METHODS TO INCREASE ALGAE BIOMASS PRODUCTIVITY IN RACEWAY POND
MONOCULTURES

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ABSTRACT

Methods to increase algae biomass productivity in raceway pond monocultures

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The economics of algae biofuels and bioproducts would be improved by increased biomass productivity. Two studies on this potential are described in this thesis – one on a locally isolated filamentous yellow-green alga and the other on a planktonic strain genetically improved via adaptive laboratory evolution.

Polycultures have been viewed as productive, stable, and, in some cases, harvestable by natural bioflocculation. Local native strains might have higher productivity than culture collection strains because they are already adapted to local outdoor conditions. In this study, the filamentous yellow-green alga *Tribonema minus* was isolated from a local volunteer polyculture. Its productivity as a monoculture was compared to a volunteer polyculture in a year of thrice-weekly samples. The study was conducted in duplicate 1,000-L, 3.5-m^2 outdoor raceway ponds fed with nitrified and filtered reclaimed wastewater. *T. minus* monocultures were more productive (17.6 ± 0.5 g/m^2-d; mean ± range) than the polyculture (13.3 ± 0.4 g/m^2-d). The *T. minus* monocultures were stable, growing for an average of 38 days before significant contamination with other algae genera, at which point the cultures were restarted. The annual average biochemical composition, in percent of ash-free dry-weight, of the *T. minus* cultures was 28.3 ± 0.4% (mean ± std. dev.) carbohydrates, 37.6 ± 0.7% proteins, and 6.1 ± 0.3% lipids. Eicosapentaenoic acid, a valuable nutritional omega-3 fatty acid, comprised 0.3% to 4% of the ash-free dry-weight and was the predominant fatty acid methyl ester measured. In summary, an alga isolated from a volunteer polyculture was more productive as a monoculture than the originating polyculture. The monoculture biomass contained a valuable nutritional fatty acid.

Scenedesmus obliquus was subjected to UV mutagenesis followed by cultivation in benchtop bubble columns at high dilution rates to select for cultures (cultigens) that grew faster than the wild-type. Fast growing cultigens were transferred to 1,350-L outdoor raceways ponds for productivity measurement. Cultigen and wild-type cultivations were conducted on reclaimed wastewater media in coastal central California for seven months. One cultigen, MBE 501, had 23% higher productivity than the *S. obliquus* wild-type (11.5 ± 0.02 vs. 9.4 ± 0.6 g/m^2-d) during July 28 -December 30, 2019. MBE 501 had been

subjected to 1:400 and 1:200 dilutions twice per week for the first two months and last five months of selection, respectively, and went through 289 generations in the lab.. Compared to a volunteer polyculture (14.4 $\pm$ 1.3 g/m^2-d), MBE 501 was not as productive on average. This study demonstrated that high dilution rates in lab cultures can select for cells that are more productive in outdoor raceways. Genetic comparison of MBE 501 and its wild-type are pending.

Keywords: algae, wastewater, bioprospecting, adaptive laboratory evolution, directed evolution, strain development, productivity

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1. Introduction

Microalgae can be feedstocks for biofuels and bioproducts, with the potential advantage of not requiring arable land or freshwater for cultivation (Burlew, 1953). Despite the high growth rate of many microalgae, the projected costs of algae biofuels are not competitive with liquid fossil fuels (Davis et al., 2011). Algae biorefineries have been suggested to reduce the cost of algae biofuel by simultaneous production of the low-value biofuels and high-value bioproducts (Brennan and Owende, 2010) such as nutritional fatty acids (e.g., eicosapentaenoic acid) and pigments (phycocyanin, astaxanthin, and other carotenoids) in addition to less valuable constituents such as crude protein. In particular, cultivation of yellow-green algae (xanthophytes) is one option for algae biorefinery because this taxon is known to produce beta-carotene and eicosapentaenoic acid—compounds with health benefits that can be sold as food supplements or used in animal feeds (Chen, 2019).

Fertilizer costs can be decreased or eliminated by using treated wastewaters containing dissolved nutrients as algae growth media. Algal assimilation of nutrients also provides wastewater treatment benefit (Oswald and Gotaas, 1954; Lundquist et al., 2010). Increasing areal productivity is another way to potentially lower production costs (Davis et al., 2011). The term "culture performance" can be used to encompass the combination of growth rate and culture stability (e.g., resistance to pathogens and weed algae). High performance cultures would have high annual average areal productivity due to rapid growth rate plus minimal culture crashes and contamination events.

Native strains are adapted to their local biotic and abiotic conditions and are expected to perform better under those conditions than nonnative culture collection strains (Sun et al., 2011). In the present study, filamentous *Tribonema minus,* was isolated from a volunteer polyculture grown in outdoor raceway ponds on reclaimed wastewater. The raceways were located in San Luis Obispo in central coastal California. The annual average productivity of *T. minus* grown in outdoor raceways was compared to volunteer

polycultures. The biochemical composition of *T.* minus cultures was measured to determine potential uses of the biomass.

T. minus is an unbranched filamentous yellow-green alga in the class Xanthophyceae. Filaments are composed of "H" shaped overlapping compartments, with each compartment containing two to three –disk-shaped chloroplasts arched against the cylindrical cell wall (). The chloroplasts contain chlorophyll *a*, chlorophyll *c*, beta-carotene, and diadinoxanthin (Wehr, 2010). *T. minus* biomass has been demonstrated as a feedstock for aquaculture feed and biofuels production. Chen et al. (2019) found that flesh quality was better for fish receiving feed containing *T. minus*. They also observed that *T. minus* produced both eicosapentaenoic acid and palmitoleic acid, both valuable for nutrition. Additionally, Wang et al. (2014) showed that *Tribonema* can be used as a feedstock for bioethanol and biodiesel production.
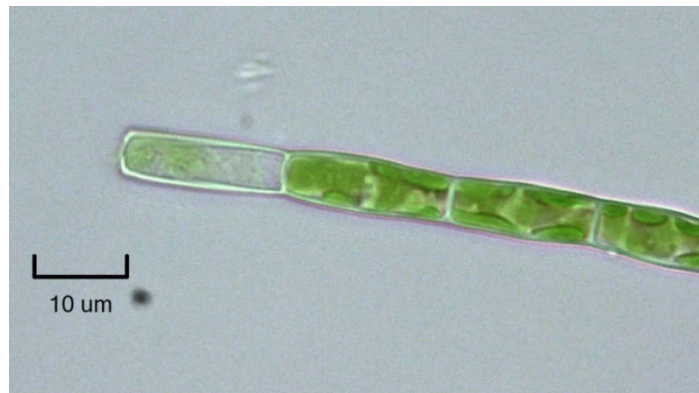
Figure 1. Micrograph of native *Tribonema minus* used in the present work. 1,000x total magnification. Filaments are unbranched with square ends.

2. Materials and Methods

This section describes the materials and methods associated with strain isolation of *T. minus*, culture propagation for inoculum production, and outdoor cultivation. The analytical methods used to measure raceway pond productivity, nutrient concentrations and biochemical composition are described. The equation used to calculate the areal productivity of outdoor raceway cultures is presented as well as the other data analysis methods used for comparison between *T. minus* and the polyculture.

2.1 Strain Isolation

Samples were obtained from municipal reclaimed wastewater raceway polyculture ponds at the San Luis Obispo Water Resource Reclamation Facility (SLO WRRF) in San Luis Obispo, California Samples were diluted 100-fold and several 100 µL aliquots were plated on solid BG-11 medium (utex.org/products/bg-11-n-medium) containing a 1:1000 dilution of an antibiotic-antimycotic mixture consisting of penicillin, streptomycin and amphotericin B (Millipore Sigma, Burlington, Mass.). Plates were incubated at room temperature under continuous illumination (100 µmol/m^{2}s^1) for 10 – 20 days. Isolated colonies were expanded on a shaker table under 200 µmol/m^{2}s^1 set at 200 rpm and using 150 mL borosilicate flasks containing 10-20 glass beads and BG-11 growth media. Strain isolated was performed by Dr. Aubrey Davis.

2.2 *T. minus* Inoculum Production

T. minus cultures for scale-up inoculum production were started indoors from agar plate cultures in five one liter glass bubble columns using 800 mL of autoclaved liquid BG-11 media. Plates were maintained at room temperature (20-25ºC) under low light conditions (50-100 µmol/m^{2}s^1) on solid BG-11 medium. Bubble columns were illuminated on a 16h:8h light/dark cycle with a light intensity of 600 100 µmol/m^{2}s^1 at temperatures between 20 and 25ºC. Once per week 600 mL of each bubble column was harvested and replaced with fresh BG-11. Bubble column cultures were completely restarted from plates every two months. Culture harvested from bubble columns was used as inoculum for four indoor 18-L acrylic flat panel photobioreactors. Bubble columns and panel photobioreactors were sparged from the bottom of the reactors with 1.5% v/v CO_2-air mixture at a flowrate which maintained complete mixing. Indoor reactors and had a typical pH of 7.5. Panels were started at a volume of 10 L with chlorinated and dechlorinated reclaimed wastewater from the SLO WRRF. Panels were topped off to 15 L after five days of growth. Panels were harvested completely each week and bleached before restart. Microscopic examination was conducted weekly on bubble column and panel cultures to ensure culture purity. Photos of the bubble columns and panel photobioreactors used for inoculum production can be found in the appendix.

Culture harvested from the panels was used as inoculum for outdoor raceway ponds.

2.3 Outdoor Pond Materials

A native volunteer polyculture was established by seeding a raceway pond with algae samples from nearby wastewater evaporation ponds at the SLO WRRF. *T. minus* cultures and the volunteer polyculture were grown in duplicate 1000-L paddle-wheel mixed raceway ponds (3.5-m^2 surface area, 0.3-m depth; RW3.5, MicroBio Engineering Inc., San Luis Obispo, Calif.) (). Raceway ponds were constructed of white high-density polyethylene resin (HDPE) and grade-316 stainless steel cross beams. The outsides of pond side walls were painted black to prevent light penetration. Pond paddle wheels were made of transparent polycarbonate. Pond depth was set at 30 cm using a 4-inch PVC standpipe (Figure 3).

Figure 2. Paddle-wheel mixed raceway ponds used in the present study. (3.5-m^2 surface area, 0.3-m depth). *Tribonema minus* and the volunteer polyculture were each cultivated in duplicate ponds.

Figure 3. Effluent standpipe used for sample collection and to maintain the 30-cm pond depth. Mixing flow in the pond was counterclockwise.

Ponds were fed fully-nitrified, granular media-filtered reclaimed wastewater (RWW) from SLO WRRF (Table 1). RWW flowed from a pressurized line provided by the WRRF to 55-gallon HPDE constant headtanks, which were connected to the ponds by 1 ¼ inch schedule 40 PVC pipe. Flow of RWW to the ponds was controlled by actuated valves (Part #EATB1150STE, Hayward Flow Control, Clemmons, North Carolina) and electronic controllers (APEX Fusion by Neptune Systems, Morgan Hill, Calif.). Ponds were sparged with pure CO_2 from 50-lb cylinders. Porous soaker hose tubing in each pond was connected to ½ inch plastic tubing mainline, and the mainline to a regulator (Taprite, Item #: C384-3741T) the CO_2 tank. Mainline pressure was set at 15 psi. The CO_2 soaker hose was zip-tied to a weighted PVC strut to keep the hose at the bottom of the pond and prevent the hose from becoming entangled in the paddlewheel. Figure __ shows a process flow diagram of the outdoor raceway pond testing site at the SLO WRRF. Figure __ shows a labelled photograph of a raceway pond.
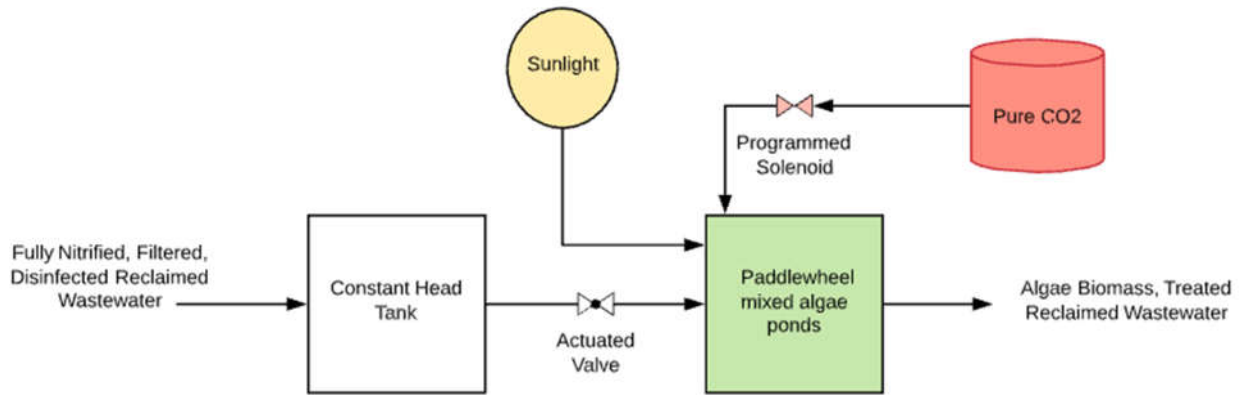
Figure 4. Process flow diagram of the outdoor raceway pond testing site at the San Luis Obispo Water Resource Reclamation Facility. Wastewater was disinfected by chlorination and was dechlorinated prior to use.
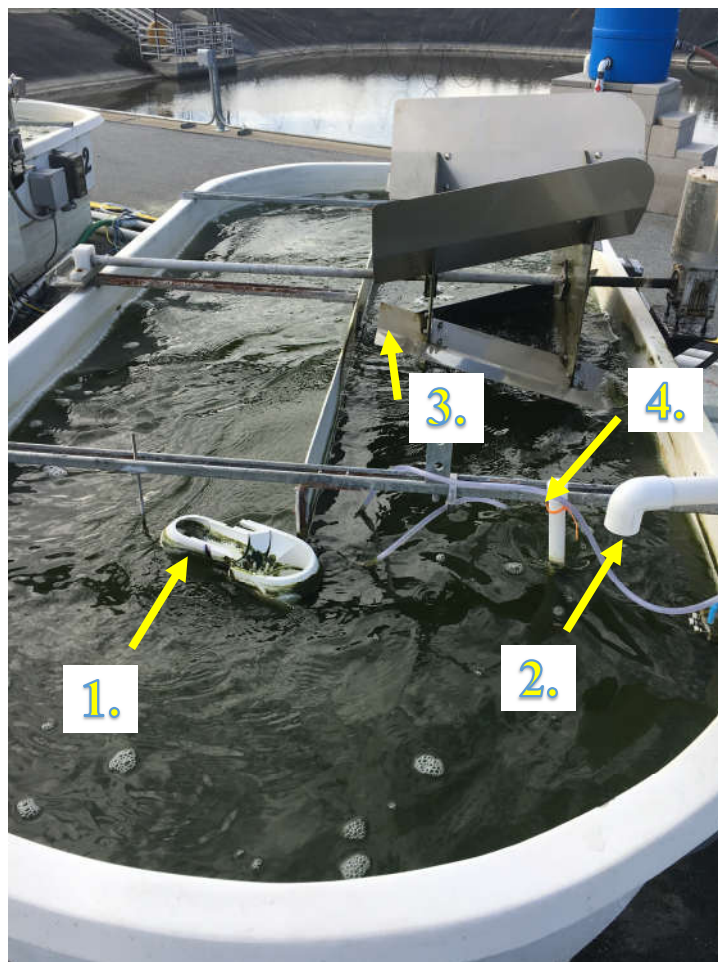
Figure 5. Photograph of an outdoor raceway pond culture. (1) The standpipe collected overflow to keep pond depth constant. (2) The pipe jutting over the sidewall of the pond provided influent reclaimed

wastewater. (3) The flow direction in the pond was counterclockwise. (4) The PVC pipe with attached clear tubing is used to hold porous CO_2 sparging tubing at the bottom of the pond.

APEX Fusion was used as the electronic system for pond management, including controlling CO_2 sparging and the reclaimed wastewater feed cycle. In situ temperature and pH probes were hooked up to the APEX Fusion system. Temperature and pH were monitored continuously, with a data point logged at the top of each hour.

Table 1. Reclaimed Wastewater Yearly Average Constituent Concentrations, May 8, 2017 to April 27, 2018. SLO WRRF data provided by Landon Mortimer.

Constituent	Average Concentration (mg/L)	Standard Deviation	n
Total Suspended Solids*	4.43	2.33	354
Ammonia Nitrogen*	0.36	1.43	354
Nitrate Nitrogen*	45.10	8.01	354
Dissolved Phosphorus**	5.43	0.77	36
5-day Biochemical Oxygen Demand**	4.85	1.92	75

* Measured by SLO WRRF   **Measured by Cal Poly

2.4 Outdoor Pond Operations

After initial seeding and start up the volunteer polyculture was not seeded with additional algae. *T. minus* raceway cultures were started using 25 L of inoculum from indoor panels for each pond. Cultures were started at a depth of 10 cm in RWW. Cultures were gradually filled to the operating depth of 30 cm over two days and were operated in batch mode at 30 cm until the cultures had a biomass concentration such that the bottom of the pond could not be seen. This density was typically 50 – 80 mg/L AFDW for *T. minus.* Not being able to see the bottom of the pond is an approximation for knowing that the greatest amount of incident photons will be absorbed by the culture and water column, and not be wasted by hitting the bottom of the pond and reflecting out of the culture. Cultures will have lower productivity if

they do not have a sufficient biomass concentration to capture the highest number of incident photons (Slocombe et al, 2016).

Once raceway pond cultures achieved sufficient density, they were operated with semi-continuous flow on an 8-hour feed cycle. Ponds received influent reclaimed wastewater daily from 08:00 to 16:00 local time with influent pulses every half hour for a total of 16 pulses per pond per day. Dosing timers were adjusted to follow Day Light Savings Time. Hydraulic residence times (HRTs) were controlled to within 10% of the desired value in weekly calibrations in which the volume of one pulse was measured. The HRT was calculated with . HRT was adjusted throughout the study with the goal of maintaining an AFDW concentration of 80 -120 mg/L. This concentration was found to be high enough to prevent washout of photoautotrophic algae cultures (R. Spierling, unpublished data). HRT of *T. minus* cultures was maintained at 2 days for the entire year, while the polyculture HRT was adjusted between 2 to 4 days ().

Equation 1. *Calculation of pond hydraulic residence time*

$$HRT = \frac{Volume}{Flow} = \frac{Average\ Depth,\ m * Pond\ area, m^2}{Measured\ Pulse\ Volume, m3/pulse * 16\ \frac{pulses}{day}}$$

Table 2. Hydraulic Residence Times used for Volunteer Polyculture Ponds

Start	End	HRT (days)
5-Jul-18	27-Dec-18	2.0
28-Dec-18	15-Jan-19	3.0
16-Jan-18	3-Mar-19	4.0
4-Mar-19	17-Mar-19	3.0
18-Mar-19	8-Jul-19	2.0

Ponds were sparged with pure CO_2 to maintain a pH below 8.0, and in situ pH probes were calibrated weekly. CO_2 flowed to ponds by a programmed solenoid, which opened after a 5-minute delay when pH exceeded 8.0. The solenoid closed after pH had fallen below 7.5. Ponds were mixed by a paddlewheel set at a speed of 25 rpm.

2.5 Sampling Methods

Raceway pond samples for ash-free dry weight (AFDW), microscopy, nitrate and phosphorus measurement were collected between 09:00 and 10:00 local time every Monday, Wednesday, and Friday. After a routine influent pulse to a pond, a plastic cup was inserted into the pond standpipe to collect an overflow sample of 1.5 L (). Samples were transferred from the plastic cup to 1.5L polypropylene sample bottles (Part # 2121-0005, Thermo Scientific™ Nalgene™, Waltham, Mass.). Sample bottles were stored in a dark cooler and transported to a Cal Poly laboratory. The samples spent between 1 to 3 hours in the cooler before analysis. Samples of 20 L were collected weekly from each pond for biochemical analysis. Samples for biochemical analysis were settled in an indoor lab at room temperature (20-25ºC) after collection for two hours followed by filtration or centrifugation to dewater the biomass, which was then freeze dried for at least two days.

2.6 Ash-Free Dry Weight Measurement

Algal concentrations as mg/L ash free dry weight (AFDW), following APHA Standard Methods 2450 (APHA, 2005), were measured in triplicate three times per week on samples collected from pond standpipe overflow. Samples collected were homogenized prior to analysis.

2.7 Raceway Pond Culture Composition

The *T. minus* pond cultures were examined weekly under the microscope to evaluate culture health, predator populations, and contamination with algae other than *T. minus*. Polyculture pond cultures were also examined for algae genera composition. Raceway pond samples were prepared on a wet mount and viewed under magnifications of 100x, 400x and 1,000x using a bright field microscope (CX41 upright microscope, Olympus Lifescience, Shinjuku, Tokyo) with microscope camera (INFINITY2-1R, Teledyne Lumenera, Ottawa, Ontario) and image capture software (Infinity Analyze and Capture, Teledyne Lumenera, Ottawa, Ontario). One wet mount slide per pond was evaluated for percent biovolume contributed by algae genera. If a *T. minus* pond culture was less than 80% pure in terms of biovolume as determined by microscopy both ponds in the set were cleaned with bleach and the culture restarted from indoor inoculum.

2.8 Raceway Pond Nutrient Concentrations

Nitrate, the predominant nitrogen species in fully-nitrified reclaimed wastewater, was measured weekly using Hach TNT 836 Nitrate vial kit and TNTplus® method 10206. Dissolved phosphorus was measured weekly on outdoor raceway pond cultures. Samples were filtered through a 0.45-micron filter prior to analysis using Hach TNT 844 Phosphorus vial kit and TNTplus® method 10209. The purpose of measuring nutrient concentrations was to ensure cultures were nitrogen and phosphorus replete.

Algae are about 8% nitrogen and 1% phosphorus in percent of AFDW. Average reclaimed wastewater nutrient concentrations were 45.1 mg/L NO_3 -N and 5.4 mg/L PO_4-P (Table 1). If raceway pond cultures were to reach twice the upper end of the goal concentration (80-120 mg/L AFDW), AFDW concentrations in the cultures would be 240 mg/L. At this concentration, algae biomass would assimilate 19.2 mg/L nitrate nitrogen and 2.4 mg/L phosphorus. At twice the goal concentration, the bulk fluid in the raceway pond cultures would still contain approximately 25.9 mg/L nitrate nitrogen and 3.03 mg/L phosphorus, indicating that cultures will be nutrient replete when a safety factor of two is applied to the goal biomass concentration.

2.9 Raceway Pond Culture Biochemical Composition

Biochemical analysis for carbohydrates, lipids as fatty acid methyl esters (FAMEs) and protein was completed using National Renewable Energy Labs methods (Laurens, 2015, Van Wychen and Laurens, 2015a, Van Wychen and Ramirez, 2015, Van Wychen and Laurens, 2015b). Samples collected from the ponds were dewatered by centrifugation and then freeze-dried for at least two days. Freeze-dried algal biomass was tested for ash, carbohydrate, lipid, and protein content. Totals solids of samples were measured after 18+ hours in a 60ºC oven to ensure a constant moisture content of less than 20%. Ash content was measured after placing samples in a 575 ºC muffle furnace for 24 hours. Total carbohydrates were tested by hydrolysis to monomers and 3-methyl-2-benzotheazolinonehydrazone (MBTH) reaction with spectrophotometry at 620 nm. Lipids were converted to FAMEs by acid-catalyzed transesterification and then quantified by GC-FID. NuCheck lipid standard (#: GLC 461 C, Nu-Chek Prep, Inc, Elysian,

Minn.) was used to determine the peak for each FAME, and match sample peaks to generate a FAMEs profile. The sum of the FAMEs profile concentrations was equal to the total FAMEs concentration. Protein was determined by combustion and elemental nitrogen conversion by a factor of 4.78. Biochemical analysis for this study was performed by Sara Leader.

2.10 Raceway Pond Productivity Calculation

Gross areal productivity of outdoor raceway cultures was calculated using AFDW, HRT and pond depth (Equation 2). Productivity values reported herein are gross areal productivity, the total biomass produced by each pond per area per time. Samples were collected from standpipe overflow, meaning that productivity values reported herein represent the biomass that would be harvested. Gross productivity of reclaimed wastewater algae cultures is nearly equal to net productivity, as AFDW contribution from influent reclaimed wastewater to the raceway cultures was negligible. In addition, productivity values reported herein are from photoautotrophic cultures, as exogenous carbon concentrations in the reclaimed wastewater are very low (Table 1). When reporting productivity, low biomass concentration values due to restart of outdoor *T. minus* cultures were excluded from reporting. Productivity calculations began when ponds reached a density of approximately 80 mg/L.

Downtime and restart of *T. minus* monocultures must be accounted for when comparing the productivity of *T. minus* and the polyculture. Total biomass produced per pond area per year factors in culture downtime as well as productivity during normal operations. To calculate the annual biomass production for each culture productivity measurements were interpolated to give each day of the year a productivity value. Samples were not taken on Tuesdays, Thursdays, or Weekends. Interpolation for no sample days was done by averaging the antecedent and subsequent measurements and setting the productivity value for the day(s) in between measurements equal to the average. Days in which *T. minus* ponds were contaminated, empty or growing up to density were counted as zero productivity, as culture downtime is not the same as a day in which cultures were operational without measurement.

Equation 2. *Calculation of outdoor raceway pond gross areal productivity*

$$Gross\ Areal\ Productivity, \frac{g}{m^2 * day} = \frac{AFDW, \frac{g}{m^3} * (Depth, m)}{(HRT, days)}$$

3. Results and Discussion

This section compares the performance of *T. minus* monocultures to the polycultures in raceway ponds. Ash-free dry-weight concentrations over time for both cultures are presented, followed by productivity. Monthly average and annual average productivities are presented for each culture, along with the average length of *T. minus* growth periods outdoors. The annual biomass produced by each culture per area was then calculated. The section concludes with the biochemical composition of raceway *T. minus* cultures and a discussion of future research areas.

3.1 Biomass concentrations

AFDW varied seasonally due to changes in local weather and HRT (**Error! Reference source not found.**). Higher HRTs were used for the local polyculture during the winter months of December and January. Both the polyculture ponds and *T. minus* ponds had AFDW concentrations below the 80 mg/L goal in December and January (), indicating that residence times should have been increased further during these months. *T. minus* cultures started in January did not reach a density of above 50 mg/L and were excluded from productivity analysis as the HRT was too low to allow the cultures to grow up to the goal density. During Fall, Spring and Summer months the ponds were operated at HRTs that kept AFDW concentrations near the goal range of 80-120 mg/L AFDW, with the exception of low AFDW data points at the beginning of *T. minus* growth periods. These start up points were excluded from productivity analysis. Notable spikes in AFDW were observed in the *T. minus* pond cultures throughout the study, the exact causes of which were not determined.
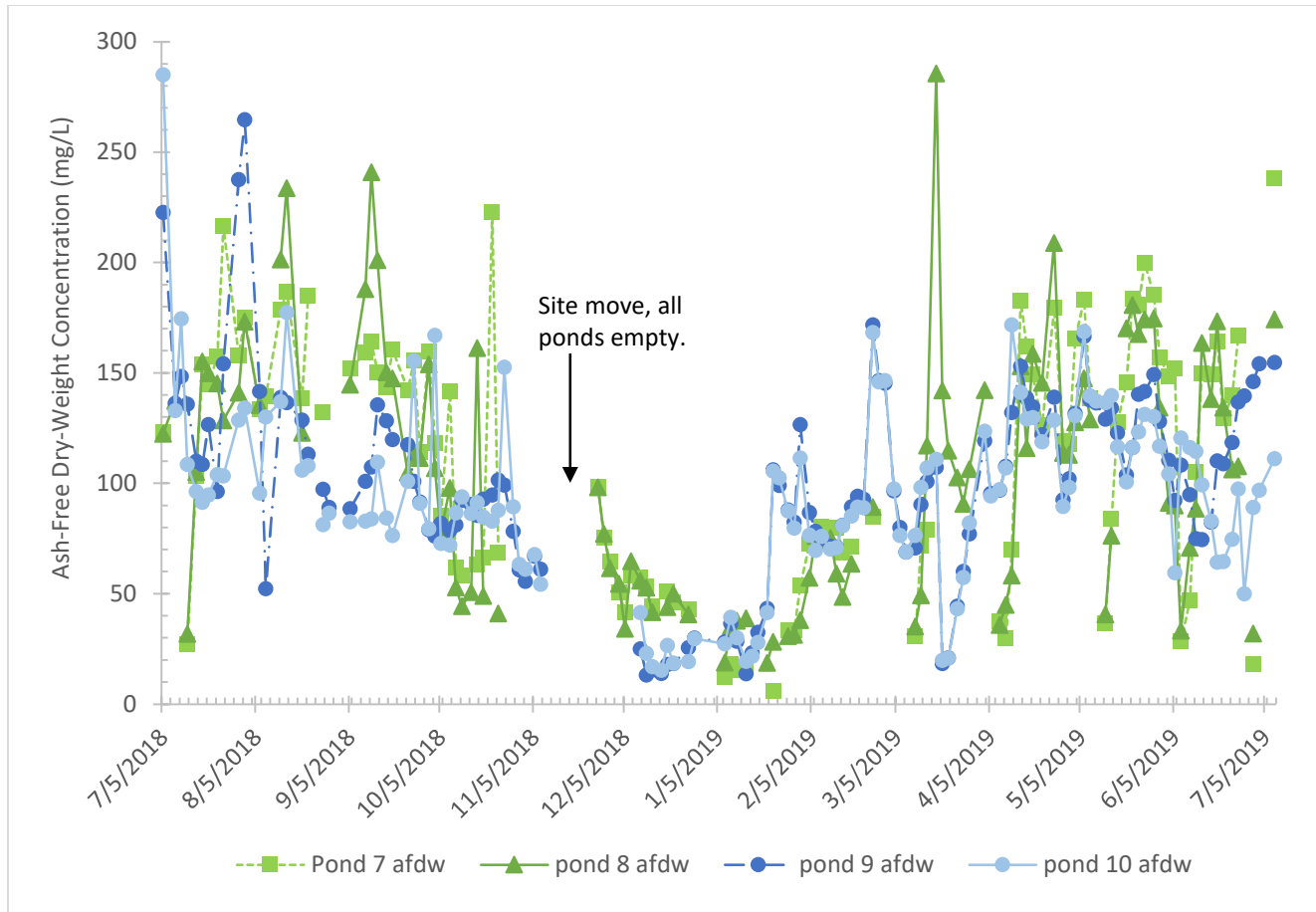
Figure 6. Ash-free dry-weight of outdoor T. minus and polyculture cultures over time. Ponds 7 and 8 are *T. minus* replicates. Ponds 9 and 10 are polyculture replicates. Each point is the average of triplicate measurements.

3.3 Productivity and Stability

Average productivity of *T. minus* cultures and the polyculture follow similar trends to those observed in AFDW over time (**Error! Reference source not found.**). This is due to the dependence of productivity on AFDW concentration (Equation *2*). *T. minus* appears to be more productive than the polyculture during most time periods. Low AFDW grow up periods of *T. minus* cultures excluded from productivity analysis are reported in the AFDW timeseries (**Error! Reference source not found.**) and these points are not reported on the productivity time series (**Error! Reference source not found.**).

In the outdoor raceways operated for a year, the annual average productivity of *T. minus* monocultures (17.6 ± 0.5 g/m²-d) was higher than that of the volunteer polyculture (13.3 ± 0.4 g/m²-d), (). Error bars are equal to ± values, and represent range of the mean of the duplication ponds.

Both *T. minus* and the native polyculture exhibit seasonal productivity differences, with greatest productivity in spring and summer. *T. minus* cultures appeared to be more productive than the polyculture for 9 months out of the year (). *T. minus* culture residence times should have been increased in January because ponds were not able to reach AFDW concentration goal, resulting in all data points being from dilute cultures (**Error! Reference source not found.**). Cultures that are too dilute will be less productive than cultures that have a high enough biomass concentration to capture as many photons as possible (Slocombe et al, 2016). Large error bars in some months shown in are due variability in AFDW between replicate ponds, not due to analytical error. Phenotypic changes in filament length in *T. minus* cultures can affect how much biomass flows over the standpipe and into the sample collection cup, which affects AFDW measurement. *T. minus* ponds were found to have an average of 10% biomass retention in the ponds (R. Anderson, data unpublished).

T. minus cultures stability was reasonably good in outdoor ponds. The interval between restarts due to contamination with other genera averaged 38 days, with a total of 10 approximately week-long restart periods. Low AFDW grow up values were excluded from productivity analysis but were included in terms of days *T. minus* cultures grew without contamination. Polyculture ponds were restarted only once, due to the need to move the raceway ponds.
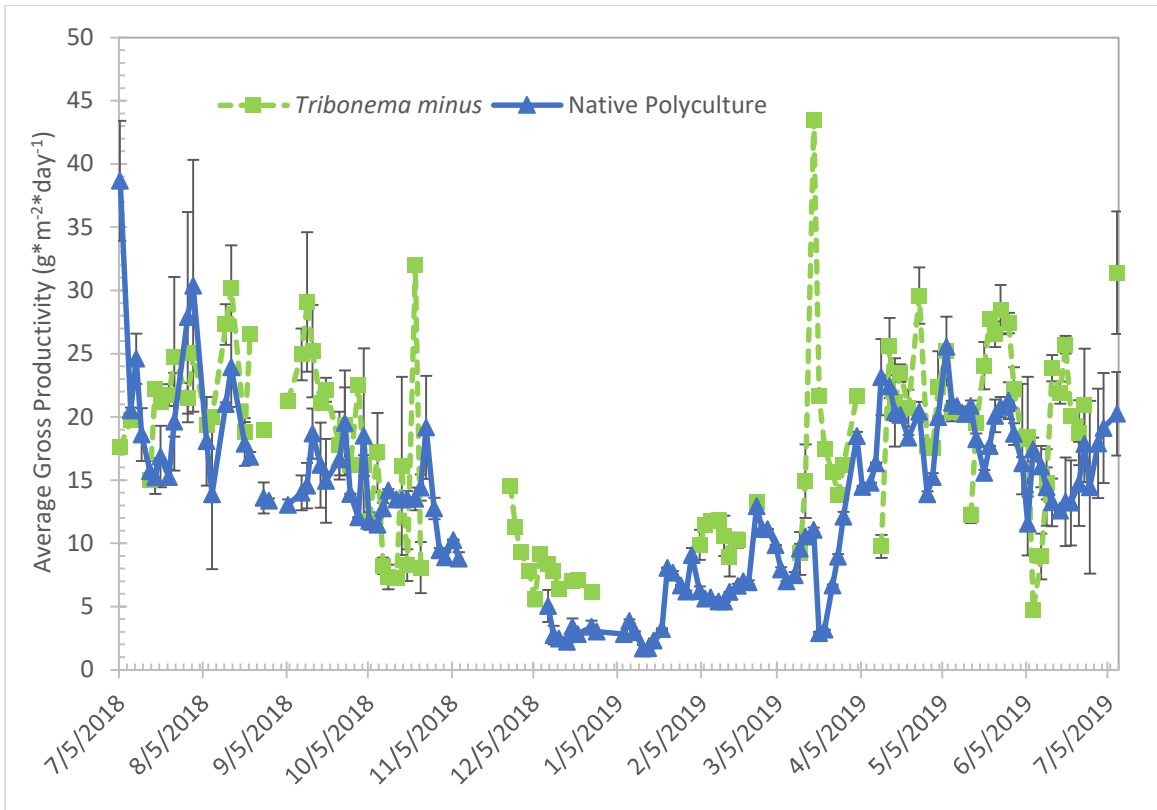
Figure 7. Average gross productivity of *Tribonema minus* and polycultures over time. Error bars represent the range of the mean of the duplication ponds. The gap in data collection in November is due to a site move.
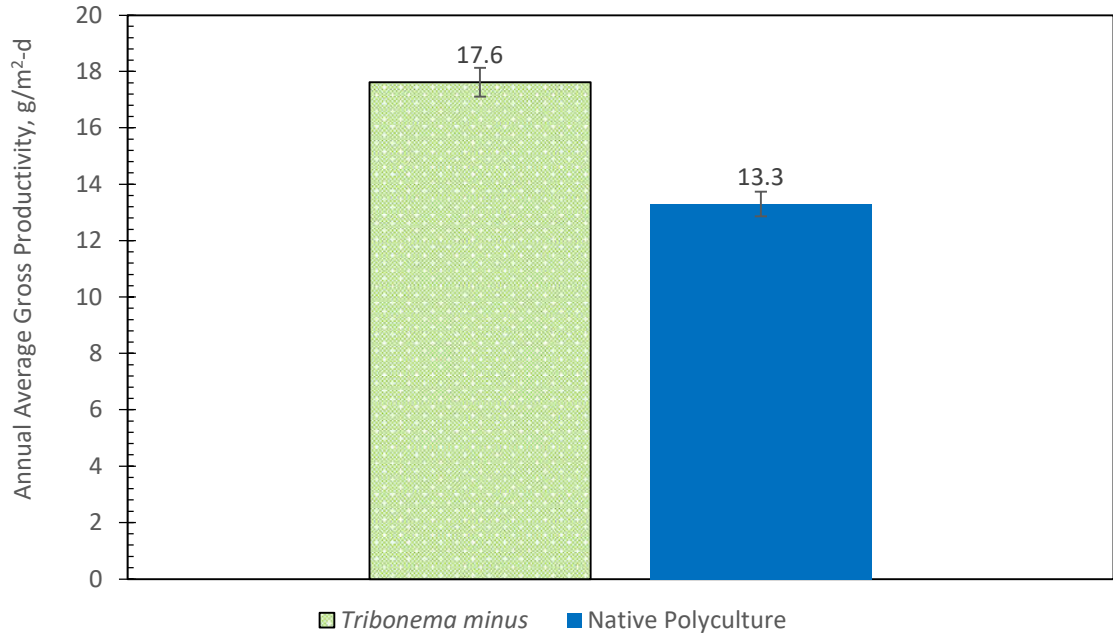
Figure 8. Average gross productivity of *Tribonema minus* and native *volunteer* polyculture outdoor pond cultures from July 5, 2018 to July 8, 2019. Error bars represent the range of the mean of the duplication ponds.

* March 18 - April 7th, 2019 values from only one *T. minus* pond
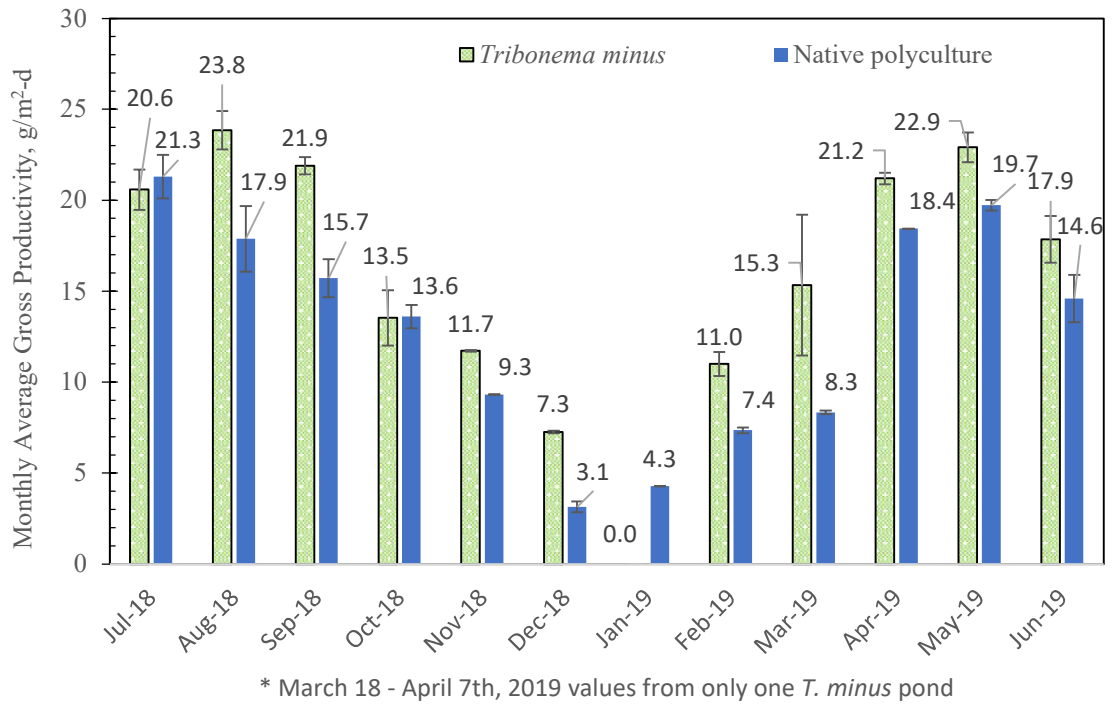
Figure 9. Monthly average gross productivities of *Tribonema minus* monocultures and volunteer polycultures. Error bars represent the range. Annual average productivities of T. minus and the polycultures were 17.6 ± 0.5 and 13.3 ± 0.4 g/m^2-d, respectively.

While *T. minus* cultures were more productive on average than the volunteer polyculture, an annual biomass production of each culture is important. Monocultures had cleaning and restart downtime due to contamination by competing strains, whereas the volunteer polyculture did not. *T. minus* monocultures produced more biomass per year than the polyculture despite *T. minus* having zero productivity days during cleaning and restart (*Table 3*). *T. minus* cultivation practices were not optimized in terms of minimizing the downtime of cultures, e.g., ponds were not restarted the same day they were cleaned, which is possible provided that dense inoculum is available. In practice, the amount of biomass produced per year could have been larger if more emphasis was placed on minimizing culture down time.

Table 3. Average ash-free dry-weight produced per area per year.

Culture	kg/(m^2·year)	Mg/(ha·year)	US Tons/(ac· year)
Tribonema	5.51 ± 0.11	55.1 ± 1.1	24.6 ± 0.5
Native Polyculture	4.66 ± 0.21	46.6 ± 2.1	20.8 ± 0.9

The most common contaminant observed in *T. minus* cultures was *Nitzschia*, a genus of pennate diatoms. The composition of the polyculture was dynamic and exhibited seasonal variation. The five most common genera observed were, in no particular order: *Chlorella*, *Scenedesmus*, *Chlorococcum*, *Actinastrum*, and *Nitzchia*. Predator populations were not observed in *T. minus* cultures during this study. Wang, et al, (2014) found that *Tribonema* cultures were resistant to predation by grazers, which could explain the lack of grazers observed during this study.

3.4 Biochemical composition of *Tribonema minus* raceway cultures.

Stable biochemical composition without much seasonal variability was observed for *T. minus* cultures (Figure *10*). This stability would like be an advantage in processing the biomass into products. Table 4 displays annual average biochemical macromolecule composition of *T. minus* biomass. Annual average *T. minus* ash content, of oven dry-weight, was 8.21 ± 2.38%. *T. minus* cultures had a high carbohydrate content averaging 28.16% of AFDW (Table 4). This indicates that *T. minus* biomass is an excellent feedstock for bioethanol production, agreeing with findings from Wang et al., 2014b. Average protein content for the year was 39.05% of AFDW, and stable lipid content was observed throughout the year averaging of 6.16% AFDW (Table 4). A lipids as FAMEs profile was generated for all sampling dates

(Figure 11). Lipids were measured as FAMEs but were not present in the biomass as such. Each FAME corresponds to a fatty acid present in the biomass. The predominant lipids in *T. minus* biomass throughout the year were eicosapentaenoic acid and palmitoleic acid, measured as methyl eicosapentaenoate and methyl palmitoleate, respectively.

Table 4. Annual average biochemical composition of reclaimed wastewater fed T. minus raceway pond cultures, in percent of ash-free dry-weight

Biochemical Macromolecule	Annual Average, % AFDW	Max. and Min., % AFDW; respectively
Carbohydrates	28.16	20.86 – 36.63
Proteins	39.05	24.09 – 45.10
Lipids as FAMEs	6.16	3.90 – 8.20

Methyl palmitoleate (C16:1-n7, palmitoleic acid), was an average of $22.13 \pm 4.31\%$ (average ± std. dev of pond replicates) of FAMEs and reached a maximum profile of 43.05% of FAMEs. As percent of AFDW, methyl palmitoleate was an average of $1.35 \pm 0.30\%$ of AFDW, with a maximum and minimum of 2.42 % and 0.87%, respectively. Methyl eicosapentaenoate (C20:5, eicosapentaenoic acid, (EPA)) was an average of $35.22 \pm 7.69\%$ of FAMEs and was the predominant FAME species observed during the study. As a percent of AFDW, methyl eicosapentaenoate was an average of $2.20 \pm 0.66\%$ of AFDW, with a maximum and minimum of 3.34% and 0.36%, respectively. No obvious correlations existed to describe the changes in biochemical composition and lipid profile. EPA is an omega-3 unsaturated fatty acid. Palmitoleic acid has application as a nutraceutical (Morse, 2015). Methyl palmitate (C16:0, palmitic acid) was also present in the FAMEs profile, and provides a potential substitute for palm oil, whose modern-day production is unsustainable (Gatti et al, 2019). Methyl palmitate was an average of $16.85 \pm 2.82\%$ of FAMEs.
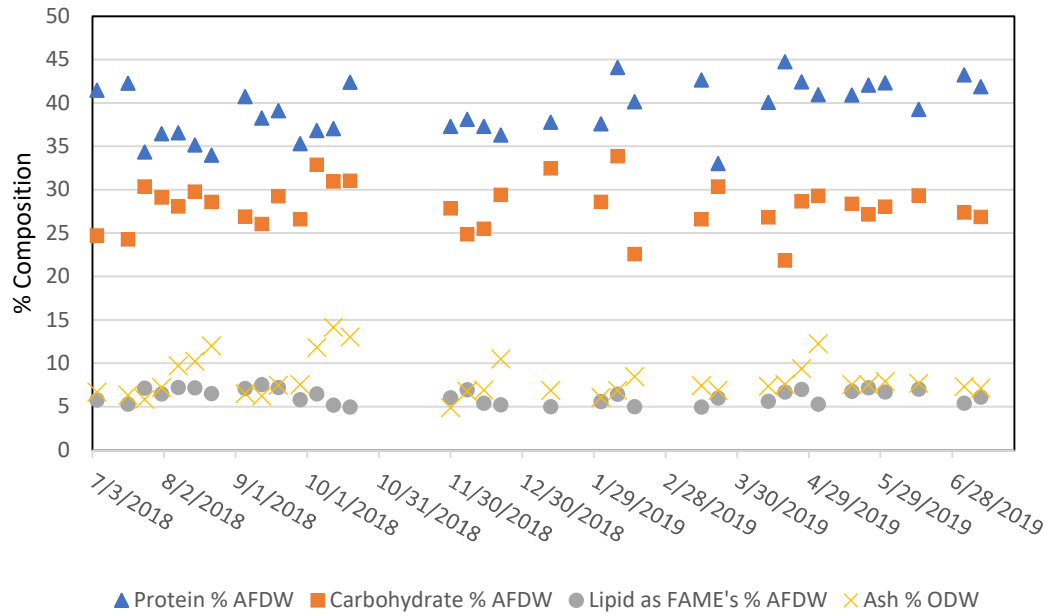
Figure 10. Annual average biochemical composition of outdoor *Tribonema minus* pond cultures in percent of ash-free dry-weight (AFDW) and ash in percent of oven dry-weight (ODW). Each data point is the average of data from duplicate ponds. No seasonal trends were observed. Annual average percent composition of ash-free dry-weight was 28.16 ± 3.23% carbohydrates, 6.16± 0.93% lipids as FAMEs, and 38.19± 3.64% protein. Annual average ash composition was 8.21 ± 2.38% of oven dry weight. n = 34 data points. Figure courtesy of Sara Leader.
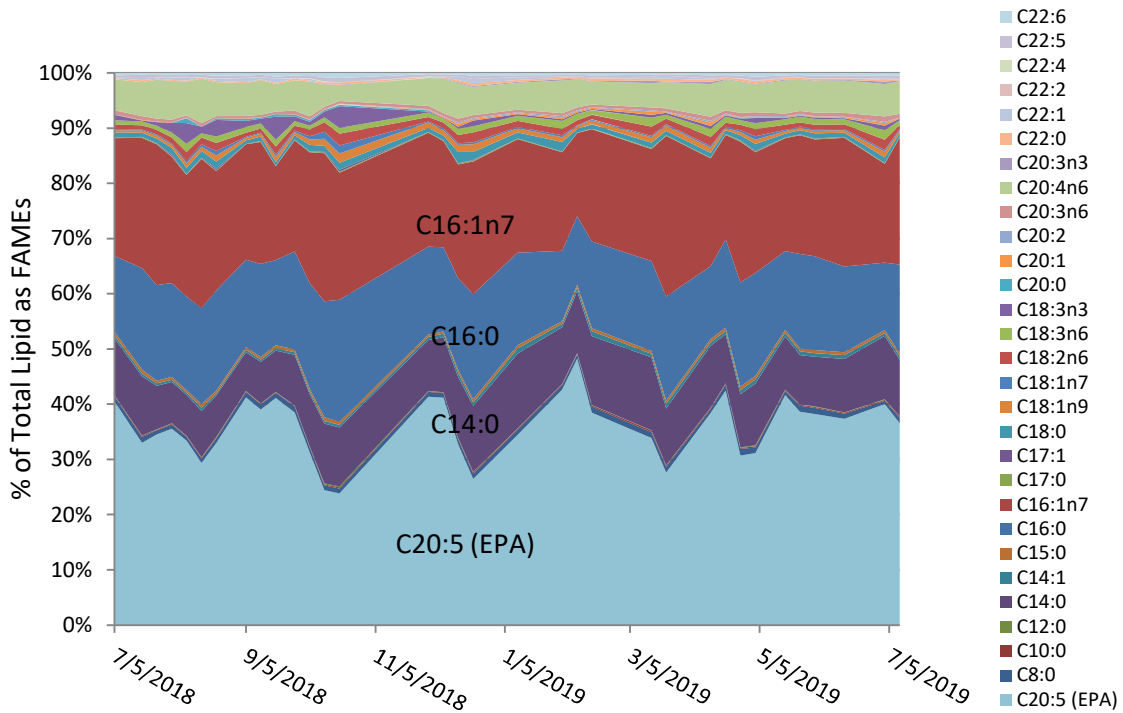
Figure 11. Profile of lipids as FAMEs in Tribonema minus outdoor pond cultures over one year. Each data point is the average of data from duplicate ponds. n = 34 data points. Figure courtesy of Sara Leader.

3.5 Future Research Involving *Tribonema minus*

While *T. minus* was found to have a consistent biomass composition and lipid profile when grown in outdoor ponds, the biomass value would be improved by increasing the content of carbohydrates and/or lipids and decreasing the content of ash and proteins Wang et al. (2013, 2014a) demonstrated that high culture concentration induced lipid accumulation, and nitrogen starvation induced carbohydrate accumulation in *Tribonema* sp. biomass grown in indoor reactors. Previous research has also indicated that nitrogen depravation does not change the fatty acid profile of *T. minus* or result in lipid accumulation (Guo et al., 2014). *T. minus* has been previously found to accumulate lipids when grown heterotrophically (Zhou et al., 2017). Zhou's group found that *T. minus* was able to grow heterotrophically and make use of ammonium, indicating that *T. minus* may be able to grow on wastewaters. By growing *T. minus* on wastewater, treatment credits might be obtained supporting algae biofuel and biorefinery concepts (Oswald et al. 1960).

4. Conclusions

Tribonema minus isolated from a volunteer polyculture was productive when grown in outdoor monocultures on reclaimed wastewater in raceway ponds. The cultures were N and P replete and sparged with CO_2 to maintain pH 7.5-8.0. An annual averaged productivity for *Tribonema minus* (17.6 ± 0.5 g/m^2-d, average $\pm$ range) appeared to be higher than that of volunteer polycultures (13.3 ± 0.4 g/m^2-d) grown on the same water. The *T. minus* monocultures grew for an average of 38 days before contamination with invasive algae genera. *T. minus* cultures produced more biomass per annum (5.51 ± 0.11 kg/m^2-yr) than the local polyculture (4.66 ± 0.21 kg/m^2-yr). Downtime during restart of *T. minus* cultures was not minimized in this study, but could be in future studies, meaning that even higher annual biomass production can be achieved.

The biochemical composition of *T. minus* cultures was stable year-round, and cultures had an average composition of $28.3 \pm 0.4\%$ carbohydrates, $37.6 \pm 0.7\%$ proteins, and $6.1 \pm 0.3\%$ lipids as FAMEs. *T. minus* cultures had an average eicosapentaenoic acid content of 2.20% of the ash-free dry-weight, and an average palmitoleic acid content of 1.35% of ash-free dry-weight. *T. minus* isolated from a volunteer polyculture grew as a fairly stable monoculture while producing easily-harvested biomass with stable desirable biochemical composition over the course of a full year.

Chapter 2. Adaptive Laboratory Evolution to Improve the Productivity of *Scenedesmus obliquus* in Raceway Ponds

Introduction

The economics of algae biofuels and bioproducts production can be improved by increasing the productivity of algal cultures (Davis et al., 2011). A way to possibly improve productivity of monocultures is adaptive laboratory evolution (ALE). In one form, ALE places cultures of microorganisms under deliberate selection pressure to make genetic changes in the population and select for a desired phenotype.. ALE has been used previously to enhance carotenoid production in microalgae (Sun et al., 2018). In the present study, ALE was used to create cultigens that had greater biomass productivity than the original wild type organism.. The goal of the study was to determine if high productivity cultigens generated in laboratory reactors would also be more productivity than the wild type when grown in outdoor raceway ponds with reclaimed wastewater as the medium.

The microalga *Scenedesmus obliquus* was chosen for ALE because full genetic sequence is known, it grows well in wastewater and it has been studied previously as a biofuel feedstock due to ability to accumulate lipids or carbohydrates. *S. obliquus* is a Chlorophyceae which grow in multi-cell coenobia (Figure 12). Although polycultures have had higher productivity than simultaneous monocultures (A. Davis, unpublished data), monocultures were used in the present study so that genetic changes might be tracked and that a cultivar with stable characteristics might be developed.

To improve the economics and environmental sustainability of algae production, use of wastewater or treated wastewater as the growth medium has been considered (Oswald and Golueke, 1960; Lundquist et al. 2010; Woertz et al. 2014). Wastewater treatment could be a revenue source in addition to that from the biomass.(Ansari et al., 2019). The present study focused on quantifying changes in areal productivity between indoor selected cultigens and wild-type *S. obliquus* monocultures cultivated in 1,350 L outdoor raceway ponds on reclaimed wastewater in coastal central California.
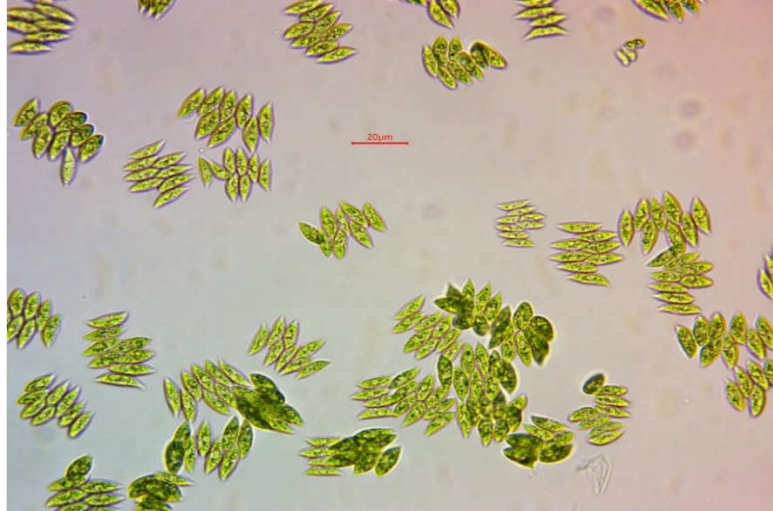
Figure 12. **Scenedesmus obliquus** *bubble column culture micrograph. 400x total magnification, scale bar is 20 microns in length.*

2. Materials and Methods

Methods used in this study for outdoor raceway sample collection, ash-free dry-weight, culture composition, nutrient measurement and productivity calculation were described in Chapter 1 sections 2.5, 2.6, 2.7, 2.8 and 2.10, respectively. This section will cover *S. obliquus* cultigen development and outline differences in inoculum production and outdoor pond operations from the previous *T. minus* chapter. Biochemical analysis of *S. obliquus* pond cultures is not presented in this study.

2.1 Cultigen Development

S. obliquus was grown with high dilution rates in laboratory bubble columns to select for high growth rate phenotypes. To increase genetic heterogeneity, ultraviolet (UV) light random mutagenesis was used. *S. obliquus* was grown in indoor 800-mL glass bubble column reactors using BG-11 growth media and 1:400 twice per week dilutions for the first two months of selection, followed by 1:200 dilutions for the last five months of selection. Wild-type *S. obliquus* cultures did not undergo intentional selection pressures, and were cultivated indoors alongside. Establishment of a cultigen occurred when that culture's productivity was measured to be improved over that of wild-type *S. obliquus* in indoor bubble column reactors. Once a cultigen was established, the cultigen was considered for outdoor cultivation. Cultigens were then

compared to each other in terms of indoor bubble column productivity, and the cultigens that were the most productive indoors were cultivated outdoors in raceway ponds.

Genomic analysis to identify differences between the wild-type and the cultigens and propose potential mechanisms behind the improvements is currently ongoing at Sandia National Laboratories in Livermore, California.

2.2 Inoculum production

Cultigens with the highest indoor bubble column productivity improvements over the wild type were scaled-up from five 800-mL bubble columns to four 18-L flat panel photobioreactors, for later inoculation into outdoor ponds. Cultigens and wild-type *S. obliquus* cultures were handled carefully using sterile method to avoid contamination. Cultigens and the wild-type were restarted from slants ever other month to avoid cultures adapting to lab conditions and undoing potential improvements selected for with outdoor cultivation in mind. The growth media used in the panel photobioreactors was chlorinated and dechlorinated reclaimed wastewater from the San Luis Obispo Water Resources Recovery Facility. RWW was treated by nitrifying activated sludge followed by granular media filtration and chlorination. (Images of photobioreactors used in this study may be found in the supplementary material.) Once in the scale-up phase, bubble column reactors were diluted weekly with 600 mL autoclaved BG-11, and the harvested biomass was used as inoculum for the larger panel photobioreactors. Deliberate selection pressures were not applied to cultigens during the scale-up phase. Panel photobioreactors were harvested completely each week and used to restart ponds if necessary. Panel photobioreactors were restarted using the volume of bubble column culture harvested from dilutions and fresh chlorinated and dechlorinated RWW. Two cultigens and the wild-type *S. obliquus* were made continuously available to restart the ponds as needed.

2.3 Outdoor Cultivation

S. obliquus cultigens and wild-type were cultivated outdoors at the SLO WRRF from May 26, 2019 to December 30, 2019 in duplicate 1,350 L, 30 cm deep raceway ponds fed with reclaimed wastewater.

Duplicate native polycultures were cultivated during the same time in 1,000 L, 30 cm deep raceway ponds. Materials used in outdoor raceway pond cultivation were described in Chapter 1, section 2.3, with the only difference being the volume of *S. obliquus* ponds.

Pond infrastructure allowed for two *S. obliquus* cultigens to be tested alongside the wild-type at one time. Wild-type *S. obliquus* and a volunteer polyculture were grown for the duration of the study to serve as productivity baselines. The remaining two pond sets were used to cultivate ALE generated *S. obliquus* cultigens that had showed promise indoors. Raceway pond cultures were started and operated as perveiously described in Chapter 1, section 2.4. All cultures had an HRT of 2-4 days. Methods used in the operation of outdoor raceway ponds were described in Chapter 1, section 2.4.

2.4 Sampling, Ash-Free Dry-Weight, Culture Composition, Nutrient Measurements and Productivity Calculation

Methods used in this study for outdoor raceway sample collection, ash-free dry-weight, culture composition, nutrient measurement and productivity calculation were described in Chapter 1 sections 2.5, 2.6, 2.7, 2.8 and 2.10, respectively.

3. Results and Discussion

Three *S. obliquus* cultigens were established indoors and tested in outdoor raceway ponds during this study. Cultigen MBE 501 was developed after 289 total lab generations and was the result of 100 remixed colonial isolates. MBE 503 was developed after 280 total lab generations, and MBE 504 was developed from MBE 503 after an additional 143 generations, for a total of 423. *S. obliquus* cultigens MBE 501 and MBE 504 were cultivated outdoors alongside the wild-type and volunteer polyculture outdoors from May 26 to September 4, 2019. The cultigen MBE 503 replaced MBE 504 on September 6, 2019, and simultaneous cultivation of MBE 501, MBE 503, wild-type and volunteer polyculture continued from September 6 to December 30, 2019. All three cultigens were observed to be stable outdoors, with MBE 501 appearing to be most productive (). The startup periods in which ponds were growing up to desired density were excluded from productivity analysis. Productivity varied over time due to weather and restarts of

each culture due to contamination. The HRT of all *S. obliquus* cultures was the same at any given time, ranging from 2 to 4 days. The volunteer polyculture HRT was not always set equal to that of the *S. obliquus* ponds. HRT needed to be increased during winter months to maintain goal AFDW concentrations in the ponds (Table 5). Growth periods of *S. obliquus* cultures were not always synchronized due to time constraints of the study. Too many resources would have been expended if all six *S. obliquus* test ponds were restarted due to one pond culture crashing or becoming contaminated.
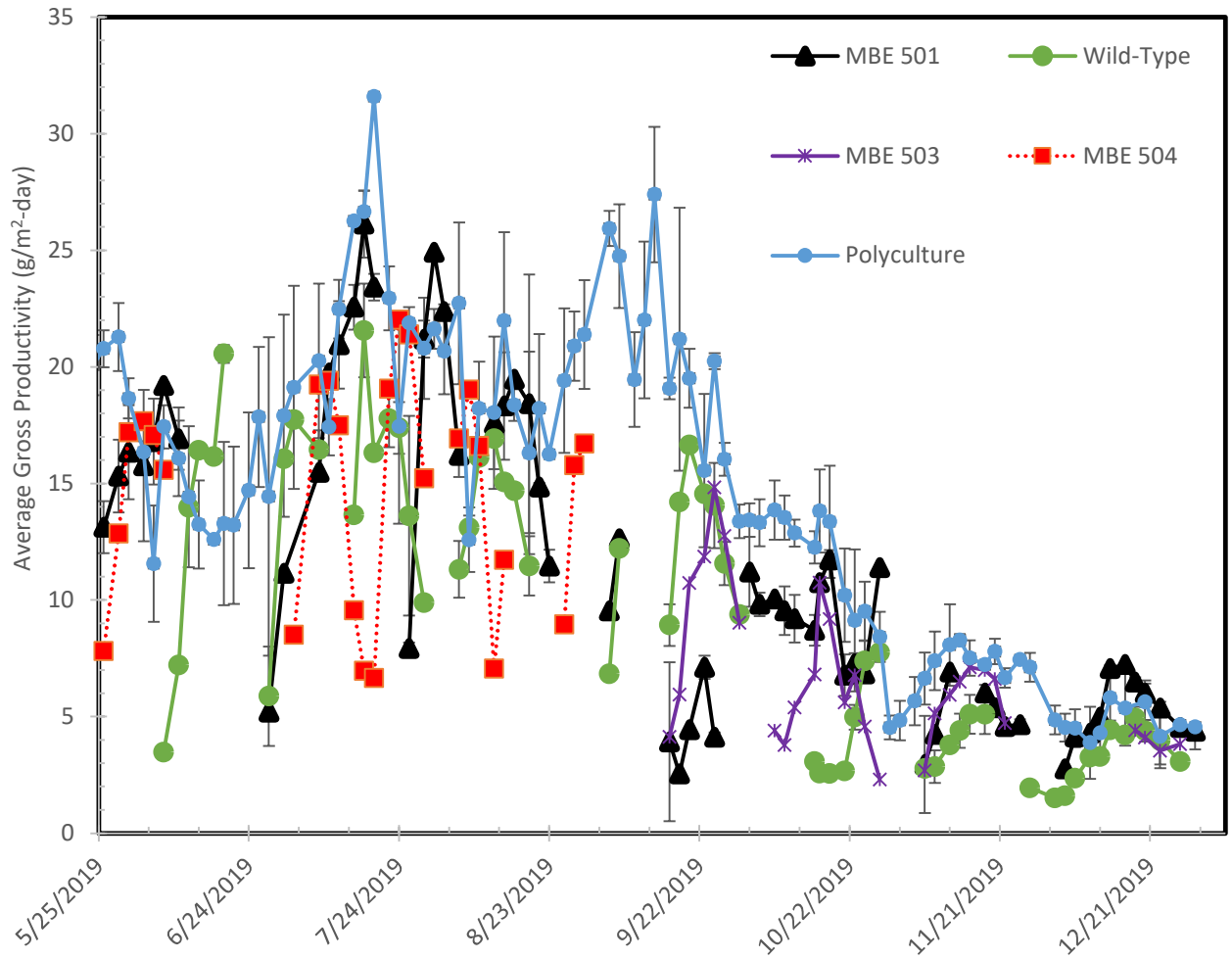

Figure 13. Time series of average gross productivity of *S. obliquus* cultigens alongside the wild-type. Repeated dips in productivity are due to restarts of the outdoor cultures. Error bars represent the range for each culture on each given date.

Table 5. Hydraulic residence time of outdoor pond cultures.

S. obliquus ponds		Polyculture ponds	
Date Range:	HRT (days)	Date Range:	HRT (days)
May 26- June 18, 2019	3	May 26 - Oct. 29, 2019	2
June 19 - Oct. 18, 2019	2	Oct. 30 - Nov. 26, 2019	3
Oct. 19 - Nov. 27, 2019	3	Nov. 17 - Dec 30, 2019	4
Nov. 28 - Dec. 30, 2019	4		

During the first outdoor testing time period from May 20 to September 4, 2019, MBE 501 had a higher average productivity (17.5 ± 0.4 g/m^2-d; mean $\pm$ range of duplication ponds) than MBE 504 (15.1 ± 0.1 g/m^2-d) and the wild-type (14.6 ± 0.8 g/m^2-d) (). The volunteer polyculture had an average productivity of 18.8 ± 1.3 g/m^2-d during this time period. The and minimum average pond productivity ranges (error bars in Figure 14) of MBE 501 and the volunteer polyculture overlap, indicated that MBE 501's most productive pond was as productive as the volunteer polyculture's least productive pond, on average. MBE 501 reached a higher maximum productivity than both MBE 504 and the wild-type, but not the volunteer polyculture **(Table 6)**. MBE 501 had higher monthly average productivities than wild-type and MBE 504 (Figure 15).. MBE 504 was not more productive than the wild-type and cultivation of MBE 504 was discontinued after September 4. A different cultigen, MBE 503, was cultivated for the remainder of the experiment alongside MBE 501 and the wild-type.

Table 6. Maximum average productivity values of outdoor cultures. Error bars illustrate the range.

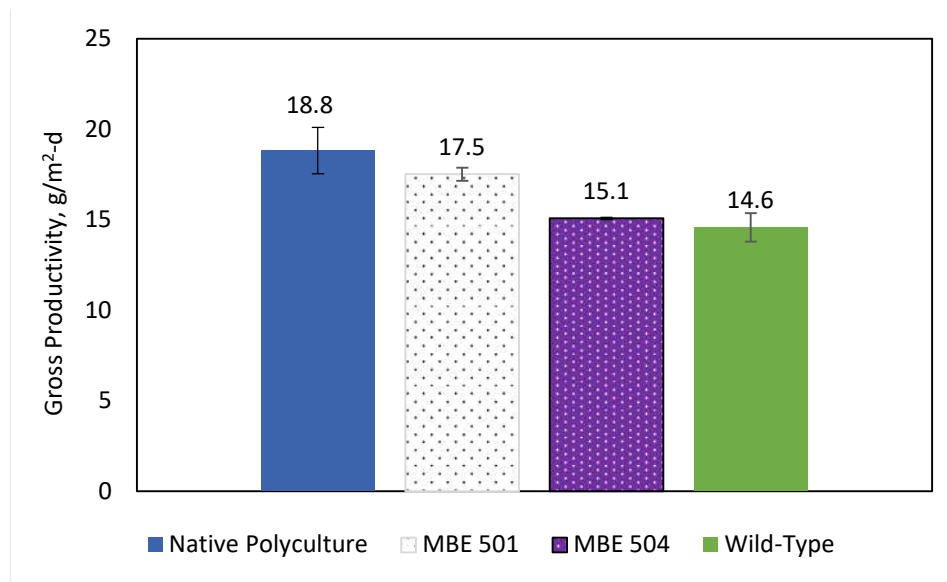| Culture	Maximum productivity (g/m^2-d)	Error	Date
MBE 501	26.13	0.26	7/17/2019
MBE 504	22.03	0.00	7/24/2019
Wild-type *S. obliquus*	21.57	1.11	7/17/2019
Polyculture	31.60	0.03	7/19/2019

Figure 14. Average outdoor pond productivities from May 26 to September 4, 2019. Error bars represent the range of the mean of the duplication ponds.

MBE 501 had higher monthly average productivities than the wild-type each month except September (Figure 15). The atypically low monthly average productivity in September for MBE 501 might be attributable to a poor growth period from mid- to late-September (Figure 17), which shows MBE 501 performing equal to or better than the wild-type at the vast majority of sampling points except during September. The start date of Figure 17, June 28th, is significant as it the first growth period in which MBE 501 and the wild-type were started at the same time outdoors. The low productivity of MBE 501 ponds during this September period can be attributed to poor start up conditions, which will be described in the following paragraphs.

MBE 501 ponds were inoculated at an average of 34 mg AFDW/L on September 11, 2019. This was a typical starting point after indoor inoculum was added to an outdoor pond at 10 cm depth. Batch operation for startup of the cultures continued until September 16, 2019, which included filling the pond to the full 30-cm depth, resulted in an AFDW concentration of 22.5 mg/L in one duplicate and 29.0 mg/L in the other. Dilution was started for the MBE 501 ponds at this time. Maximum AFDW for this growth period was achieved on September 23, 2019 at 36.9 and 56.5 mg/L for the duplicates and occurred due to contamination of the culture with *Nitzschia* sp., as the low culture concentration presumably allowed contaminants to move into the culture.

Concentrations of AFDW achieved during this time period were far below the goal concentration of 80-120 mg/L AFDW. The culture was too dilute after typical batch start-up operation and should have been given a longer growth time in batch mode to reach an adequate concentration prior to starting dilution. While typical times to reach the goal concentration are useful for planning purposes, the lesson of this time period of MBE 501 cultivation is that times required for proper culture start up can vary, and that diluting an MBE 501 culture too soon can result in washout of the culture. Due to the error made in the cultivation of MBE 501, this growth period was excluded from all averaging and analysis.

During the time period in which MBE 503 was cultivated, September 6 to December 30, MBE 501 had a higher productivity (7.1 ± 0.1 g/m^2-d) than the wild-type (6.0 ± 0.3 g/m^2-d), and MBE 503 (6.8 ± 0.6 g/m^2-d) was as productive as both MBE 501 and the wild-type (Figure 16). MBE 503 was improved on average during some months. MBE 503 was more productive than the wild-type during October, November and December and was more productive than MBE 501 in November (Figure 15). Further testing is needed to determine the if MBE 503 is improved over the wild-type and to determine whether MBE 501 or MBE 503 is the most productive.
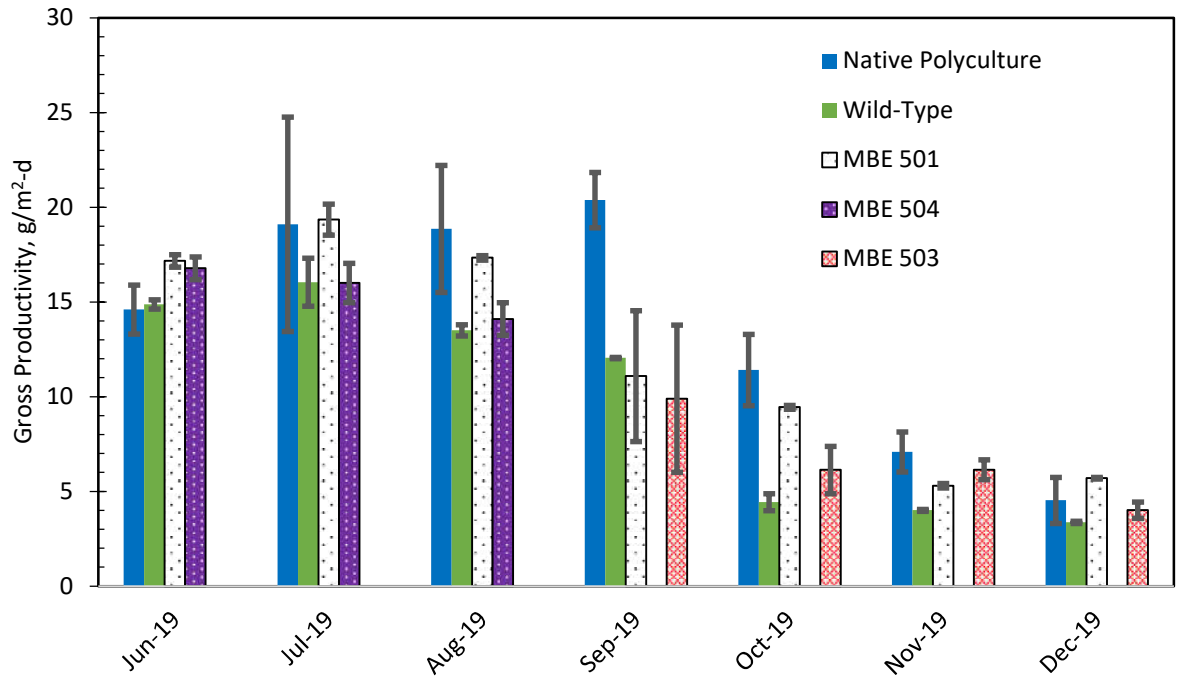
Figure 15. Monthly average productivities for S. obliquus cultigens, wild-type, and native polyculture. Error bars represent the range of the mean of the duplication ponds.
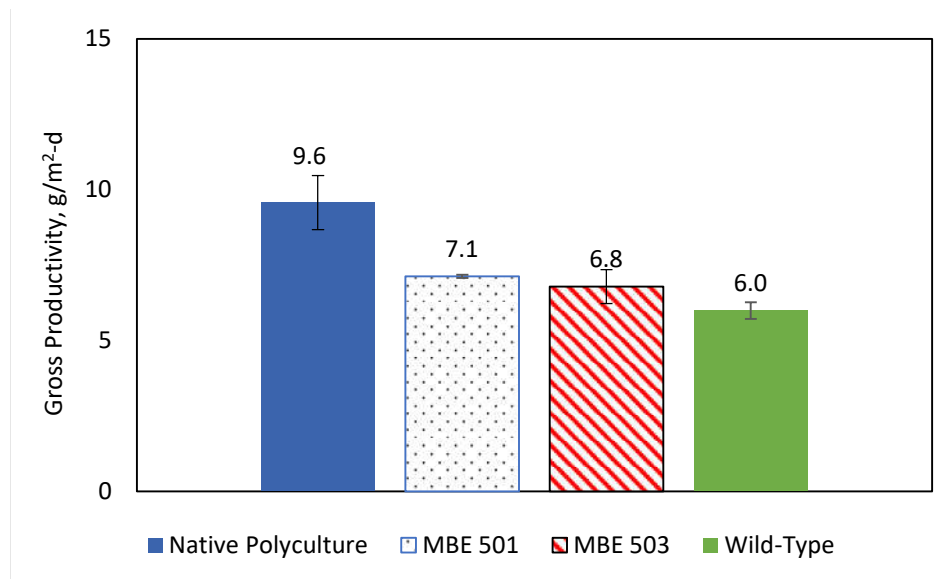

Figure 16. Average productivity of pond cultures from September 6 to December 20, 2019. Error bars represent the range of the mean of the duplication ponds.

Over the entire experiment, from June 28 to December 30, 2019, MBE 501 had a higher average productivity (11.5 ± 0.02 g/m^2-d) than the wild-type (10.4 ± 0.6 g/m^2-d) and had a lower average productivity than the volunteer polyculture (14.4 ± 1.3 g/m^2-d), (Figure 19). June 28 is significant is it is the first time in the study where MBE 501 and wild-type cultures were started outdoors simultaneously and allows for the best direct comparison. MBE 501 performed on par with the native polyculture in terms of monthly average productivity in the months of June, July, August and December of 2019, and MBE 501 had a higher monthly average productivity than the wild-type in all months except September (Figure 15). MBE 501's productivity improvement over the wild type are visible in a time series, with September's growth period as a notable exception (Figure 17). MBE 501 did briefly match the productivity of the polyculture on some dates in early June, mid-July, mid-August, and mid-December (Figure 18). While improving the productivity of an individual strain over that strain's wild-type was demonstrated, this improvement was not great enough to exceed the productivity of a volunteer polyculture.
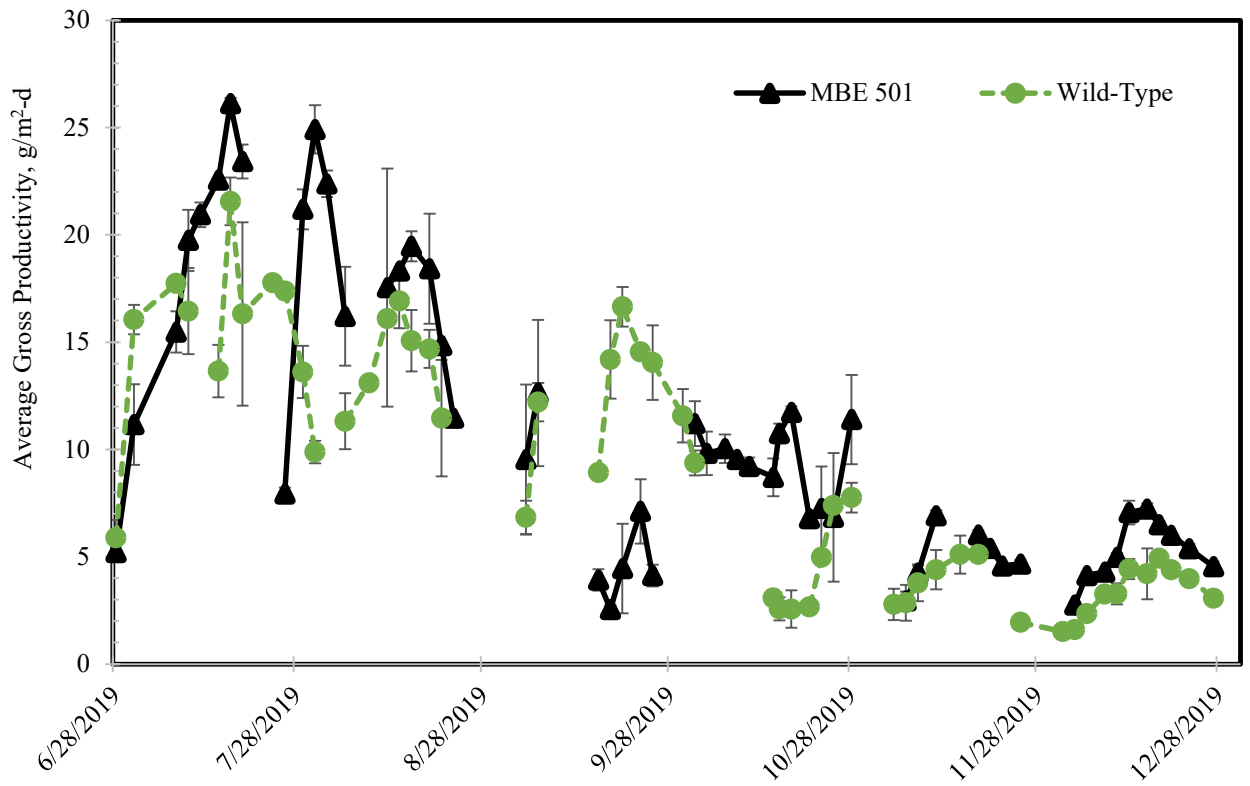
Figure 17. Average productivity of MBE 501 and wild-type S. obliquus. June 28, 2019 was the first good growth period when both strains were restarted simultaneously. Error bars represent the range of the duplication ponds.
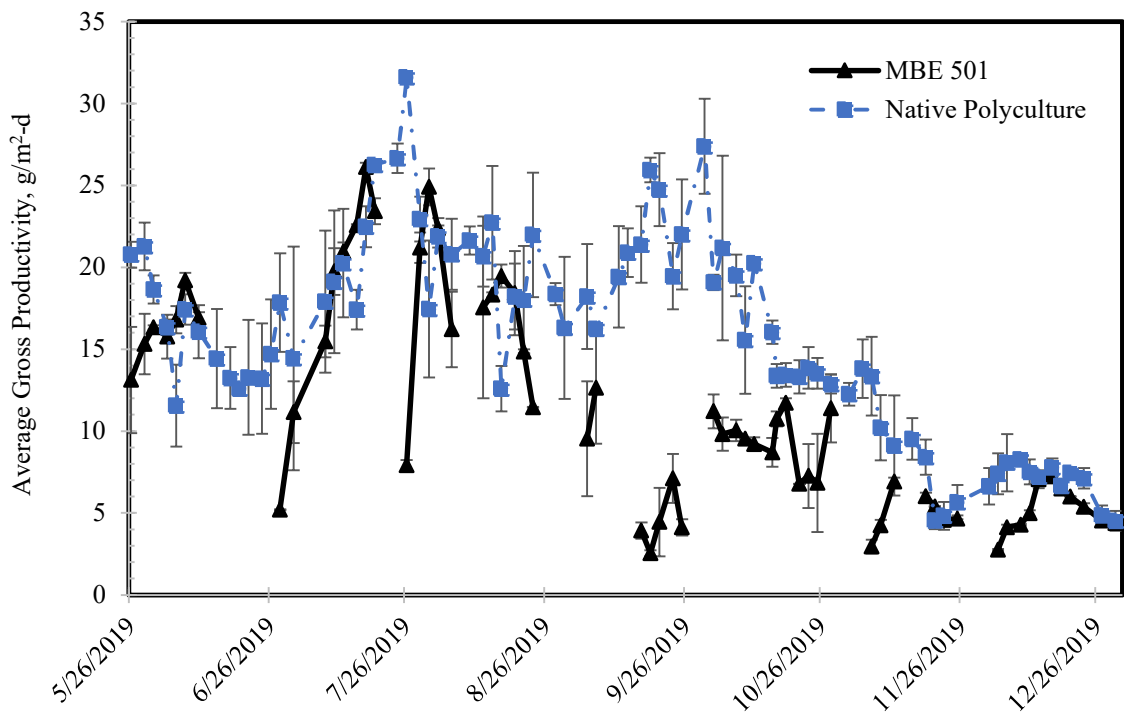
Figure 18. Productivity of MBE 501 and the native polyculture over time. Error bars represent the range of the duplication ponds.

The amount of biomass produced by each strain can give additional insight into which strain performed better outdoors. During the time period June 28 to December 30, 2019, productivity measurements for MBE 501 and the wild-type were interpolated for days in which no productivity measurement was taken because of Tuesdays, Thursdays, weekends and holidays. The sum of productivity measurements was used to calculate total biomass produced per area for each strain during the experiment. Days in which ponds were contaminated, empty due to cleaning, or growing up to density in batch mode were counted as a productivity of zero. MBE 501 was calculated to have produced 1,326.4 ± 108.0 g AFDW, and wild-type produced 1,056.3 ± 87.5 g AFDW (Table 7). The mid- to late-September period was included for MBE 501 for the biomass production comparison despite challenges in outdoor cultivation. MBE 501 was on average more productive during both outdoor cultivation periods and produced more biomass.

Figure 19. Average productivity of the polyculture, MBE 501 and S. obliquus wild-type between June 28 and December 30, 2019. Error bars represent the range of the mean of the duplication ponds.
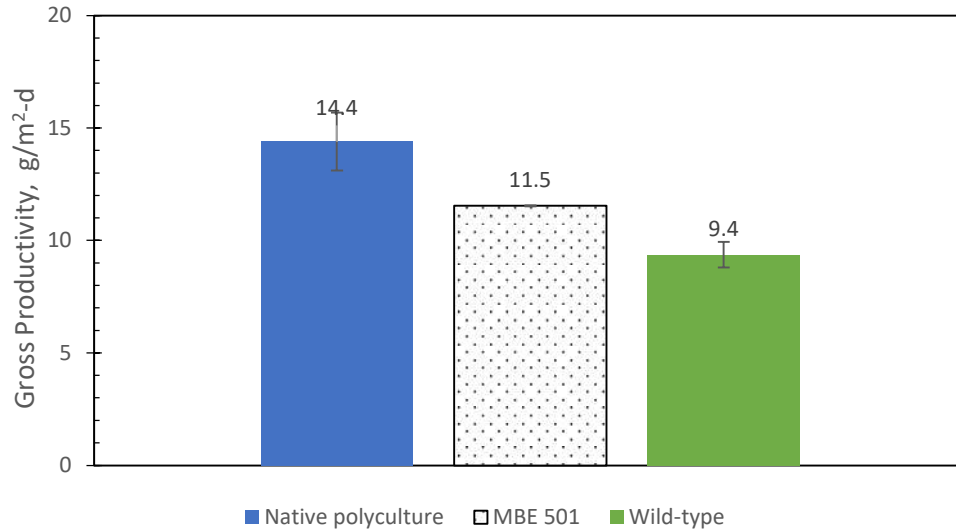
Biomass production was affected by both culture stability and productivity. MBE 501 cultures were grown outdoors for 126 days and wild-type for 117 days, excluding culture grow up time outdoors when ponds were not producing biomass. MBE 501 cultures were restarted nine times and wild-type cultures restarted eight times between June 28, 2019, and December 30, 2019 (Figure 17). Culture up-time at desired concentration outdoors was not optimized for this experiment, but certainly could be by more frequent production of high-density inoculum. If MBE 501 biomass production was normalized to the wild-type number of operational days, MBE 501 still would have produced 1,231.7 g AFDW (1,326.4 multiplied by the ratio of wild-type's 117 operational days to MBE 501's 126 days), which when coupled with MBE 501's higher average productivity (Figure 19) informs that MBE 501's larger amount of AFDW produced was not due simply to more operational days outdoors.

Table 7. Interpolated total biomass produced by MBE 501 and wild-type *S. obliquus* between June 28 and December 30, 2019.

	MBE 501		Wild-Type	
	Average	Range	Average	Range

Biomass produced (g/m²)	1,326.4	216	1056.3	175
Number of operational days	126	N/A	117	N/A

MBE 501 was more productive on average than the wild-type and did perform on par with the volunteer polyculture in terms of productivity during some growth periods (Figure 18). This result indicates that MBE 501, or other improved cultigens grown in monoculture, have the potential to match the productivity of polycultures in outdoor cultivation. One advantage that the native polyculture has over improved strains, or other pure cultures, is the lack of restarts required. This being said, consistent biomass or genera composition is not available when cultivating a volunteer polyculture. An area of future research to improve the productivity of outdoor algae cultures is the augmentation of native polycultures with improved cultigens such as MBE 501. Isolation and subsequent cultivation of local strains is also an avenue to improve productivity in addition to selective enrichment (Wilke, et al., 2011), and the two methods could be combined in the future to realize a strain that is highly productive for a given climate.

In summary, cultigen MBE 501 produced more biomass than the wild-type and was more productive. The mechanism behind the improvements were unconfirmed, but pathways for improvement of algal productivity in mass culture have been explored and identified in the past (Polle et al., 2002), and genomics work regarding this study is currently underway at Sandia National Labs. Future research includes testing of the improved *S. obliquus* cultigen on primary wastewater, which could improve biomass production as *S. obliquus* has been shown to grow heterotrophically (Abeliovich & Weissman, 1978) and could lead to economically viable wastewater treatment and biofuel feedstock production (Ansari et al., 2019).

4. Conclusions

Adaptive laboratory evolution techniques were used to create *S. obliquus* cultigens MBE 501, MBE 503 and MBE 504. These cultigens were then grown outdoors in raceway ponds on reclaimed wastewater with an HRT of 2 to 4 days. From June 28 to December 30, 2019 MBE 501 was more productive (11.5 ± 0.02 g/m²-d) than the wild type (10.4 ± 0.6 g/m²-d), but was not as productive as a volunteer polyculture (14.4 ± 1.3 g/m²-d). MBE 501 was calculated to have produced more biomass (1.326 kg/m²) during outdoor testing than the wild type (1.056 kg/m²). MBE 501 cultures were occasionally as productive as the native

polyculture but were not as productive on average. MBE 504 was found to not be improved over the wild-type, and further testing is needed to determine if MBE 503 is improved. This study demonstrated that high dilution rates in lab cultures can select for cells that are more productive in outdoor raceways.

References

Abeliovich, A., & Weisman, D. (1978). Role of heterotrophic nutrition in growth of the alga Scenedesmus obliquus in high-rate oxidation ponds. *Applied and Environmental Microbiology*, *35*(1), 32–37. doi: 10.1128/aem.35.1.32-37.1978

Ansari, F. A., Ravindran, B., Gupta, S. K., Nasr, M., Rawat, I., & Bux, F. (2019). Techno-economic estimation of wastewater phycoremediation and environmental benefits using Scenedesmus obliquus microalgae. *Journal of Environmental Management*, *240*, 293–302. doi: 10.1016/j.jenvman.2019.03.123

APHA. Standard methods for the examination of water and wastewater, 21sted. Washington, DC, New York: American Public Health Association; 2005.

Benemann, J., Woertz, I., & Lundquist, T. (2012). Life Cycle Assessment for Microalgae Oil Production. *Disruptive Science and Technology*, *1*(2), 68–78. doi: 10.1089/dst.2012.0013

Brennan, L., & Owende, P. (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*, *14*(2), 557–577. doi: 10.1016/j.rser.2009.10.009

Burlew, J.S. (1953). *Algal culture from laboratory to pilot plant*. Carnegie Institute of Washington, Washington, D.C., Publication 600, pp. 357.Chen W, Wang Y, Han D, et al. Two filamentous microalgae as feed ingredients improved flesh quality and enhanced antioxidant capacity and immunity of the gibel carp (Carassius auratus gibelio). Aquacult Nutr. 2019;00:1–11. https://doi.org/10.1111/anu.12930

Corpet, F., Multiple sequence alignment with hierarchical clustering. Nucleic acids research, 1988. **16**(22): p. 10881-10890.

Davis, R., Aden, A., & Pienkos, P. T. (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy*, *88*(10), 3524–3531. doi: 10.1016/j.apenergy.2011.04.018

Edgar, R.C., MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research, 2004. **32**(5): p. 1792-1797.

Gatti, R. C., Liang, J., Velichevskaya, A., & Zhou, M. (2019). Sustainable palm oil may not be so sustainable. *Science of The Total Environment*, *652*, 48–51. doi: 10.1016/j.scitotenv.2018.10.222

Guo, F., Wang, H., Wang, J., Zhou, W., Gao, L., Chen, L., … Liu, T. (2014). Special biochemical responses to nitrogen deprivation of filamentous oleaginous microalgae Tribonema sp. *Bioresource Technology*, *158*, 19–24. doi: 10.1016/j.biortech.2014.01.144

Huang, X. and A. Madan, CAP3: A DNA sequence assembly program. Genome research, 1999. **9**(9): p. 868-877.

Larkin, M.A., et al., Clustal W and Clustal X version 2.0. bioinformatics, 2007. **23**(21): p. 2947-2948.

Laurens, L.M.L. *Summative Mass Analysis of Algal Biomass-Integration of Analytical Procedures.* NREL/TP-5100-60943. Golden, CO: National Renewable Energy Laboratory, 2015. http://www.nrel.gov/docs/fy16osti/60943.pdf.

Maistro, Silvia, et al. "Phylogeny and taxonomy of xanthophyceae (stramenopiles, chromalveolata)." Protist 160.3 (2009): 412-426.

Morse, N. (2015). Are some health benefits of palmitoleic acid supplementation due to its effects on 5′ adenosine monophosphate-activated protein kinase (AMPK)? *Lipid Technology*, *27*(12), 278–281. doi: 10.1002/lite.201500061

Neofotis, P., et al., Characterization and classification of highly productive microalgae strains discovered for biofuel and bioproduct generation. Algal Research-Biomass Biofuels and Bioproducts, 2016. **15**: p. 164-178.

Oswald, W.J., and Gotaas, H.B. (1954). Photosynthesis in Sewage Treatment. *Paper presented before the Sanitary Engineering Division, American Society of Civil Engineers, New York, N.Y., (October 1954). Reprinted in Transactions of the American Society of Civil Engineers, Volume 122, (1957).*

Oswald, W.J., (1995). Ponds in the Twenty-first Century. *Water Science and Technology*, 38(12), 1-8.

Polle, J., Kanakagiri, S., Jin, E. S., Masuda, T., & Melis, A. (2002). Truncated chlorophyll antenna size of the photosystems a practical method to improve microalgal productivity and hydrogen production in mass culture. *International Journal of Hydrogen Energy*, *27*(11-12), 1257–1264. doi: 10.1016/s0360-3199(02)00116-7

Reyes, L. H., Gomez, J. M., & Kao, K. C. (2014). Improving carotenoids production in yeast via adaptive laboratory evolution. *Metabolic Engineering*, *21*, 26–33. doi: 10.1016/j.ymben.2013.11.002

Slocombe, S. P., Benemann, J. R., Weissman, J., & Nielsen, R. (2016). Chapter 2. Algae Growth Kinetics and Productivity. In *Microalgal production for biomass and high-value products* (pp. 21–58). Boca Raton, FL: CRC Press.

Sun, X.-M., Ren, L.-J., Bi, Z.-Q., Ji, X.-J., Zhao, Q.-Y., & Huang, H. (2018). Adaptive evolution of microalgae Schizochytrium sp. under high salinity stress to alleviate oxidative damage and improve lipid biosynthesis. *Bioresource Technology*, *267*, 438–444. doi: 10.1016/j.biortech.2018.07.079

Van der Auwera, Gert, and Rupert De Wachter. "Complete large subunit ribosomal RNA sequences from the heterokont algae Ochromonas danica, Nannochloropsis salina, and Tribonema aequale, and phylogenetic analysis." Journal of molecular evolution 45.1 (1997): 84-90.

Van Wychen, S.; Laurens, L.M.L. *Determination of Total Carbohydrates in Algal Biomass.* NREL/TP-5100-60957. Golden, CO: National Renewable Energy Laboratory, 2015. http://www.nrel.gov/docs/fy14osti/60957.pdf.

Van Wychen, S.; Laurens, L.M.L. Determination of Total Solids and Ash in Algal Biomass. NREL/TP-5100-60956. Golden, CO: National Renewable Energy Laboratory, 2015. http://www.nrel.gov/docs/fy14osti/60956.pdf.

Van Wychen, S.; Ramirez, K.; Laurens, L.M.L. *Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by* in situ *Transesterification.* NREL/TP-5100-60958. Golden, CO: National Renewable Energy Laboratory, 2015. http://www.nrel.gov/docs/fy14osti/60958.pdf.

Wang, H., Gao, L., Chen, L., Guo, F., & Liu, T. (2013). Integration process of biodiesel production from filamentous oleaginous microalgae Tribonema minus. *Bioresource Technology*, *142*, 39–44. doi: 10.1016/j.biortech.2013.05.058

Wang, H., Ji, B., Wang, J., Guo, F., Zhou, W., Gao, L., & Liu, T. Z. (2014a). Growth and biochemical composition of filamentous microalgae Tribonema sp. as potential biofuel feedstock. *Bioprocess and Biosystems Engineering*, 37(12), 2607–2613.

Wang, H., Ji, C., Bi, S., Zhou, P., Chen, L., & Liu, T. (2014b). Joint production of biodiesel and bioethanol from filamentous oleaginous microalgae Tribonema sp. *Bioresource Technology*, *172*, 169–173. doi: 10.1016/j.biortech.2014.09.032

Wehr JD (2010) Xanthophyta and phaeophyta. In: Algae: source to treatment manual of water supply practices M57. American Water Works Association, Denver, pp 271–287 [ISBN 978-1-58321-787-0].

White, T.J., et al., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 1990. **18**(1): p. 315-322.

Wilkie, A. C., Edmundson, S. J., & Duncan, J. G. (2011). Indigenous algae for local bioresource production: phycoprospecting. *Energy for sustainable development*, *15*(4), 365-371.

Woertz, I. C., Benemann, J. R., Du, N., Unnasch, S., Mendola, D., Mitchell, B. G., & Lundquist, T. J. (2014). Life cycle GHG emissions from microalgal biodiesel–a CA-GREET model. *Environmental science & technology*, *48*(11), 6060-6068.

Zhou, W., Wang, H., Chen, L., Cheng, W., & Liu, T. (2017). Heterotrophy of filamentous oleaginous microalgae Tribonema minus for potential production of lipid and palmitoleic acid. *Bioresource Technology*, *239*, 250–257. doi: 10.1016/j.biortech.2017.05.045