are distinct from this strain in major characteristics of morphology, cell production, lipid production throughout the cycle, etc. When UTEX 1644 was ordered recently, the strain was a *D. salina* so the contaminant has been removed. Here, we refer to the unique strain as *D. viridis* CONTAM.

5.3 Materials and Methods

5.3.a Cultures

The *Dunaliella* isolate was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX). This strain was first cultured in Artificial Seawater medium (ASW, made using f/2-Si nutrients; Guillard 1975, Tompkins *et al.* 1995) as designated by the culturing facility. However, ASW contains soil water with undefined constituents. Its nutrient components (f/2-Si) were similar to those of L1-Si medium. Therefore, L1-Si was selected for use in this study.

The *Dunaliella* isolate was purified using a combination of differential centrifugation and flow cytometry (Coulter® EPICS Altra, Hialeah, Florida) to remove bacterial contamination (Guillard 2005, Kawachi and Noël 2005, Chapter 1 and methods therein). Replicate stock sub-cultures (n = 3) were grown in L1-Si at 25 mL volume (n = 3) under an optimal salinity of 60 and an initial pH of 8.3-8.4 (see Chapter 3), at 23˚C and a light intensity of ~180 $\mu Em^{-2}sec^{-1}$ (light source, ProLume® compact fluorescent bulbs) under a 12-hr : 12 hr light : dark (L:D) photoperiod.

Cell densities and population growth as cell production were assessed from stock culture subsamples preserved in acidic Lugol's solution (1% final concentration; Vollenweider *et al.* 1974). Samples were analyzed within 1-2 days after collection using Palmer-Maloney chambers (Wetzel and Likens 2000) under light microscopy at 200x magnification using an Olympus BH-2 light microscope (Germany).

The mass culturing facility consisted of a series of growth tubes, each 1.21 m height x 30 cm diameter, made of lightweight 0.1-cm polymer fiberglass with ~90% transparency.

Each tube held a maximum volume of ~175 L. Light banks (Standard Utilitech 48 ½ in fluorescent light, Lowes, Mooresville, North Carolina) provided ~160 to 180 µE/m^2/sec for each tube. The temperature was maintained at 23 ± 1 ^oC. Tubes were sampled daily between 0800 and 0900 hr for temperature, pH (Oakton Waterproof pH Testr 30 Pocket pH Tester, Oakton Instruments, Vernon Hills, Illinois), and cell counts as described above.

In preparation for mass culturing, tubes were filled with deionized water (DI) adjusted to salinity 30 and pH (8.3-8.4) with Coralife® salts (CL; Franklin, Wisconsin) + each of parts ProLine® A/B nutrient mixtures (F/2; Aquatic Eco-Systems, Apopka, Florida) (Guillard 1975). Stock culture was grown by inoculating ~3 L volume (salinity 30 or 60, adjusted with CL + L1-Si nutrients) (Hydrolab MiniSonde 4, Hach Company, Loveland, Colorado) with ~300 mL of culture (i.e. a 10% inoculum) under growth conditions described above. Once the stock reached late exponential growth (as determined by cell counts), it was added to a freshly prepared mass culture tube (~15 L stock culture in ~150 L prepared medium) and grown as stock for the mass culture experiments. The total number of tubes and volume prepared varied by experiment (described below). Cultures were grown to a density of 10^6 cells/mL within 7-10 days depending on the the experimental conditions.

5.3.b Auto-flocculation through adjustment to high pH

The auto-flocculation experiment tested *Dunaliella* culture grown in bench-scale or mass culture at salinity 30 or 60 (pH 8.3-8.4), then adjusted to pH 10 with 2N and/or 6N NaOH (Fisher Scientific, Fair Lawn, New Jersey) (n = 3 replicates for controls and salinity x NaOH treatments).

At bench scale, the culture used for this experiment was grown at ~3.5 L volume as described above, at salinity 30 or 60. At late exponential phase (i.e. maximum cell production, as determined by cell counts), the culture was poured into a 5-L pitcher, thoroughly mixed, and adjusted to a pH ~10 with either 2N or 6N NaOH. A ~500 mL subsample was removed prior to adjustment as a control. After adjustment, remaining culture was evenly distributed into ~500 mL subsamples. All replicates were sampled for pH and cell counts. In addition, samples for lipid analysis (total FAs) were taken at 30 sec, 1 hr, 2 hr, 3 hr, and 24 hr after pH adjustment. Samples were centrifuged (3,000 RPM, 704 x *g*, 10 min - CL2 Centrifuge, ThermoScientific, Milford, Massachusetts) and the pellets were immediately frozen at -80˚C until FA analysis (within 7-10 days for all experiments; L. Dean, U.S. Department of Agriculture, NCSU, personal communication).

Preliminary results at bench-scale volume indicated that high pH (10) x high salinity (60) resulted in an efficient harvesting mechanism, so mass cultures used for this experiment were grown in salinity 60. The mass cultures were grown as described above (n = 3 for controls and treatments; 150 L volume). Controls were initially at pH 8.3-8.4; treatment cultures (n = 3) were adjusted to an initial pH 10 using 6N NaOH. The cultures were grown until senescence (~21 days). Samples (~500 mL) were centrifuged at ~3 day intervals and immediately frozen at -80˚C until total FA analysis.

5.3.c Electro-flocculation

The culture for this experiment was grown in ~3.5 L volume (n = 3) at salinity 30 under the conditions described above until late exponential phase. Each culture was then mixed and divided into ~500 mL subsamples; each treatment subsample was mixed

thoroughly while adding 0.5 mL of the settled flocculant, prepared as described below, per L of culture. Controls (~500 mL) did not receive flocculant addition. The pH and salinity were measured in all replicates, and sampled for cell counts. In addition, samples for lipid analysis (total FAs) were taken at 30 sec, 5 min, 30 min, 1 hr, 2 hr, and 24 hr after the addition of flocculant. Samples were centrifuged (3,000 RPM, 704 x *g*, 10 min) and immediately frozen at -80˚C until analysis of total FA analysis.

Iron (Fe) and aluminum (Al) anodes were used to determine flocculant production efficiencies. In 150-mL batches, medium (salinity 30, made with DI + NaCl) was added to a 250-mL beaker with electrodes placed on opposite sides. To create a Fe anode, the positive lead was connected to a stainless steel electrode, and vice versa to create an Al anode. The water was thoroughly mixed using a magnetic stir bar and the following currents were applied: 0.1, 0.4, 0.7, and 1.0 V for 5, 10, 15, or 20 min. After the electrodes were removed, stirring speed was increased to remove any foam accumulation and the pH was measured immediately and 72 hr after settling. Pre-washed glass fiber filters (Whatman, UK, 0.2 µm pore size) were pre-weighed and flocculant samples filtered using a vacuum. The flocculant samples were dried for 24 hr. In all, 33 samples, including a control where no current was applied, were collected. Each sample was weighed and the net weight determined from the control sample. The theoretical weight (*m*) of each hydroxide was calculated using Faraday's second law of electrolysis:

$$m = It\frac{M}{Fz}$$

where the Faraday Constant (F) = 96,485 C/mol, electrons used in reaction (z) = 3, and molar mass (M) = 78 g/mol for $Al(OH)_3$ or 106.8 g/mol for $Fe(OH)_3$. Harvesting efficiency of electro-flocculation was tested on *Dunaliella* with $AL(OH)_3$ flocculant prepared by running 3.0V for 15 min (parameters changed due to geometry of electrodes: 105 cm^2 total area; 3.8 cm distance between electrodes). This flocculant was used in a process we refer to here as indirect electro-flocculation.

5.3.d Hollow-fiber filtration

This experiment tested harvest of *Dunaliella* culture that was concentrated from 10 L (n = 3, repeated 3 times) or 30 L (n = 3, repeated 2 times). For two of the 10 L experiments, three 4-L flasks were each filled with ~3 L (salinity 30) and each was inoculated with ~300 mL culture grown under the conditions described above until late exponential phase. For the last 10 L experiment replicate, a mass culture tube was filled with 30 L of medium (salinity 30) and inoculated with ~3 L of *Dunaliella* culture. This tube was grown under conditions described above, until late exponential phase. For the 30 L experiments, three mass culture tubes (n = 2) were filled with 30 L of medium (salinity 30) and inoculated with ~3 L of *Dunaliella* culture. Tubes were grown until late exponential phase.

Replicates were then harvested using hollow-fiber filtration. Briefly, 10 L or 30 L culture volume was added to a carboy of appropriate volume with a spigot at the bottom that served as a reservoir for the culture during cell concentration. Algal cultures exited the carboy through the spigot into a recirculating system consisting of appropriate tubing and a peristaltic pump that was plumbed into the hollow-fiber ultrafilter unit (Optiflux F200A, Fresenius Medical Care, Lexington, Massachusetts), similar to systems described previously

by Simmons *et al.* (2001) and Hill *et al.* (2007). The algal cultures were recirculated through the hollow-fiber unit, with algal cells retained in the recirculating retentate and culture medium free of cells exited the hollow-fiber unit as filtrate. Once the recirculating culture volumes were reduced to the hold-up volume of the tubing/hollow-fiber unit, the tubing was removed from the spigot of the carboy and the resulting algal concentrate was pumped into appropriate sample containers using the peristaltic pump within the system. Subsamples of the concentrated algal samples were taken for cell counts, and pH and salinity were measured for both the concentrate and filtrate.

After the culture was harvested, 50 mL was sub-sampled from the concentrate for a short-term salinity stress experiment (n = 3; see Chapter 3 and methods therein). Control pellets and treatment pellets were re-suspended with 50 mL of L1-Si medium at salinity 30 and salinity 90, respectively. All samples were taken 30 sec after treatment (see Chapter 3). Samples were centrifuged (3,000 RPM, 704 x *g*, 10 min) and immediately frozen at -80˚C until analysis of total FAs.

5.3.e Total FAs

Direct transesterification (DT; Griffiths *et al.* 2010; see Chapter 1 for details) was used to convert the lipids in the biomass to FA methyl esters, hereafter referred to as total FAs, which were then directly quantified using gas chromatography (GC) with a flame ionization detector (FID). For this technique, we used a combination of acidic and basic transesterification catalysts. Briefly, 0.5 M methanolic KOH was added to each sample, homogenized, and heated to 85˚C. Then, boron trifluoride (BF_3, 14% in MeOH, Sigma Aldrich®, St. Louis, Missouri) was added and the samples were reheated to 85˚C. Equal

volumes of water and hexane were then added and the samples were allowed to separate into layers by density. The lighter hexane layer containing the neutral lipids was removed and analyzed with a GC HP5890 series II equipped with a FID (Hewlett Packard, USA) and a 7673A autosampler (Alpha Omega Technologies, Inc., New Jersey, USA). Separation was achieved in an Rtx-2330 capillary column (Hewlett Packard). The temperature was programmed to include an initial 3 min at 60˚C, and then was increased to 230˚C at a rate of 4˚C/min. Both injector and detector temperatures were set at 265˚C. Injections were performed under the splitless mode (Sparkman *et al.* 2011). Data acquisition and analysis were completed using the GC Chemstation Rev. A.08.03 (847) software. Calculations for total FAs were completed using the relative response factor (RRF) method (Sparkman *et al.* 2011; L. Dean, USDA, Raleigh NC, personal communication), and area ratios were compared to the internal standard (KEL-FIM-FAME-5 Mixture, Matreya, LLC, Pennsylvania).

5.4 Results and Discussion

5.4.a Culture harvest via auto-flocculation experiment

Auto-flocculation through adjustment to high pH was compared at bench (~3.5 L) and mass culture (~150 L) scales. By adjusting the initial pH to 10 using 2N or 6N NaOH, *Dunaliella* cells flocculated to the bottom of the container within 24 hr, regardless of culture volume (Fig. 5.1 A-E). Salinity significantly affected how the cultures responded to pH adjustment. At salinity 60 in the bench-scale experiment, substantial NaOH (2N or 6N; ~25 mL compared to < 1 mL for cultures grown at salinity 30) was required to adjust the pH to

10. Within 30 sec after adjustment, the cells became completely surrounded by a glycerol-like substance, making cell counts difficult. At mass culture scale, *Dunaliella* populations grown at salinity 60, then adjusted to pH 10 did not recover from flocculation (Fig. 5.1 A-B). At 24 hr, the pH 10-adjusted cultures grown at salinity 60 were at pH 9.6 (Fig. 5.2 A), whereas adjusted cultures grown at salinity 30 were at pH 9.8; thus, the pH was not as stable at the higher salinity (Fig. 5.2 B).

Total FA content was ~20 to 25% (% total by dry weight) in both control and pH-adjusted cultures; thus, the imposed pH stress did not enhance lipid production. This auto-flocculation technique recovered 95% or more of the algal biomass at both salinities (Fig. 5.1 C-E), as reported from other studies (Horiuchi *et al.* 2003, Granados *et al.* 2012). Although auto-flocculation was shown to be effective in harvesting *Dunaliella*, the addition of NaOH made the culture medium unsuitable for reuse.

5.4.b Culture harvest via electro-flocculation

Production of hydroxide ($Al(OH)_3$ or $Fe(OH)_3$ was predictable and there was a positive correlation between energy consumption and flocculant mass produced (Fig. 5.3), consistent with Faraday's law. More variation was observed at higher amperages and longer times, and the differences between theoretical and measured values increased, indicating that the hydroxide caused the precipitation of additional elements out of solution. In addition, washing to remove the adsorbed salts in the flocculant samples was not completed before drying, which likely contributed to increased differences between theoretical and measured values. When producing hydroxide for application to harvest of *Dunaliella* cultures, it was advantageous to allow the hydroxide to settle and reach equilibrium. Adding 0.5 mL

hydroxide to culture at 1 L volume (1 x 10^6 cells/mL) was ideal for complete harvest of the biomass. Mixing of the culture was necessary to maximize contact between the hydroxide flocculant and culture. The pH of the culture when flocculant was added was around ~9.8, but had decreased to ~9.1 by 24 hr. Thus, the addition of the electro-flocculant resulted in decreased pH as the total cells/mL decreased due to flocculation (Fig. 5.4).

The separation of flocculant production from application to the culture resulted in an efficient harvesting mechanism (> 95% biomass recovery). Both auto-flocculation and indirect electro-flocculation required an extra step for dewatering (by centrifugation) before FA analysis could be completed. Total FA content remained at ~20% (dry weight basis) for control and flocculated cultures. The culture medium would not have been reusable unless the additional particles had been removed, which was not attempted here. Overall, indirect electro-flocculation was an effective harvesting mechanism, but the stressors involved did not enhance lipid production.

5.4.c Culture harvest via hollow-fiber filtration

The 10-L and 30-L cultures were concentrated ~50-fold and ~150-fold, respectively, resulting in final concentrate volumes of ~200 mL. Processing times for 10-L and 30-L cultures were approximately 20 and 60 min, respectively. Regardless of culture volume, total FAs significantly increased after filtration (47-51% versus 35-39% before filtration; Table 5.1, $p = 0.006$, Student's *t* test). The average salinity for both the concentrate and filtrate was ~30, indicating that the filtering process did not remove salts from the culture medium. The pH was higher in the filtrate than the concentrate (9.86 vs. 9.06, respectively) Careful

examination of the filtrate under light microscopy (200x) revealed only 1-2 cells; thus, it was inferred that hollow-fiber filtration had recovered more than 99% of the algal biomass.

To assess the efficacy of the filtrate as recycled medium, *Dunaliella* was grown in both bench scale (~100 mL) and mass culture (~150 L), testing the filtrate as reused culture medium. Growth was comparable in fresh versus reused medium. We also tried reusing the culture filtrate three times at the mass culture scale, with similar success and no observed detrimental effects to the cells. There was no need to add nutrients because nitrogen (as nitrate, NO_3^-) and phosphorus (as phosphate, PO_4^{3+}) were available at concentrations required for growth (~18,000 ± 697 µg/L NO_3^-; 1350 ± 340 µg/L PO_4^{3+}, n = 3). The pH was considerably higher (9.8) in the reused medium than the optimal pH we had determined for population growth (cell production) (8.0 - 8.4). Nevertheless, the *Dunaliella* inoculum from stock culture sustained similar cell production as control cultures in L1-Si at initial pH 8.3-8.4.

Lastly, a short-term salinity stress experiment (see Chapter 3) was completed on the concentrate in bench scale volume (50 mL; n = 3) by adjusting the salinity to 90 after dewatering the concentrate by centrifugation. The total FAs-to-biomass ratio of the salinity-adjusted concentrate was higher than that of the pre-filtered culture. Salinity stress has been shown to increase FAs in *Dunaliella* (Takagi *et al.* 2006, Chapter 3). Here, however, the combination of filtration plus a second dewatering step via centrifugation, together with the elevated salinity, may have stressed the cells to such an extent that lipid production did not occur.

5.5 Conclusion

Auto-flocculation and electro-flocculation were efficient harvesting mechanisms (> 95% biomass recovery), while hollow-fiber filtration resulted in even more efficient harvest (> 99% biomass recovery). Beyond harvest of the algal biomass, these techniques required an additional dewatering step, centrifugation, for full recovery of the product of most interest from a biofuel perspective, FAs. Flocculation methods rendered the medium unsuitable for reuse, unless methods can be developed for the removal of the added NaOH or flocculant. Among the three harvesting techniques tested with *Dunaliella*, hollow-fiber filtration is advantageous because it does not require adding chemicals to the culture. As another advantage, the filtrate was directly reusable as culture medium without having to supplement with nutrients. And, since filtration does not add chemicals or metals to the culture, hollow-fiber filtration avoids catalyst contamination concerns in the downstream processing of the algal biomass and lipids.

5.6 References

Avron, M., Ben-Amotz, A. (Eds.), 1992. *Dunaliella*: Physiology, Biochemistry, and Biotechnology. CRC Press, Boca Raton (Florida). 256 pp.

Ben-Amotz, A., Polle, J.E.W., Rao, D.V.S. (Eds.), 2009. The Alga *Dunaliella*: Biodiversity, Physiology, Genomics and Biotechnology. Science Publishers, Enfield (New Hampshire). 555 pp.

Borowitzka, M.A., 1992. Algal biotechnology products and processes – matching science and economics. J. Appl. Phycol. 4, 267-279.

González-López, C.V., Acién-Fernández, F.G., Fernández-Sevilla, J.M., Sánchez-Fernández, J.F., Cerón-García, M.C., Grima, E.M., 2009. Utilization of the cyanobacteria *Anabaena* sp. ATCC 33047 in CO_2 removal processes. Bioresour. Technol. 100, 5904-5910.

Granados, M.R., Acién, F.G., Gómez, C., Fernández-Sevilla, J.M., Grima, E.M., 2012. Evaluation of flocculants for the recovery of freshwater microalgae. Bioresour. Technol. 118, 102-110.

Griffiths, M.J., van Hille, R.P., Harrison, S.T.L., 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 45, 1053-1060.

Grima, E.M., Belarbi, E.-H., Acién-Fernández, F.G., Medina, A.R., Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol. Adv. 20, 491-515.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York (New York). pp. 29-60.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234-236.

Guillard, R.R.L., 2005. Purification Methods for Microalgae. In: Anderson, RA (Ed.), Algal Culturing Techniques, Elsevier Academic Press, Burlington (Massachusetts). pp. 117-132.

Hill, V.R., Kahler, A.M., Jothikumar, N., Johnson, T.B., Hahn, D., Cromeans, T.L., 2007. Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter tap water samples. Appl. Environ. Microbiol. 73, 4218-4225.

Horiuchi, J.-I., Ohba, I., Tada, K., Kobayashi, M., Kanno, T., Kishimoto, M., 2003. Effective cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control. J. Biosci. Bioeng. 95, 412-415.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621-639.

Kawachi, M, Noël, M-H (2007) Sterilization and Sterile Technique. In: Anderson, RA (Ed.), Algal Culturing Techniques, Elsevier Academic Press, Burlington (Massachusetts). pp. 65-81.

Mallick, N., 2002. Biotechnological potential of immobilized algae for wastewater N, P and metal removal: a review. BioMetals 15, 377-390.

Miyamoto, K., Wable, O., Benemann, J.R., 1988. Vertical tubular reactor for microalgae cultivation. Biotechnol. Lett. 10, 703-708.

Moulton, T.P., Borowitzka, L.J., Vincent, D.J., 1987. The mass culture of *Dunaliella salina* for β-carotene: from pilot plant to production plant. Hydrobiologia 151/152, 99-105.

Richmond, A., E.W. Becker., 1986. Technological Aspects of Mass Cultivation – A General Outline In: Richmond, A. (Ed.), CRC Handbook of Microalgal Mass Culture. CRC Press, Inc., Boca Raton (Florida). pp. 245-264.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100-112.

Roessler, P.G., 1990. Environmental control of glycolipid metabolism in microalgae: commercial implications and future research directions. J. Phycol. 26, 393-399.

Shimizu, Y., Okuno, Y.-I., Uryu, K., Ohtsubo, S., Watanabe, A., 1996. Filtration characteristics of hollow fiber microfiltration membranes used in membrane bioreactor for domestic wastewater treatment. Water Res. 30, 2385-2392.

Simmons, O.D. III., Sobsey, M.D., Heaney, C.D., Schaefer, F.W. III., Francy, D.S., 2001. Concentration and detection of *Cryptosporidium* oocysts in surface water samples by method 1622 using ultrafiltration and capsule filtration. Appl. Environ. Microbiol. 67, 1123-1127.

Şirin, S., Trobajo, R., Ibanez, C., Salvadó, J., 2012. Harvesting the microalgae *Phaeodactylum tricornutum* with polyaluminum chloride, aluminum sulphate, chitosan and alkalinity-induced flocculation. J. Appl. Phycol. 24, 1067-1080.

Sparkman, O.D., Penton, Z.E., Kitson, F.G., 2011. Gas Chromatography and Mass Spectrometry: A Practical Guide, 2nd Edition. Elsevier Inc., Burlington (Massachusetts). 611 pp.

Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J. Biosci. Bioeng. 101, 223-226.

Tompkins, J., DeVille, M.M., Day, J.G., Turner, M.F., 1995. Culture Collection of Algae and Protozoa, Catalog of Strains, Ambleside (United Kingdom). 204 pp.

Vandamme, D., Foubert, I., Meesschaert, B., Muylaert, K., 2010. Flocculation of microalgae using cationic starch. J. Appl. Phycol. 22, 525-530.

Vollenweider, R.A., Talling, J.F., Westlake, D.F. 1974. A Manual on Methods for Measuring Primary Production in Aquatic Environments, 2nd Edition. Blackwell Scientific, Oxford. 225 pp.

Wetzel, R.G., Likens, G.E., 2001. Limnological Analyses, 2nd Edition. W.B. Saunders, New York (New York). 429 pp.

Xu, L., Wang, F., Li, H.-Z., Hu, Z.-M., Guo, C., Liu, C.-Z., 2010. Development of an efficient electroflocculation technology integrated with dispersed-air flotation for harvesting microalgae. J. Chem. Technol. Biotechnol. 85, 1504-1507.

Table 5.1 – Total FAs before and after hollow-fiber filtration, considering data from the 10 L and 30 L sample volumes collectively (means ± 1SE).

	% Total FAs ± 1 SE
Before Filtration	36.0 ± 1.4
After Filtration	49.1 ± 1.5

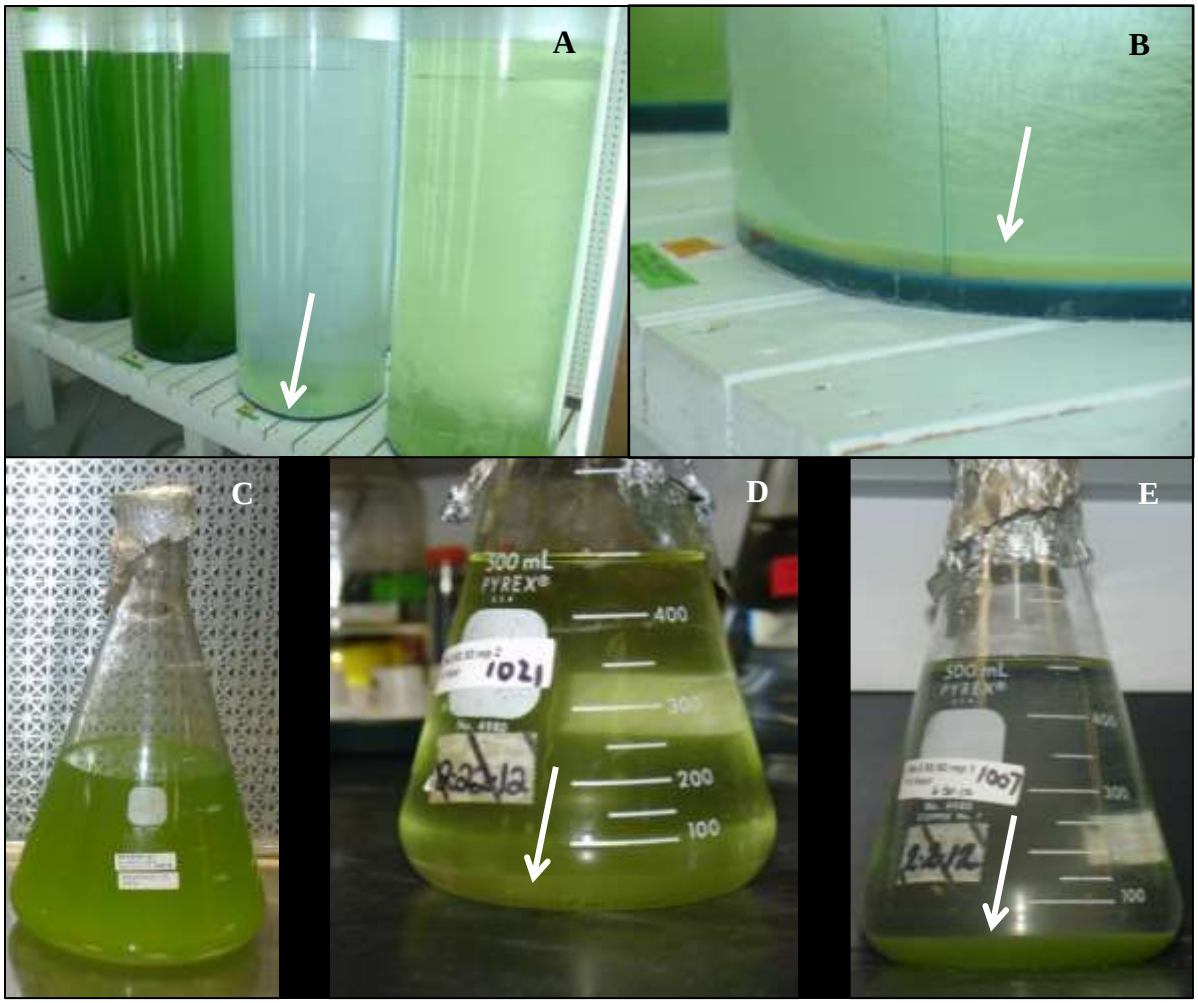

Figure 5.1 – *Dunaliella* culture after auto-flocculation at either A,B) mass culture or C-E) bench-scale. White arrows indicated the layer of *Dunaliella* cells at the bottom of each container. A,B) Photographs depicting effect of high pH on mass culture tubes: A1 and A1 tubes did not receive pH adjustment, A3 tube was grown at salinity 60 and adjusted to pH 10, and A4 tube was grown at salinity 30 and adjusted to pH 10; B) Layer of cells flocculated at the bottom of tube A3. C-E) Photographs depicting how quickly cells settle from C) before high pH adjustment, to D) 2 hr after addition of 6N NaOH, and E) 24 hr after the addition of 6N NaOH.

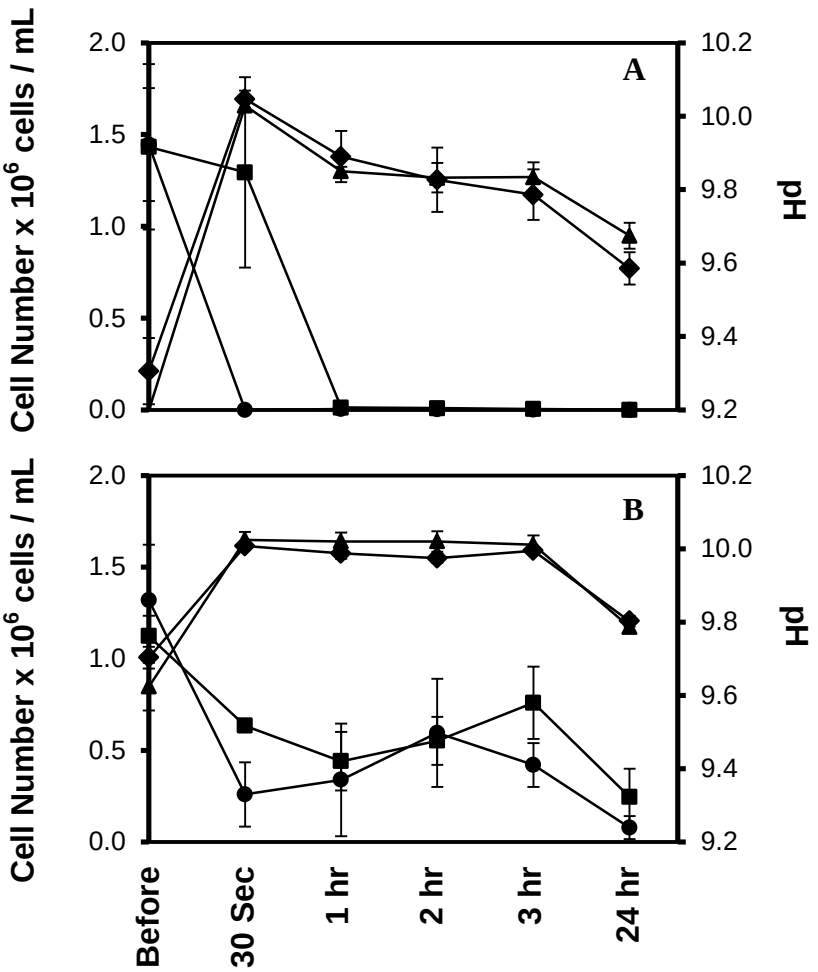

Figure 5.2 – Auto-flocculation - The effect of pH adjustment by 2N or 6N NaOH on cell production and pH of bench scale cultures grown at A) salinity 60 or B) salinity 30. Cell production in the 2N (■) and 6N (●) NaOH treatments generally decreased over time after pH adjustment. The pH in both the 2N (♦) and 6N (▲) decreased within 24 hr at either salinity. Controls and treatments were sampled before and after adjustment (30 sec, 1 hr, 2 hr, 3 hr, and 24 hr). Data are given as means ± 1 SE (n = 3).

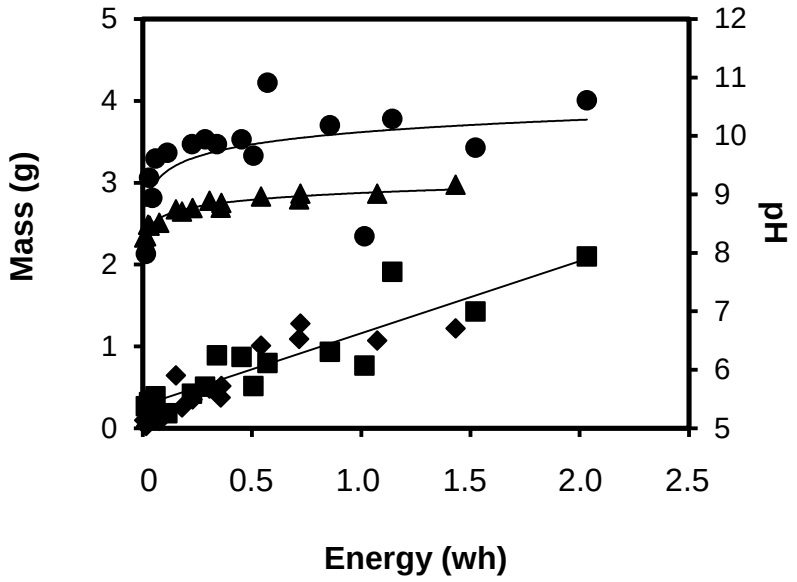

Figure 5.3 – Electro-flocculation - Measured hydroxide mass (g) and initial pH values for iron (Fe) and aluminum (Al) in response to energy consumption in watt-hours (wh). Al mass (♦) and Fe mass (■) increase with increasing energy consumption. Al pH (▲) and Fe pH (●) reached equilibrium as power consumption increased. The watt-hour scale normalizes the data for various amperages and times.

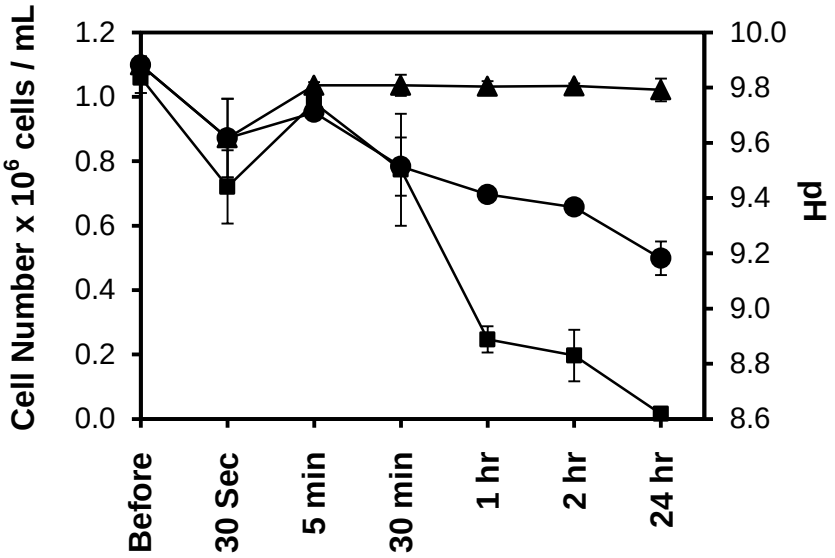

Figure 5.4 – Electro-flocculation- Cell number and pH of cultures when the flocculant was added (at addition) and at the final sampling time (30 sec, 5 min, 30 min, 1 hr, 2 hr, 24 hr). pH at addition (▲) was around 9.8 for all samples and the pH at the sampling time (●) decreased over time. Cell count (■) decreased over time to almost zero by 24 hr. Data are given as means ± 1 SE (n = 3).

6. CONCLUSION

The current research has provided valuable information for the renewable biofuel industry. Fourteen strains within four species of *Dunaliella* were investigated. Strains were purified using a combination of flow cytometry and differential centrifugation to remove potential bacterial contamination. Conditions such as salinity (30 or 60), medium type (L1-Si), and initial pH (8.0 – 8.4) were optimized for each strain. Growth curves were then produced based upon population growth as cell production, and the strains were tested for lipid content. Seven strains that exhibited high cell production, high lipids, or ideally both conditions, were selected for environmental stressor experiments to further enhance neutral lipid production.

Both qualitative and quantitative techniques were optimized for the isolation and accurate quantification of total fatty acid (FA) content of *Dunaliella* spp. In order to compare results to the literature, an accurate wet-weight-to-dry-weight conversion factor had to be determined because samples were not dried before analysis. The best wash method tested for these saltwater organisms was determined to be 0.5 M ammonium formate. Both Karl Fischer titration, which directly analyzes intracellular water content, and the use of ammonium formate wash yielded a 95% conversion factor.

A rapid, qualitative screen for neutral lipid content was developed using the fluorescent dye Nile Red (NR). The use of NR cannot be generalized for all microalgae and must be optimized depending upon the species and strain used. Optimized conditions for NR use with *Dunaliella* included concentration and type of solvent, concentration of NR, and the excitation and emission wavelengths. In order to be considered a semi-quantitative method

rather than as a qualitative screen, further refinements such as the calibration of total neutral lipids from extraction methods to lipid standards (Elsey *et al.* 2007) and additional factors such as cell wall composition, chlorophyll, or lipid membranes that may interfere with fluorescence background, would need to be considered (Chen *et al.* 2009). Preliminary experiments using NR indicated that once the cells reached onset of senescence, neutral lipids were produced throughout the day (12:12 L:D) with no clear maximum early, mid-way through, or late in the light period.

Several methods were rigorously compared for quantifying lipid content as total fatty acids (Chapter 1). Direct transesterification (DT) was superior for use with the typically small samples of this microalga. Advantages of DT over the conventional methods of Folch (1957) or Bligh and Dyer (1959) included combining the extraction and transesterification steps in a one-tube, one-step process as well as the ability to accurately quantify neutral lipids on small sample sizes. In addition, this method was further improved by sequentially adding both a basic and acidic catalyst, considered imperative for samples that were not dried before processing (Griffiths *et al.* 2010). Extraction is an energy-intensive process. Combining the extraction and purification processes in a one-step, one-tube technique that converts oil-bearing biomass into biodiesel will greatly reduce this cost (Hidalgo *et al.* 2013). These procedures were developed based upon the literature and optimized for use in *Dunaliella* cells and should not be generalized for all microalgae; however, a similar approach can be taken to analyze potential strains of *Dunaliella* or other promising algal candidates as feedstock for the renewable biofuel industry.

Dunaliella strain curation exemplifies a major, ongoing problem throughout phycology, incorrect identification of algal strains in commercial culture clearinghouses and perpetuation in the literature. Moreover, the definition of "species" in many microalgae is a topic of ongoing debate (Guiry 2012). As an initial step in this research, the identification of the 14 purified strains was checked using a combination of morphological (size, biovolume), population-level (cell production), and sequencing data ITS and 18S regions of the rRNA (Chapter 2). Both inter- and intraspecific variation was observed within and among these strains and species of *Dunaliella*. Such variation in microalgae is well documented in the literature (Wood and Leatham 1992, Lakeman and Cattolico 2007). Lakeman *et al.* (2009) completed an in-depth review of regarding the species concept as it relates to microalgae. They concluded that an algal strain should not be considered a single snapshot of an algal population, because strains are dynamic and ever-changing in laboratory populations (Lakeman *et al.* 2009). The analysis presented in Chapter 2 indicated that 11 of the 14 strains had been incorrectly named in culture clearinghouses. The reason(s) for the high intra- and interspecific variation in morphological, population-level, and molecular traits cannot be fully explained from this study. It is suggested that a multi-gene molecular analysis (Lane *et al.* 2006) be used to further elucidate the relationship of these 14 *Dunaliella* strains. A combination of genes from the nucleus (18S and ITS), chloroplast (RUBISCO, intergenic spacers), and mitochondrion (NADH dehydrogenase) can be investigated alone and in combination as taxonomic indicators (Lane *et al.* 2006). The chloroplast DNA (cpDNA) contains intergenic spacers similar to the ITS region found in the nuclear DNA and has been investigated to determine interspecific relationships among plants (Taberlet *et al.* 1991, Kim

et al. 1999). The mitochondrial DNA (mtDNA) is also useful for reconstructing phylogenies because the genes are generally more variable than nuclear genes such as small subunit (SSU) rDNA (Saccone *et al*. 2000). The use of multiple markers will further clarify the phylogenetic relationships determined from this work.

Additional genes of interest (GOI) that could be sequenced to determine variation among strains include those involved in cell size or cell production. Both characteristics showed high variation among strains of *Dunaliella*. Additional biochemical characteristics (see Borowitzka and Siva 2007 and methods therein) could also be investigated, but will be time-consuming because many of these properties are observed under various culturing conditions. In order to complete an analysis with GOI will necessitate sequencing the full genome or transcriptome. Sequencing the transcriptome facilitates *de novo* assembly for species lacking full genome information, including *Dunaliella* (Rismani-Yazdi *et al.* 2011; Wong *et al.* unpublished data).

The unique strain, *D. viridis* COMTAM, and *D. tertiolecta* UTEX 1000 initially were selected as potential "best strains" for potential use in sustainable biofuel production. These two strains exhibited high growth rates (i.e. > 1 $x10^7$ cells/mL for *D. viridis* CONTAM and > 6 x 10^6 cells/mL for the much larger-celled *D. tertiolecta* UTEX 1000. Both strains also showed high lipid production (i.e. > 40% by dry weight). Moreover, the low optimum salinity for growth of *D. tertiolecta* UTEX 1000 (30, versus 60 for *D. viridis* CONTAM) would have minimized the cost of culture media. After several months, however, this strain unfortunately lost its ability to produce high amounts of neutral lipids, and when an additional culture of this strain was obtained; it grew slowly and had poor lipid production.

Thus, it was not possible to consider this *D. tertiolecta* strain for further work. This phenomenon of losing certain characteristics has been documented in the literature (see Burkholder et al. 2005, Burkholder and Glibert 2006, and references therein). For example, Martins *et al.* (2004) discovered that when two sub-clones of a previously toxic *Alexandrium lusitanicum* strain were grown in separate laboratories for 12 years, one strain remained toxic while the other had lost its ability to produce the toxin.

This research examined effects of environmental stressors on *Dunaliella* including *rapid response* (30 sec to ≤ 5 min) wherein cell size, shape, structure, and ion concentration change; *short-term response* (up to 2 hr) whereby the osmotic pressure is balanced by regulating glycerol concentration, and *long-term* response (≥ 24 hr) which includes stress-induced gene expression and accumulation of salt-induced proteins (Chen and Jiang 2009) (Chapter 3). Adaptation here refers to physiological adjustments to surrounding conditions or a hereditary alteration enabling the organism to adjust to its surroundings (Nielson and Jorgensen 1968, Lakeman *et al.* 2009). *Dunaliella* has been shown to adapt physiologically to changes in its environment, for example, by producing glycerol in response to increasing salt (Ben-Amotz *et al.* 2009), or by changing FA composition in response to high CO_2 (Muradyan *et al.* 2004). Over longer time frames, such as the long-term experiments completed here, epigenetic adaptation may occur and this would allow for changes in gene regulation or expression (Lakeman *et al.* 2009).

Lipid production by seven selected strains of *Dunaliella* was enhanced using various environmental stressors. Simple high salinity stress yielded the maximum total FA content (up to 65% by dry weight) in some strains when compared to the controls (~10-25%)

(Chapter 3). However, intra-specific as well as inter-specific variation was observed during these experiments. For example, under long-term salinity stress, 4 of 7 strains increased in total FA content in response to high salinity, whereas 2 strains increased total FAs under low salinity stress. One strain, *D. viridis* CONTAM, had maximal amounts of total FAs at its optimal salinity of 60. During short-term salinity stress experiments, total FA content increased significantly in 4 tested strains within sec to min after exposure to salinity stress.

Osmoregulation is an important mechanism when *Dunaliella* strains are exposed to hyperosmotic stress. The osmolyte glycerol significantly increased 30 to 60 min after exposure to high salinity. In addition, the glycerol biosynthesis gene, glycerol-3-phosphate dehydrogenase or *GPDH*, was expressed during a similar time frame. This work suggests that a proportion of the glycerol produced in response to hyperosmotic shock was incorporated in the backbone of triacylglycerides (TAGs). The glycerol cycle is reversible, however, and the total amounts of intermediate products signal to the cell whether to synthesize additional glycerol, or metabolize this product to form starch, sugars, or pyruvate (Taiz and Zeiger 2006, Chen and Jiang 2009, Shariati and Hadi 2011).

Additional conditions such as high pH x low salinity, low pH, and continuous light x CO_2 yielded near maximal total FA content in 4 tested strains (56%, 43%, and 42%, respectively)(Chapter 4). Nutrient limitation (20% nitrogen and/or phosphorus) and 12:12 (light:dark photoperiod) x CO_2 did not enhance total FA production (23% and 31%, respectively). N deficiency was expected to increase neutral lipids in part because storage and membrane lipids do not contain N (Roessler 1990). Studies of *Dunaliella* under low nitrogen stress have yielded varying results and, in general, strains within the species *D.*

bardawil, D. primolecta, D. tertiolecta, and *D. viridis* do not increase total lipid content in response to N deficiency (Shifrin and Chisholm 1981, Ben-Amotz *et al.* 1985, Uriarte *et al.* 1993, Adam 1997, Gordillo *et al.* 1998, present study). In this work, results were strain-specific and intraspecific variation was observed throughout the experiments (Chapters 3 and 4). Gardner *et al.* (2011) investigated the chlorophyte *Scenedesmus* spp. and found that when this strain was exposed to both high pH and nutrient deficiency, neutral lipids increased. In this study as mentioned, nutrient limitation did not increase neutral lipids in the strains studied, but high pH alone resulted in an increase in total FAs in *D. viridis* CONTAM. Lastly, during the enriched CO_2 x continuous light study, the lack of a dark cycle to break down carbon products likely allowed for excess available C as CO_2 to be incorporated by *D. viridis* CONTAM into FAs.

Commercialization of mass culture harvesting techniques has not yet been proven economically feasible. In this research, three methods were compared as potential harvesting mechanisms including auto-flocculation, indirect electro-flocculation, and hollow-fiber filtration (Chapter 5). Auto-flocculation and indirect flocculation had > 95% biomass recovery compared to > 99% recovery by hollow-fiber filtration. In addition, the filtrate produced by hollow-fiber filtration was successfully reused as culture medium at both small batch and mass culture scale. An economic analysis was beyond the scope of this work. Such an analysis would require the incorporation of each of these harvesting technologies at a full production scale including both capital and operational costs. Hollow-fiber filtration concentrated ~ 30 L down to 250 mL and the pump consumed 0.123 kwh on average, which is approximately $0.01 worth of electricity. The energy required to produce flocculant in the

indirect electro-flocculation technique required 0.015 kwh or $0.15 worth of electricity. Energy requirements were not calculated for auto-flocculation because the NaOH was available in the laboratory without the need to create more chemical. A complete economic analysis would require the cost of initial materials, labor, electricity requirements, etc. at mass culture capacity.

This study investigated how to obtain both high cell production and high neutral lipid production using environmental stressors. At mass culture scale, algae can be grown in tubes or open-pond systems. Vertical tubes provide high surface-to-volume ratios similar to that of small batch cultures (Miyamoto *et al.* 1988). In addition, a vertical design allows for efficient gas exchange and mixing of culture. High efficiency of CO_2 use is possible by adjusting either air flow or CO_2 flow rates, which allows for a variety of experimental factors to be tested such as crossing bubbling CO_2 with and without continuous light. In contrast, open raceway ponds are limited by depth in order to maximize light intensity (Chaumont 1993). Closed photobioreactors (PBR) allow for more control over the physical, chemical, and biological environment of the cultures compared to open pond systems (Miyamoto *et al.* 1988). Batch or closed systems assume that production of cells is limited due to factors such as depleting nutrients in the medium composition and light intensity becoming limiting due to self-shading. Open-pond systems are less expensive to build and operate and typically more durable than a PBR system as well as typically having a larger production capacity (Mata *et al.* 2010). Despite the many advantages of PBRs, at present open-pond systems are more cost-effective even though more land area is required and contamination can be an issue (Mata *et al.* 2010).

As mentioned, because *Dunaliella* species are saltwater organisms, they can more easily outcompete potential bacterial contaminants than many freshwater species. To be economically feasible, sustainable biofuel production with *Dunaliella* would require growing mass cultures adjacent to coastal areas or in salt lakes so that saltwater will be readily available as culture medium. The water would need to be analyzed to ensure that the salt content (qualitative and quantitative) was conducive for high cell production. An in-depth review of the elemental composition of many commercially available synthetic mixes of seawater salts was completed by Atkinson and Bingman (2011). In general both Coralife® (Franklin, Wisconsin, used in this study) and Instant Ocean (Blacksburg, Virginia) have similar elemental cation composition. Coralife® was used in these mass culture experiments largely due to the lower cost at mass quantities. Due to their "weedy" nature and so as not to interfere with other coastal activities, such as aquaculture, *Dunaliella* can also be grown commercially in salt lakes or highly polluted waters. In fact, certain strains of *Dunaliella*, including *D. salina* have been successfully mass cultured for the product of β-carotene (Ben-Amotz 1987, Moulton *et al.* 1987, Olmos-Soto *et al*. 2002, Ben-Amotz *et al.* 2009). The use of these waters could potentially increase the economic potential of the area that would otherwise not be developed. Also, by eliminating the land requirement that would be necessary for open-pond systems or housing PBRs, land in these areas can be made available for agricultural crops or livestock required to feed the population. Even with the necessity of producing renewable fuels, the value of the product is presently not high. I suggest that in combination with mass culturing microalgae for high lipid content, selection of strains should also include the possibility of other high value products such as β-carotene.

This research examined the high intraspecific and interspecific variation among 14 strains of *Dunaliella*. This variation was observed in all of the studied traits, including morphology (cell size, biovolume), population growth as cell production, sequencing information, total FAs, and glycerol production. Optimization and development of lipid analysis techniques was especially important for this study and can be used in other research for use of microalgae in biofuel production. Of these 14 strains, one unique strain, *D. viridis* CONTAM, maintained high cell production regardless of light regime and high amounts of total FAs throughout its growth cycle. Simple high or low salinity stress yielded maximal total FAs for 13 of the 14 strains, but *D. viridis* CONTAM also produced high amounts of lipids when exposed to high pH or continuous light x enriched CO_2. Lastly, optimal harvest of large culture volumes using hollow-fiber filtration was shown to be very promising in a proof-of-concept experiment for potential use in commercializing biofuel production from *Dunaliella*. This technique can be easily adapted for use with other microalgae as well.

6.1 References

Adam, M.S., 1997. Metabolic response of the halotolerant green alga *Dunaliella bardawil* to nitrogen : phosphorus ratios in batch culture. Folia Microbiol. 42, 357-360.

Atkinson, M.J., Bingman, C. 2011. Elemental composition of commercial seasalts. Journal of Aquariculture and Aquatic Sciences. VIII, 39-43.

Avron, M., Ben-Amotz, A. (Eds.), 1992. *Dunaliella*: Physiology, Biochemistry, and Biotechnology. CRC Press, Boca Raton (Florida). 256 pp.

Ben-Amotz, A., Tornabene, T.G., Thomas, W.H., 1985. Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol. 21, 72-81.

Ben-Amotz, A., 1987. Effects of irradiance and nutrient deficiency on the chemical composition of *Dunaliella bardawil* Ben-Amotz and Avron (Volvocales, Chlorophyta). J. Plant Physiol. 131, 479-487.

Ben-Amotz A, Polle JEW, Rao DVS (Eds), 2009. The Alga *Dunaliella*: Biodiversity, Physiology, Genomics and Biotechnology. Science Publishers, Enfield (New Hampshire). 555 pp

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

Borowitzka, M.A., Siva, C.J., 2007. The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. J. Appl. Phycol. 19, 567-590.

Burkholder, J.M., Gordon, A.S., Moeller, P.D., Law, J.M., Coyne, K.J., Lewitus, A.J., Ramsdell, J.S., Marshall, H.G., Deamer, N.J., Cary, S.C., Kempton, J.W., Morton, S.L.,

Rublee, P.A. 2005. Demonstration of toxicity to fish and to mammalian cells by *Pfiesteria* species: comparison of assay methods and multiple strains. Proc. Natl. Acad. Sci. (U.S.A.) 102, 3471-3476.

Chaumont, D. 1993. Biotechnology of algal biomass production: a review of systems for outdoor mass culture. J. Appl. Phycol. 5, 593-604.

Chen, H., Jiang, J.G., 2009. Osmotic responses of *Dunaliella* to the changes of salinity. J. Cell Physiol. 219, 251-258.

Chen, W., Zhang, C., Song, L., Sommerfeld, M., Hu, Q., 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods 77, 41-47.

Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.Y.S., Salley, S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour. Technol. 102, 1649-1655.

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126-131.

Elsey, D., Jameson, D., Raleigh, B., Cooney, M.J., 2007. Fluorescent measurement of microalgal neutral lipids. J. Microbiol. Methods 68, 639-642.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Gardner, R., Peters, P., Peyton, B., Cooksey, K.E., 2011. Medium pH and nitrate concentration effects on accumulation of triglycerol in two members of the chlorophytes. J. Appl. Phycol. 23, 1005-1016.

Glibert, P.M., Burkholder, J.M. 2006. The complex relationships between increasing fertilization of the earth, coastal eutrophication and proliferation of harmful algal blooms. In: Granéli, E., Turner, J. (Eds.), The Ecology of Harmful Algae. Springer-Verlag, New York (New York), pp. 341-354.

Gordillo, F.J.L., Goutx, M., Figueroa, F.L., Niell, F.X., 1998. Effects of light intensity, CO_2 and nitrogen supply on lipid class composition of *Dunaliella viridis*. J. Appl. Phycol. 10, 135-144.

Griffiths, M.J., van Hille, R.P., Harrison, S.T.L., 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 45, 1053-1060.

Guiry, M.D. 2012. How many species of algae are there? J. Phycol. 48, 1057-1063.

Hidalgo, P., Toro, C., Ciudad, G., Navia, R. 2013. Advances in direct transesterification of microalgal biomass for biodiesel production. Rev. Environ. Sci. Biotechnol. 12, 179-199.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621-639.

Kim, S.-C., Crawford, D.J., Jansen, R.K., Santos-Guerra, A. 1999. The use of a non-coding region of chloroplast DNA in phylogenetic studies of the subtribe *Sonchinae* (Asteraceae: Lactuceae). Pl. Syst. Evol. 215, 85-99.

Lakeman, M.B., Cattolico, R.A. 2007. Cryptic diversity in phytoplankton cultures is revealed using a simple plating technique. J. Phycol. 43, 662-674.

Lakeman, M.B., von Dassow, P., Cattolico, R.A. 2009. The strain concept in phytoplankton ecology. Harmful Algae. 8, 746-758.

Lane, C.E., Mayes, C., Druehl, L.D., Saunders. G.W. 2006. A multi-gene molecular investigation of the kelp (Laminariales, Phaeophyceae) supports substantial taxonomic re-organization. J. Phycol. 42, 493-512.

Mata, T.M., Martins, A.A., Caetano, N.S. 2010. Microalgae for biodiesel production and other applications: a review. Renewable and Sustainable Energy Reviews. 14, 217-232.

Miyamoto, K., Wable, O., Benemann, J.R. 1988. Vertical tubular reactor for microalgae cultivation. Biotechnol Letters. 10, 703-708.

Moulton, T.P., Borowitzka, L.J., Vincent, D.J., 1987. The mass culture of *Dunaliella salina* for β-carotene: from pilot plant to production plant. Hydrobiologia 151/152, 99-105.

Muradyan, E.A., Klyachko-Gurvich, G.L., Tsoglin, L.N., Sergeyenko, T.V., Pronina, N.A. 2004. Changes in lipid metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO_2 concentration. Russian Journal of Plant Physiology 51, 62-72.

Nielsen, E. S., Jorgensen, E.G. 1968. The adaptation of plankton algae. Physiologia Plantarum 21, 401-413.

Olmos-Soto, J., Paniagua-Michel, J., Contreras, R., Trujillo, L., 2002. Molecular identification of β-carotene hyper-producing strains of *Dunaliella* from saline environments using species-specific oligonucleotides. Biotechnol. Letters 24, 365-369.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Bibby, K., Peccia, J. 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: Pathway description and gene discovery for production of next-generation biofuels. BMC Genomics 12, 148.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100-112.

Roessler, P.G., 1990. Environmental control of glycolipid metabolism in microalgae: commercial implications and future research directions. J. Phycol. 26, 393-399.

Saccone, C., Gissi, C., Lanave, C., Larizza, A., Pesole, G., Reyes, A. 2000. Evolution of the mitochondrial genetic system: an overview. Gene 261, 153-159.

Shariati, M., Hadi, M.R. 2011. Microalgal biotechnology and bioenergy in *Dunaliella*. Prog. Molec. Environ. Bioeng. 22, 483-506.

Shifrin, N.S., Chisholm, S.W., 1981. Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. J. Phycol. 17, 374-384.

Taberlet, P., Gielly, L., Pautou, G., Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of the chloroplast DNA. Plant Molecular Biology 17, 1105-1109.

Taiz, L., Zeiger, E., 2006. Plant Physiology. 4th Edition. Sinauer Associates, Inc., Sunderland (Massachusetts). 764 pp.

Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J. Biosci. Bioeng. 101, 223-226.

Uriarte, I., Farías, A., Hawkins, A.J.S., Bayne, B.L., 1993. Cell characteristics and biochemical composition of *Dunaliella primolecta* Butcher conditioned at different concentrations of dissolved nitrogen. J. Appl. Phycol. 5, 447-453.

Wong, G. K.-S., Carpenter, E., Sauer, M.-L., Sederoff, H., Transcriptomes of four *Dunaliella* species in the 1KP project (unpublished data; http://www.onekp.com/index.html).

APPENDICES

Appendix A – ITS2 pairwise comparison matrix generated using MEGA5 for all 14 *Dunaliella* strains.

	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Dunaliella_parva_CCAP19/10	0.00	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Dunaliella_salina_CCAP19/3	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Dunaliella_bardawil_ATCC30861	0.10	0.10	0.10	0.10		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Dunaliella_salina_LB200	0.10	0.10	0.10	0.10	0.08		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
Dunaliella_salina_CCAP19/18	0.10	0.10	0.10	0.10	0.08	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.01
Dunaliella_parva_LB1983	0.10	0.10	0.10	0.10	0.08	0.00	0.00		0.01	0.01	0.01	0.01	0.01	0.01
Dunaliella_parva_CCAP19/9	0.09	0.09	0.09	0.09	0.08	0.06	0.06	0.06		0.00	0.00	0.00	0.00	0.00
Dunaliella_tertiolecta_LB999	0.09	0.09	0.09	0.09	0.08	0.06	0.06	0.06	0.00		0.00	0.00	0.00	0.00
Dunaliella_primolecta_LB1000	0.09	0.09	0.09	0.09	0.08	0.06	0.06	0.06	0.00	0.00		0.00	0.00	0.00
Dunaliella_tertiolecta_CCMP364	0.09	0.09	0.09	0.09	0.08	0.06	0.06	0.06	0.00	0.00	0.00		0.00	0.00
Dunaliella_parva_CCAP19/26	0.09	0.09	0.09	0.09	0.08	0.06	0.06	0.06	0.00	0.00	0.00	0.00		0.00
Dunaliella_tertiolecta_CCMP1320	0.09	0.09	0.09	0.09	0.08	0.06	0.06	0.06	0.00	0.00	0.00	0.00	0.00	
Dunaliella_tertiolecta_CCAP19/24	0.09	0.09	0.09	0.09	0.08	0.06	0.06	0.06	0.00	0.00	0.00	0.00	0.00	0.00

6.2 Appendix B

Appendix B – Phylogenetic tree generated using data from Assunção *et al.* 2012 and the 14 strains of *Dunaliella* used in this research. Each strain is in bold and the corresponding clade has been highlighted.