Environmental Stressors and Lipid Production in *Dunaliella* spp. II. Nutrients, pH, and light under optimal or low salinity

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Abstract

Fourteen strains within four species of the halophytic microalgal genus, *Dunaliella* were rapidly screened for cellular lipid content during senescence using an adapted Nile Red technique. Four promising strains that exhibited both rapid population growth and high lipid content, including three strains of *Dunaliella tertiolecta* and one strain of *D. viridis*, were selected to attempt to enhance lipid production as total fatty acids (FAs) in bench-scale cultures (0.5 to 3.5 L) under environmental stressors including nutrient limitation or deprivation of inorganic nitrogen (nitrate, N) and/or phosphorus (P), pH stress (control - early senescent cultures, versus pH 10 or pH 7), a 12-hr : 12-hr light:dark (L:D) photoperiod versus continuous light, and enrichment versus no enrichment with carbon dioxide ($\pm$ 4-hr pulse daily of bubbling with CO₂). These strains 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 *Dunaliella viridis* UTEX ZZ1150 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, low pH (7), and continuous light + pulsed CO₂ 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 ± pulsed CO₂, cells likely incorporated the excess carbon as FAs and other products. Low N and/or P under a 12:12 L:D photoperiod or continuous light, and a 12:12 L:D with pulsed CO₂, did not significantly enhance total FA production. High intraspecific and interspecific variation in growth and lipid production was found in *Dunaliella* in response to each environmental stressor, suggesting fundamental metabolic differences.

Keywords: *Dunaliella*, CO₂, continuous light, lipids, nitrogen, pH, phosphorus, photoperiod

1. Introduction¹

Microalgae have high surface area-to-volume ratios, facilitating rapid responses to environmental stressors (Becker, 1994; Rodolfi et al., 2009; Sharma et al., 2012). They can rapidly alter their lipid metabolism in terms of 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, 1997; Azachi et al., 2002; Takagi et al., 2006). *Dunaliella* spp. (Chlorophyceae), as promising candidates for sustainable biofuel production, have been studied in detail with regard to salinity stress and its effect on increasing total fatty acids (FAs) (Azachi et al., 2002; Oren, 2005; Takagi et al., 2006; Ben-Amotz et al., 2009; Mixson et al., submitted).

¹ FAs, fatty acids; FID, flame ionization detector; GC, gas chromatography; NR, Nile Red; TAGs, triacylglycerides

Lipids generally are considered to provide a storage function that helps to enable the cell to survive adverse environmental conditions or stressors (Fields et al., 2014). In Part I of this work (companion paper, Mixson et al. submitted), we focused in detail on effects of salinity stress alone. Here we investigated the effects of various abiotic stressors on total FA production in four strains of *Dunaliella* within two species. The environmental stressors included low nutrients or nutrient deprivation, low and high pH stress, and two different light regimes with/without pulsed CO₂ under optimal or low salinity.

Deprivation of macronutrients such as nitrogen and phosphorus can significantly enhance lipid accumulation, depending on the species, strain, and other environmental conditions (Fields et al. 2014). Lack of nitrogen limits protein synthesis which is needed for most cellular functions including photosynthesis and cell division, and cells compensate by using alternative metabolic pathways for inorganic carbon fixation such as fatty acid synthesis (Msanne et al., 2012). When cell growth and division are impeded by lack of nutrients, the supply of the major electron acceptor for photosynthesis, NADP⁺, can be depleted. FA synthesis consumes NADPH and ATP, and maintains the NADP⁺ pool (Brown et al., 2009; Sharma et al., 2012; Fields et al., 2014). 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; Yilancioglu et al., 2014 – *Dunaliella salina*). 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. Shifrin and Chisholm (1981) completed an in-depth study on the effects of N limitation on total lipids within species of chlorophytes, and 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). Arias-

Forero et al. (2013) reported a N-limiting threshold, as a N:P ratio of 14:1 by weight, for stimulation and accumulation of triglycerides; on the other hand, Song et al. (2016) found that a strain of *Dunaliella tertiolecta* had maximal lipid content at an atomic N:P ratio of 4:1 when nitrate or urea was the nitrogen source. P limitation in microalgal chlorophytes has decreased lipid content in some taxa (e.g., *Nannochloris*, *Tetraselmis*) and increased lipid content in others (e.g., *Chlorella kessleri*) (El-Sheek and Rady, 1995; Widjaja et al., 2009). The influence of P limitation on lipid production in *Dunaliella* is poorly known, as are the effects of combined N+P limitation.

Use of pH stress to induce microalgal accumulation of lipids has been applied to other chlorophytes (Guckert and Cooksey, 1990; Gardner et al., 2011; Santos et al., 2012). 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). The pH experiments conducted here 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 Mixson et al. submitted). The nutrient limitation variable indicated by Gardner et al. (2011) was eliminated in those experiments, so that any increase in total FAs was in response to pH stress.

Carbon (C) accounts for almost 50% of the overall dry weight of microalgal biomass (Becker 1994) and, of course, carbon is required for lipid biosynthesis (Palmqvist et al., 1988;

Spalding, 2008). Microalgae synthesize various intracellular C compounds from DIC uptake during the light period (Kaplan et al., 1986; Carvalho and Malcata, 2004). Algae commonly are cultured in a 12:12 (light, 12-14 hr; dark, 10-12 hr) light:dark (L:D) photoperiod. Although most algae grow poorly or cease growth in continuous light (Carvalho and Malcata 2004; Lorenz et al., 2005), 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). This information was extended by examining the response of two other *Dunaliella* strains to increased pulses of CO₂ under 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 and mass culture experiments. 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.

2. Materials and Methods

2.1 Strains, Stock Cultures, and Cell Counts – Fourteen strains of *Dunaliella* were visually screened for visually high neutral lipid content with a fluorescent Nile Red dye technique specially adapted for use with *Dunaliella* spp. (Mixon, 2013). Of these, five initially were selected for use in the experiments. These 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). They included *D. tertiolecta* strains CCAP 19/9 ($120 \pm 6 \mu\text{m}^3$, mean ± 1 standard deviation [SD], n = 30 cells), CCAP 19/24 ($132 \pm 6 \mu\text{m}^3$), UTEX 999 ($163 \pm 9 \mu\text{m}^3$),

and UTEX 1000 ($168 \pm 8 \mu\text{m}^3$), and *D. viridis* UTEX ZZ1150 ($74 \pm 5 \mu\text{m}^3$) (Table 1; and see Mixson et al. submitted). The strains were purified using a combination of differential centrifugation and flow cytometry (Beckman Coulter® EPICS Altra, NCSU) to remove bacterial contamination (Guillard, 2005; Kawachi and Noël, 2005), as described in Mixson (2013) and Mixson et al. (submitted). 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, 2010) under light microscopy at 200x using an Olympus BH-2 light microscope.

ITS2 sequence data for each of the five strains indicated that they fell 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, “*Dunaliella salina* strain UTEX 1644” was found to have been a *D. viridis* contaminant of that culture (Mixson et al., 2014). The strain, of unknown origin, proved to be unique in major features including cell size, population growth as cell production, sustained growth in continuous light, and high lipid production throughout the cell cycle. Based on that information, the strain was not simply a contaminant from the other *Dunaliella* cultures. Strain UTEX 1644 was ordered in 2013 from the UTEX collection by another laboratory, and was confirmed as *D. salina* rather than this unique strain. Thus, the unique strain sent to us by the UTEX collection in 2009 was a contaminant that can no longer be obtained under the number, UTEX 1644. As the research revealed that this strain is of special interest for its growth and lipid production, this strain was deposited at the UTEX collection (*D. viridis* strain UTEX ZZ1150). The other strains are referred to here by the species name indicated from the ITS2 sequence data (*D. tertiolecta*), along with the original strain numbers. The strains included *D. tertiolecta* CCAP 19/9 (120 ± 6

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4 139 μm^3 , mean $\pm$ 1 SD, n = 30 cells), CCAP 19/24 ($132 \pm 6 \mu\text{m}^3$), UTEX 999 ($163 \pm 9 \mu\text{m}^3$), and
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6 140 UTEX 1000 ($168 \pm 8 \mu\text{m}^3$); and *D. viridis* UTEX ZZ1150 ($74 \pm 5 \mu\text{m}^3$) (Mixson et al.,
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8 141 submitted; Table 1).
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11 Preliminary work indicated that the *Dunaliella* strains grew well in Erdschreiber's (Føyn
12 142 1934), Artificial Seawater (ASW, made using f/2-Si nutrients; Guillard, 1975; Tompkins et al.,
13 143 1995), and L1-Si medium (Guillard and Hargraves, 1993), at salinity 30 or 60 depending on the
14 144 strain. Erdschreiber's and ASW media contained soil water with undefined constituents, and
15 145 nutrient components of f/2-Si were similar to those in L1-Si. Therefore L1-Si was selected for
16 146 use in this study. Once growth was established for strains in the source media (Table 1; salinity
17 147 30 or 60), each culture was transferred into a series of increasing proportions of L1-Si and the
18 148 source medium (after Lorenz et al., 2005) until the cells finally were acclimated to 100% L1-Si
19 149 medium. Cell production for each strain was compared in its source medium versus L1-Si
20 150 medium. All strains attained comparable or higher maximum cell production in L1-Si medium
21 151 (Mixson et al. submitted). In a second series of transfers, cultures were adjusted by salinity $\pm$ 5
22 152 to 10 (for each adjustment) using NaCl (YSI 3200 Conductivity Instrument, Yellow Springs,
23 153 Ohio, U.S.A.), and tested for population growth rate (as cell production) to determine the optimal
24 154 salinity for each strain (Mixson et al. submitted). An analogous approach was applied to identify
25 155 the optimum pH for each strain, using incremental changes of 0.1 pH unit (PerpHecT LogR
26 156 meter 350, Boston, Massachusetts, U.S.A.) from an initial pH of 7.5 to 8.5. Accordingly, three
27 157 strains (*D. tertiolecta* CCAP 19/9, UTEX 999, and UTEX 1000) were grown at an optimal
28 158 salinity of 30 and optimal pH of 8.0-8.4 for cell production; the other two strains (*D. tertiolecta*
29 159 CCAP 19/24 and *D. viridis* UTEX ZZ1150) were grown at their optimum salinity of 60 and
30 160 optimal pH of 8.0 to 8.4. Stock culture for each strain was grown at 23°C and $\sim 180 \mu\text{E}/\text{m}^2/\text{sec}$
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(light source, ProLume® compact fluorescent bulbs) under a 12-hr : 12-hr light : dark (LD) photoperiod.

To check species identifications, strains were purified using a combination of differential centrifugation and flow cytometry (Beckman Coulter® EPICS Altra, Hialeah, Florida, U.S.A.) to remove bacterial contaminants (Guillard, 2005; Kawachi and Noël, 2005; Mixson et al., submitted). Cultures were plated on 1% Agar M (Sigma Aldrich®, St. Louis, Missouri, U.S.A.) or 1% Phytoblend (Caisson Laboratories, Logan, Utah, U.S.A.) to promote algal growth or bacterial growth, respectively. Subsequent plating was completed aseptically on a monthly basis until each strain was purified.

2.2 Mass Cultures – The mass cultures consisted of series of growth tubes, each 1.21 m height x 30 cm diameter, made of lightweight 0.1-cm polymer fiberglass which provided ~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 fluorescent light banks (Standard Utilitech, 123.2 cm lights) that provided ~160 to 180 $\mu\text{E}/\text{m}^2/\text{sec}$ for each tube.

The temperature for these experiments was maintained at $23 \pm 1^\circ\text{C}$. 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, U.S.A.), and cell counts (see 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, U.S.A.) and pH (8.3-8.4) with Coralife® salts (CL; Franklin, Wisconsin, U.S.A.) + ~19.35 mL each of ProLine® A and B nutrient mixtures (F/2 following Guillard, 1975; Aquatic Eco-Systems, Apopka, Florida, U.S.A.). 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 the 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 (~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 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 a given tube was ~165 L including culture. The total number of tubes prepared varied by experiment. Cultures of *Dunaliella* 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.

2.3 Low Nitrogen and/or Phosphorus – In bench-scale preliminary trials, *D. viridis* UTEX ZZ1150 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 P deprivation (P as PO_4^{3-} P, added as 10% or 0% of nutrient-replete conditions, respectively). Nutrient deprivation was included to enable comparison of these results to other studies that used nutrient-free medium in an attempt to enhance total lipid production in *Dunaliella* (e.g. Shifrin and Chisholm, 1981; Davidi et al., 2012). Preliminary trials were also conducted using NH_4^+ as the N source, but NH_4^+ N resulted in poor growth, so the experiments included nitrate as the inorganic N source.

In the preliminary trials, 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 g fresh weight (fr wt) per mass culture tube on day 5 (at optimal salinity 30), and maximal lipid production of 29.4 g fr wt per tube on day 11. By comparison, *D. viridis* UTEX ZZ1150 attained maximal biomass of 42.7 g fr wt per tube occurred on day 7 (at optimal salinity 60), and

maximal lipid production was 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 was eliminated from further considered 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, below the practical quantitation limits of the analytical methods used); 10% limitation (10% of the control, replete media concentration(s) of N and/or P added); and a low N and/or P condition (20% of the nutrient-replete concentration(s) in controls). Replicates (bench scale only, ~500 mL volume, n = 4) were grown for one week under the described conditions. Two replicates were then moved to continuous light (CL) for an additional week. The remaining two replicates per treatment were maintained under a 12:12 L:D photoperiod for the additional week. Subsamples were taken at 0800 hr daily until onset of senescence, and cell production quantified as in Section 2.1. At the end of the experiment, samples (~ 450 mL) were centrifuged (3,000 RPM, 10 min – CL2 Centrifuge, ThermoScientific, Milford, Massachusetts, U.S.A.) 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).

2.4 Long-Term pH Stress – Strain *D. viridis* UTEX ZZ1150 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 and pH readings. Subsamples (~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) cultures at each salinity level, allowing for determination of the effect of pH alone on FA production.

2.5 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* CCAP 19/9, CCAP 19/24, and UTEX 999, and *D. viridis* UTEX ZZ1150. Low or no growth as cell production was tracked for cultures grown at an initial pH of 2, 4, 6, or 12. From those observations, low and high pH treatments were selected 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 above until cultures reached early senescence.

Each replicate culture was evenly distributed into seven sterile 50-mL centrifuge tubes by centrifuging (3,000 RPM, 10 min) ~500 mL culture and adding the pellet to a 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 (Mixson et al. submitted), 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. NR (9-diethylamino-5H-benzo(α)phenoxazine-5-one (Sigma-Aldrich) is used to reveal the presence of neutral lipid droplets (Pinkart et al., 1998; Yao et al., 2016). In the method used here, 10 µL of NR (0.625 µg/mL dissolved in 100% MeOH) was added to slides (slightly heated on a hotplate set to low) containing 2 drops of culture with cells that had been immobilized on 1.3% Type V

agarose (Sigma Aldrich). Cells of each strain/treatment were photographed at 600x using an Olympus AX 70 microscope (excitation, 488 nm; emission, 545-655 nm) and an Olympus DP70 camera. Samples were centrifuged (3,000 RPM, 10 min) and immediately frozen at -80°C until total FA analysis.

2.6 CO₂ x Light Regime – The strain *Dunaliella viridis* UTEX ZZ1150 was tested in bench-scale preliminary trials (~500-mL cultures) and was shown to produce neutral lipids under both a 12:12 L:D photoperiod and CL based on fluorescence microscopy with NR-stained cells as described above. Effects of continuous versus pulsed bubbling of CO₂ were also tested. Continuous bubbling resulted in pH readings below neutral (~6.5-7.0), which was detrimental to cell growth. Therefore, CO₂ was bubbled for a daily pulse 4 hr between 0010 and 1400 hr when highest photosynthetic activity was expected.

Stock culture of *D. viridis* UTEX ZZ1150 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 culture stock A; Section 2.2). After one week the culture was in exponential growth phase (~8.50 x 10⁵ 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), CO₂ 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 tubes served as controls and were sampled following the same regime as in days 1-5.

A second experiment in a separate set of mass culture tubes was set up similarly; then continuous light (CL) was imposed for all eight tubes from day 6 through the remainder of the

experiment. Four tubes also received 4 hr of bubbling CO₂ (during 1000 – 1400 hr) beginning at day 6.

Control tubes did not receive CO₂ enrichment in either experiment. For both experiments (12:12 L:D ± pulsed CO₂, and CL ± pulsed CO₂), a ~500 mL subsample was taken between 0800 – 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. Pellets were stored at -80°C until FA analysis.

2.7 Total FAs – Direct transesterification (DT; Griffiths et al., 2010; Mixson et al., submitted) was performed to convert saponifiable lipids to FA methyl esters (hereafter, 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; samples were then homogenized and heated to 85°C. Then, BF₃ (14% in MeOH) was added and samples were re-heated to 85°C. Equal volumes of water and hexane were added and the samples were allowed to separate into layers. The hexane layer, containing neutral lipids, was removed and analyzed with a GC HP5890 series II GC equipped with a FID (Hewlett Packard, refurbished by Primera Scientific LLC, Princeton, New Jersey, U.S.A.) and a 7673A autosampler (Alpha Omega Technologies, Inc., refurbished by Primera Scientific LLC). 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, and injections were performed under the splitless mode. Data acquisition and analysis were completed using 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, North Carolina, U.S.A., personal communication), and area ratios were compared to the internal standard (KEL-FIM-FAME-5 Mixture, Matreya, LLC, State College, Pennsylvania, U.S.A.).

2.8 Statistics – Data were analyzed by either a one-way or two-way ANOVA with repeated measures (SAS v.9.2 - SAS Institute, Inc., 2009). 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.

3 Results

3.1 Low N and/or P – Cell production by *D. viridis* UTEX ZZ1150 under N or P deprivation or 10% limitation (i.e., 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 controls.

Maximum cell production for the nutrient-replete cultures was 2.8×10^6 cells/mL under 12:12 L:D and 2.3×10^6 cells/mL under CL (Fig. 1). Replicates grown in the lower nutrient regime (20% of nutrient-replete conditions) attained maximal cell production of 1.8×10^6 cells/mL in both light regimes. Cultures in the lower nutrient regime (20% N, 20% P, or 20% N+P relative to nutrient-replete conditions) under 12:12 L:D consistently had a lower pH than cultures grown under CL (Table 2). Whether in 12:12 L:D or CL, nutrient-replete controls were similar in pH throughout the growth phase (Table 2). Overall, low nutrient stress as N and/or P (20% of replete conditions) did not promote an increase in total FAs under the 12:12 L:D regime or CL; thus, total FA content in *D. viridis* UTEX ZZ1150 was comparable in both light treatments under the low nutrient regimes (Fig. 2).

3.2 Long-Term pH Stress – Adjusting the pH in exponentially growing culture revealed a potentially useful harvesting mechanism (also see Mixson et al. 2014). Addition of 2N or 6N sodium hydroxide (NaOH) to the culture medium resulted in flocculation of *Dunaliella* cells to the bottom of the flasks or mass culture tubes (Fig. 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). 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 ($\sim 1.25 \times 10^6$ cells/mL) by ~day 18, whereas control mass cultures peaked in mean cell production ($\sim 2 \times 10^6$ cells/mL) by ~day 12. Comparison of total FAs for the control mass cultures grown in salinity 30 and an initial pH of 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).

In the control mass cultures (salinity taken from 60 to 30; no pH adjustment), total FAs were highest on day 12 ($41.7 \pm 6.6\%$; % total FAs ± 1 standard error [SE]) in comparison to day 3 ($9.6 \pm 1.7\%$) ($p < 0.001$) (Table 3). The treatment mass cultures (salinity taken from 60 to 30; pH adjusted to 10) behaved similarly; total FA content increased significantly on day 9 in comparison to day 3 ($p = 0.014$) (Table 3). At day 9, tubes at salinity 60 taken to salinity 30 and adjusted to pH 10 yielded the maximum total FA content ($55.6 \pm 3.1\%$) when compared to FA content on other days in that treatment, or to the FA content of control mass cultures (Fig. 5A).

To ensure that osmotic stress did not affect total FA content, control mass cultures (salinity taken from 60 to 60, or from 60 to 30; no pH adjustment) were compared. A similar trend in total FA production was observed for control mass cultures transferred from salinity 60 to 60, wherein total FAs increased throughout the growth phase (Fig. 5B). For control mass

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4 353 cultures transferred from salinity 60 to 60, total FAs increased significantly on days 9, 12, and 14
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6 354 in comparison to their total FA content on day 3 ($p = < 0.001$ to 0.019) (Table 3). Within the
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8 355 mass cultures transferred from salinity 60 to 30, total FAs increased significantly on all days in
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10 356 comparison to FA content on day 3 ($p < 0.001$ to 0.003) (Table 3). There was no significant
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12 357 difference in total FA content between control mass cultures transferred from salinity 60 to 60
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14 358 versus from salinity 60 to salinity 30 on each sampling date (Fig 5B). Thus, hypo-osmotic stress
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16 359 did not affect total FA content.
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23 361 **3.3 Short-Term pH Stress** – As expected, responses to short-term pH stress were strain-specific.
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25 362 *Dunaliella tertiolecta* CCAP 19/9 significantly increased in total FAs when exposed to pH 7, but
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27 363 only after 24 hr of exposure to pH 10 (Fig. 6, left panels). After resuspension in pH 7 medium,
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29 364 there was a significant increase in total FAs at all time periods from 30 sec to 24 hr ($p = 0.001$ to
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31 365 0.015) (Table 4). However, the maximum total FA content was only $19.9 \pm 0.8\%$, similar to the
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33 366 control total FA content ($15.3 \pm 1.5\%$). After resuspension in pH 10 medium, there was a
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35 367 significant increase in total FAs at 24 hr ($p = 0.046$) (Table 4). The second tested strain, *D.*
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37 368 *tertiolecta* CCAP 19/24, increased total FA content when exposed to pH 7 or pH 10 media (Fig.
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39 369 6, right panels). Maximum total FA content ($35.6 \pm 1.1\%$) was measured 30 sec after exposure to
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41 370 pH 7, and was higher than the total FA content of controls ($30.8 \pm 0.6\%$) (Fig. 6, upper right
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43 371 panel). After resuspension in pH 10 medium, *D. tertiolecta* CCAP 19/24 significantly decreased
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45 372 in total FA content at 30 sec, 1 hr, and 24 hr ($p = 0.001$ to 0.049) (Table 4). The third strain, *D.*
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47 373 *tertiolecta* UEX 999, did not significantly change in total FA content when it was exposed to pH
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49 374 7 medium, but significantly increased total FA content at 30 sec, 5 min, and 24 hr after exposure
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51 375 to pH 10 medium ($p = 0.006$ to 0.035) (Table 4; Fig. 7, left panels). The maximum total FA
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content for this strain was $23.9 \pm 1.2\%$ at 30 sec when resuspended in pH 10 medium, whereas control cultures had $18.6 \pm 1.5\%$ total FA content.

The *D. viridis* UTEX ZZ1150 strain significantly *decreased* in total FA content ($p = 0.005$ to 0.021), in comparison to its FA content on day 3, when it was exposed to either pH 7 (except at 5 min when there was a significant increase to $43.3 \pm 5.5\%$; $p = 0.004$) or pH 10 medium (Table 4; Fig. 7, right panels). When resuspended in pH 10 medium, this strain significantly decreased its total FA content at every time period tested from 30 sec to 24 hr ($p < 0.001$ to 0.003) (Table 4).

3.4 CO₂ Pulses x Light Regime – The pH decreased from ~ 9.3 to ~ 8.0 (12:12 L:D) (Fig. 8A) or from ~ 9.8 to ~ 8.4 (CL) (Fig. 8B) when mass cultures received pulsed CO₂ enrichment. Maximum cell production of mass-cultured *Dunaliella viridis* UTEX ZZ1150 under 12:12 L:D or continuous light (CL) occurred on day 11 (Fig. 8). Tubes under 12:12 L:D $\pm$ CO₂, or under CL without pulsed CO₂, attained maximal cell production of 1.4×10^6 cells/mL, and maximal cell production was comparable in mass cultures under CL + pulsed CO₂ (1.9×10^6 cells/mL) by day 11 (Fig. 8). Mass cultures under CL + pulsed CO₂ were much darker green in color than cultures under 12:12 L:D $\pm$ pulsed CO₂, or under CL without pulsed CO₂ (Fig. 9).

Total FAs increased throughout growth to senescence of mass-cultured *D. viridis* UTEX ZZ1150 (Fig. 10). The total FA content significantly increased at days 9 and 12, in comparison to day 6, in mass cultures grown under 12:12 L:D $\pm$ pulsed CO₂ ($p < 0.001$ to 0.003), and in cultures grown under CL $\pm$ pulsed CO₂ ($p < 0.001$ to 0.002) (Table 5). By day 12, mass cultures in CL+ pulsed CO₂ had a maximal total FA content of $42.8 \pm 8.2\%$, significantly higher than that of cultures in CL without pulsed CO₂ ($22.6 \pm 0.1\%$) ($p = 0.026$) (Table 6, Fig. 10B). Overall, cultures with pulsed CO₂ enrichment had significantly higher total FAs than cultures without

pulsed CO₂, whether in a 12:12 L:D or CL regime ($p = 0.009$) (Table 6). By day 12, comparison of cultures with pulsed CO₂ revealed that this *D. viridis* strain had significantly higher maximal total FA content in CL ($42.8 \pm 8.2\%$) than in 12:12 L:D ($31.5 \pm 1.8\%$) ($p = 0.049$) (Table 6, Fig. 10).

4 Discussion

4.1 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 NH₄⁺ over other N forms, with NO₃⁻ taken up only after NH₄⁺ is depleted (Kaplan et al., 1986; Glibert et al., 2015). Yet, NH₄⁺ can be inhibitory or toxic to cells when used as the sole source of N (Kaplan et al., 1986; Glibert et al., 2015) and, thus, has been referred to as a “paradoxical” nutrient (Britto and Kronzucker, 2002). The threshold of toxicity responses to NH₄⁺ is species-specific, and also can be strain-specific (Glibert et al., 2015). In this study, NH₄⁺ appeared to inhibit both of the tested *Dunaliella* strains even at the lowest concentrations used, and resulted in poor growth.

N deficiency might have been expected to increase lipid accumulation, partly because storage and membrane lipids do not contain N (Roessler, 1990). These lipids can continue to be synthesized while synthesis of N-containing compounds such as proteins and nucleic acids is slowed or stopped as N becomes limiting (Roessler 1990). Inorganic N limitation can 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). Studies with *Dunaliella* strains have yielded varying results, as shown for various other microalgae (e.g., Shifrin and Chisholm, 1981; Lombardi and Wangersky, 1995, and references therein). Some

strains within *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 strains within *D. salina* and *D. tertiolecta* have produced *more* lipids (Weldy and Huesemann, 2007; Chen et al., 2011; Davidi et al., 2012; Arias-Forero et al., 2013). Here, several strains of *D. tertiolecta* and the unique strain *D. viridis* UTEX ZZ1150 *did not* increase neutral lipids in response to low N stress.

Luxury consumption of P by microalgae is a well-known phenomenon (Beardall et al., 2001; Wetzel 2001, and references therein). 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 *Phaeodactylum tricornutum* significantly increased its total FA content in medium without P in comparison to its total FA content in P-replete medium, but this response has not been found in *D. tertiolecta* (Siron et al., 1989; present study) or *D. viridis* UTEX ZZ1150 (present study).

4.3 Long-term pH Stress – A promising harvesting mechanism was revealed when *Dunaliella* cultures were adjusted from pH 8.3-8.4 to pH 10. Spontaneous flocculation or auto-flocculation occurred due to the sharp increase in pH (see Richmond and Becker, 1986). The *Dunaliella* cells excreted sufficient glycerol to enhance their flocculation and removal from the water column (Mixson et al., 2014). The combination of high salinity (60) and pH adjustment to 10 led to no recovery of the culture from flocculation/settlement. By contrast, in media with salinity 30 and 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 FA content to ~56%, whereas the total FA content of controls was only ~29%. Gardner et al. (2011) reported that the chlorophycean, *Scenedesmus* sp. produced appreciable lipids in alkaline medium (pH > 9); however, this observation was also correlated with N depletion. In this study, *D. viridis* UTEX ZZ1150 did not increase total FA content in response to N limitation, suggesting that high pH alone increased total FA content in this mass-cultured *D. viridis* strain.

4.4 Short-term pH Stress –Responses to short-term pH stress were strain-specific but, in general, increases in total FA content were not observed. After exposure to pH 7, only *Dunaliella tertiolecta* CCAP 19/9 significantly increased in total FA content at every time point (30 sec to 24 hr) (note that *D. viridis* also increased total FA production, but only 5 min after exposure; at every other time point, this strain significantly decreased total FA content in response to pH 7). Exposure of cultures to low or high pH led to variation among strains in total FA content as a short-term response. Except for *D. tertiolecta* CCAP 19/9 (at pH 10) and *D. tertiolecta* UTEX 999 (at pH 7), the imposed short-term pH stress led to a *decrease* in total FA content in comparison to the total FA content of controls. It was expected that pH 10 stress would stimulate FA production, as reported for other chlorophycean microalgae (e.g. *Scenedesmus* spp.; Gardner et al., 2012). As explained above, however, *Dunaliella* spp. have differed in responses to environmental stressors relative to other chlorophyceans. It should also be noted that 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 CO₂ x Light – In this experiment, total FAs accumulated throughout the growth cycle for mass-cultured *Dunaliella viridis* UTEX ZZ1150, with highest production by early senescence. Most microalgae have been found to accumulate most lipids during stationary phase or senescence rather than during active growth (Henderson and Sargent, 1989; Hodgson et al., 1991; Brown et al., 1996; Pond and Harris, 1996; Hu et al., 2008 and references therein; Yoo et al., 2010). Aging or senescence of algal cultures is accompanied by a change in lipid biosynthesis pathways from the chloroplast or other cellular membranes to neutral storage lipids (Qunju et al., 2015). Additionally, most algae that have been tested do not grow well in continuous light (e.g., Carvalho and Malcata, 2004; Lorenz et al., 2005; Sforza et al., 2012). 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).

Mass-cultured *D. viridis* UTEX ZZ1150 grown under CL+ pulsed CO₂ had maximal total FA content by day 12 (42.8%) (Fig. 10). In marked contrast to what is known for most microalgae that have been examined, in CL these cultures continually maintained photosynthesis (Mixson, 2013). Without a dark cycle to break down carbon products, the excess carbon as CO₂ apparently was converted by *D. salina* cells into FAs. Gardner et al. (2012) investigated adding sodium bicarbonate (NaHCO₃) to N-depleted cultures. Adding bicarbonate triggered FA production in N-deprived *Scenedesmus* spp. under 14:10 L:D. Here, *D. viridis* UTEX ZZ1150 did not respond to stress imposed by low N and/or P. The combination of CL (continuous light) and pulsed CO₂ enrichment significantly increased total FA production by *D. viridis* UTEX ZZ1150 when compared to cultures grown under CL alone, or in a 12:12 L:D period ± enriched CO₂.

Mass cultures of *D. viridis* UTEX ZZ1150 that received both CL and pulsed CO₂ had much more green pigment than cultures under 12:12 L:D ± pulsed CO₂, or cultures under CL without pulsed CO₂. It was hypothesized that the additional available CO₂ resulted in enhanced chlorophyll *a* production, as has been found in other work. For example, Tripathi et al. (2001) found that certain strains of *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae), *Scenedesmus obliquus* (Chlorophyta, Chlorophyceae), and the cyanobacteria *Spirulina*, *Nostoc*, and *Stigonema* exhibited a 2- to 3-fold increase in both chlorophyll and carotenoid content when cells were exposed to 2% CO₂ after 21 days and 10 days, respectively. Another experiment investigated the effects of CO₂ deprivation on chlorophyll content in the cyanobacterium *Synechococcus lividus* (Miller and Hold 1977). Within 100 hr of CO₂ depletion, the chlorophyll *a* content significantly declined below that of control (CO₂-replete) cultures. When the CO₂-depleted cultures were given CO₂ for 48 hr, the chlorophyll *a* content increased to comparable levels as in the control cultures.

As mentioned, this strain originated in the CAAE as a contaminant in a UTEX *D. salina* culture, and grew well in mass culture under continuous light with maintenance of high lipid production (Mixson, 2013). Under both low- and high-salinity stress in additional experiments with both bench-scale and mass cultures (Mixson, 2013; Mixson et al., submitted), *D. viridis* UTEX ZZ1150 also grew well under continuous light, unlike 13 other tested strains within five *Dunaliella* spp. In other work the same strain, provided by the CAAE, was tested in small batch cultures along with one strain each of three other *Dunaliella* species, for response to changes in light duration x growth temperature (Srirangan et al. 2015). Unlike the strains representing the other three *Dunaliella* species, the rate of cell division in *D. viridis* UTEX ZZ1150 increased under continuous light. A shift from 25° to 35°C under continuous light did not significantly alter

the rate of cell division, but increased triacylglycerol content per cell several-fold. Lauric acid was incorporated into triacylglycerol after 24 hr in continuous light.

5 Conclusion

Environmental stressors of high pH + low salinity, low pH (short-term), and CL with pulsed CO₂ significantly enhanced lipid production as total FAs, on average, by 56%, 43%, and 42%, respectively, in comparison to the lipid content of controls (23%). In contrast to reports for other chlorophytes, low or depleted N and/or P, high pH alone, and 12:12 L:D with pulsed CO₂ in these *Dunaliella* strains did not increase total FA content. 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 CO₂ under continuous light promoted higher total FA production by the unique strain *D. viridis* UTEX ZZ1150 during active growth, rather than only during senescence as for most microalgae. High intraspecific variation in both growth and neutral lipid production was observed for the *Dunaliella tertiolecta* strains under each environmental stressor. Future research can build upon this study to further elucidate the complex relationships among environmental stressors and total FA production, toward harnessing *Dunaliella* as a source of sustainable biofuel. Of the strains tested here, the unique strain *D. viridis* UTEX ZZ1150 was shown to sustain both high maximal cell production and high total FA content in mass culture under continuous light or L:D periods, both during active growth and senescence. Among many published experiments testing lipid production among multiple species and strains of *Dunaliella*, this aberrant *D. viridis* strain, found as a contaminant of a commercial culture, appears to hold exceptional promise for sustainable biofuel production.

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Figure Legends

Fig. 1. Cell production of *Dunaliella viridis* UTEX ZZ1150 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 (●) with A) 12:12 L:D photoperiod and B) continuous light (CL). Data are given as means $\pm$ 1 SE (n = 2).

Fig. 2. Total FAs (as percent dry weight, %) in *Dunaliella viridis* UTEX ZZ1150 under N-replete 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 (CL) for control (nutrient-replete) versus low N and/or low P conditions. Data are given as means $\pm$ 1 SE (n = 2).

Fig. 3. Photographs showing cell flocculation at either A) mass culture or B) bench scale in response to high pH. Arrows indicate the layer of *Dunaliella* cells at the bottom of each container.

Fig. 4. Photograph of mass culture tubes in the long-term pH stress experiment after 9 days: A) and B) control cultures, both grown in salinity 60; C) culture at salinity 60 and high pH – *Dunaliella* cells flocculated to the bottom of the containers after high pH adjustment and did not recover; D) culture at salinity 30 and high pH - *Dunaliella* cells flocculated after high pH adjustment, but recovered and cell production increased by day 9.

Fig. 5. (A) Long-term pH stress: Total FAs (as percent by dry weight, %) in *Dunaliella viridis* UTEX ZZ1150, 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. The data 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 $\pm$ 1 SE ($n = 3$). **(B)** Long-term pH stress and hypo-osmotic stress: Total FAs (as percent dry weight, %) in *Dunaliella viridis* UTEX ZZ1150, comparing controls (no salinity adjustment) to the treatment (salinity adjusted from 60 to 30). Samples were taken in 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 given as means $\pm$ 1 SE ($n = 3$).

Fig. 6. Short-term pH stress: Total FAs (as percent by dry weight, %) in (left panels) *Dunaliella tertiolecta* CCAP 19/9 (left panels) and (right panels) *D. tertiolecta* CCAP 19/24 (right panels). Upper panels show response to low pH stress (initial pH adjusted from 9 to 7); lower panels show response to high pH stress (initial pH adjusted from 9 to 10). Significant differences from controls (= Before) are indicated by an asterisk ($p \leq 0.05$). Data are given as means $\pm$ 1 SE ($n = 3$).

Fig. 7. Short-term pH stress: Total FAs (as percent by dry weight, %) in *D. tertiolecta* UTEX 999 (left panels) and *D. viridis* UTEX ZZ1150 (right panels). Upper panels show response to low pH stress (initial pH adjusted from 9 to 7); lower panels show response to high pH stress (initial pH adjusted from 9 to 10). Significant differences from controls (= Before) are indicated by an asterisk ($p \leq 0.05$). Data are given as means $\pm$ 1 SE ($n = 3$).

Fig. 8. Mean cell production and pH values for mass culture of *D. viridis* UTEX ZZ1150 comparing responses in two light regimes, with vs. without pulsed CO₂ enrichment: Cell production with (●) and without (◆) pulsed CO₂, and pH values with (▲) and without (■) pulsed CO₂ with A) 12:12 L:D photoperiod or B) continuous light (CL). Arrows indicate where subsamples were taken for total FA analysis. The dashed vertical line indicates initiation of pulsed enrichment with 4 hr of CO₂ bubbling

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

Fig. 9. Photos showing the appearance of mass cultures in the pulsed CO₂ x light regime experiment: A) 12:12 L:D photoperiod or B) continuous light (CL). Tubes that received pulsed CO₂ enrichment are indicated.

Fig. 10. Total FAs (as percent by dry weight, %) for mass-cultured *D. viridis* UTEX ZZ1150 $\pm$ pulsed CO₂: A) Cultures grown under 12:12 L:D; and B) cultures grown under continuous light (CL) after 6 days. Samples were taken in mid-exponential (day 6), late-exponential (day 9) growth phases, and in early senescence (day 12). Cultures were compared $\pm$ pulsed CO₂ enrichment. Significant differences from day 6 are indicated by an asterisk ($p \leq 0.05$). Data are given as means $\pm$ 1 SE (n = 4).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Table 1

Species Name *	Commercial Source and Strain Number	Biovolume (μm^3 , mean ± 1 SE, n = 30)	Maximum Cell Production (10^6 cells/mL ± 1 SE; n = 3) in L1 - Si medium
<i>Dunaliella tertiolecta</i>	CCAP 19/9	120 $\pm$ 6	6.2 $\pm$ 0.9
<i>Dunaliella tertiolecta</i>	CCAP 19/24	132 $\pm$ 6	3.4 $\pm$ 0.4
<i>Dunaliella tertiolecta</i>	UTEX 999	4.4 $\pm$ 0.8	4.4 $\pm$ 0.8
<i>Dunaliella tertiolecta</i> **	UTEX 1000	168 $\pm$ 8	6.6 $\pm$ 0.8
<i>Dunaliella viridis</i>	UTEX ZZ1150	10.0 $\pm$ 0.6	10.0 $\pm$ 0.6

* We refer here to the *D. tertiolecta* strains by the species name indicated from our ITS2 sequence data, and not by the strain number. The *D. viridis* strain was a contaminant from a commercial culture, and was deposited by the NCSU Center for Microbial Sciences in the UTEX collection.

** This strain inexplicably stopped growth and lipid production, so its use in the study was discontinued.

Table 2

Treatment	pH after 10 days under 12:12 L:D	pH after 3 days under CL
Control	9.39	9.43
N+P	9.2	9.45
P	9.38	9.55
N	9.41	9.64

Treatment	pH after 10 days under 12:12 L:D	pH after 3 days under CL
Control	9.25	9.43
N+P	8.43	8.99
P	8.70	9.24
N	8.80	9.27

Table 3

Treatment	Day	Total FAs (%) (mean ± 1 SE)	LS Mean Diff	p value
Salinity 60 to 30 (unadjusted)	3	9.6 ± 1.7		n.a.
	6	29.8 ± 7.0	20.19	0.003
	9	28.6 ± 8.0	18.95	0.004
	12	41.7 ± 6.6	32.09	< 0.001
	14	39.8 ± 4.6	30.23	< 0.001
Salinity 60 to 30 (adjusted)	3	19.2 ± 2.5		n.a.
	6	33.8 ± 7.6	14.59	n.s.
	9	55.6 ± 3.1	36.44	0.014
	12	46.1 ± 15.6	26.86	0.031
Salinity 60 to 60 (unadjusted)	3	13.0 ± 4.6		n.a.
	6	18.4 ± 5.9	5.38	n.s.
	9	23.7 ± 3.6	10.64	0.019
	12	26.5 ± 4.4	13.49	0.007
	14	37.7 ± 1.2	24.66	< 0.001

Table 4

Strain	pH	Time Point	Mean $\pm$ 1 SE (%)	LS Mean Diff	p value
<i>D. tertiolecta</i> CCAP 19/9	10	Control	14.5 $\pm$ 1.7		n.a.
		30 sec	18.9 $\pm$ 1.8	4.44	n.s.
		5 min	18.9 $\pm$ 0.8	4.40	n.s.
		30 min	17.6 $\pm$ 1.2	3.09	n.s.
		1 hr	19.1 $\pm$ 5.2	4.61	n.s.
		2 hr	17.8 $\pm$ 2.9	2.86	n.s.
		24 hr	19.7 $\pm$ 6.0	5.25	0.046
	7	Control	15.3 $\pm$ 2.7		n.a.
		30 sec	19.6 $\pm$ 3.3	4.37	0.004
		5 min	18.7 $\pm$ 1.7	3.41	0.015
		30 min	19.6 $\pm$ 2.4	4.37	0.004
		1 hr	19.5 $\pm$ 3.1	4.23	0.005
		2 hr	20.5 $\pm$ 2.3	5.25	0.001
		24 hr	19.6 $\pm$ 1.3	4.35	0.004
<i>D. tertiolecta</i> CCAP 19/9	10	Control	30.2 $\pm$ 3.6		n.a.
		30 sec	28.0 $\pm$ 1.4	-2.18	0.049
		5 min	30.3 $\pm$ 0.7	0.11	n.s.
		30 min	28.4 $\pm$ 3.8	-1.76	n.s.
		1 hr	25.0 $\pm$ 0.9	-5.23	0.001
		2 hr	29.5 $\pm$ 1.7	-0.24	n.s.
		24 hr	26.9 $\pm$ 1.2	-3.25	0.01
	7	Control	30.8 $\pm$ 1.1		n.a.
		30 sec	35.6 $\pm$ 1.9	4.82	0.081
		5 min	32.0 $\pm$ 2.5	1.19	n.s.
		30 min	32.5 $\pm$ 12.8	1.69	n.s.
		1 hr	30.8 $\pm$ 4.3	0.02	n.s.
		2 hr	29.6 $\pm$ 0.6	-1.18	n.s.
		24 hr	29.9 $\pm$ 4.8	-0.90	n.s.
<i>D. tertiolecta</i> UTEX 999	10	Control	18.7 $\pm$ 2.6		n.a.
		30 sec	23.9 $\pm$ 2.1	5.22	0.006
		5 min	22.5 $\pm$ 4.2	3.83	0.025
		30 min	19.9 $\pm$ 2.9	1.26	n.s.
		1 hr	20.1 $\pm$ 4.3	1.40	n.s.
		2 hr	21.7 $\pm$ 3.6	3.08	n.s.
		24 hr	22.2 $\pm$ 1.6	3.50	0.035
	7	Control	21.3 $\pm$ 5.0		n.a.

Table 5

Method	Day	Total FAs (mean ± 1 SE, %)	LS Mean Diff	p value
12:12, no CO ₂	6	15.8 ± 2.3		n.a.
	9	22.8 ± 1.3	7.01	0.003
	12	27.2 ± 1.8	11.36	< 0.001
12:12, no CO ₂	6	14.3 ± 2.9		n.a.
	9	27.9 ± 2.5	13.59	< 0.001
	12	31.5 ± 1.8	17.20	< 0.001
CL, no CO ₂	6	13.0 ± 1.6		n.a.
	9	19.6 ± 2.2	6.52	0.001
	12	22.6 ± 1.2	9.58	< 0.001
CL with CO ₂	6	14.2 ± 1.5		n.a.
	9	33.2 ± 5.9	19.07	0.011
	12	42.8 ± 8.2	28.67	0.002

Table 6

Day	Comparison			Total FAs (%) (means)		LS Mean Diff
9	12:12, no CO ₂	vs.	12:12 with CO ₂	22.8	27.9	– 6.58
	CL, no CO ₂	vs.	CL with CO ₂	19.6	33.2	– 12.50
	12:12, no CO ₂	vs.	CL, no CO ₂	22.8	19.6	0.49
	12:12, with CO ₂	vs.	CL with CO ₂	27.9	33.2	– 5.48
12	12:12, no CO ₂	vs.	12:12 with CO ₂	27.2	31.5	– 5.84
	CL, no CO ₂	vs.	CL with CO ₂	22.6	42.8	– 19.10
	12:12, no CO ₂	vs.	CL, no CO ₂	27.2	22.6	1.79
	12:12 with CO ₂	vs.	CL with CO ₂	31.5	42.8	– 11.50
12	12:12	vs.	CL	29.3	32.7	– 9.67
	no CO ₂	vs.	with CO ₂	24.9	37.2	– 24.90