Evaluation of Algal Biomass Production on Vertical Aeroponic Substrates

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ABSTRACT

Large scale algal biomass production has focused primarily on the Open Pond (OP) and Photobioreactor (PBR) systems, but to date neither system has been able to produce algae biofuel in a financially viable manner. For this reason, a novel aeroponic substrate based cultivation system was evaluated to determine if it could produce significant quantities of biomass without a negative impact on the lipid productivity and fatty acid profile compared to the two traditional systems. This vertical substrate system produced significant areal yields (exceeding 15 g biomass m⁻² floor space d⁻¹) assuming a 1:1 lab scale to full scale conversion and significant harvest concentrations (exceeding 4.94 g biomass L⁻¹), resulting in reduced energy inputs and increased financial return. In addition to productivity increases, the aeroponic nature of this substrate system did not negatively affect the fatty acid composition of the cultivated biomass, thus demonstrating the promising potential for using substrate based systems to produce biofuel, nutraceuticals and feed for fisheries and various other applications.

INTRODUCTION

For the production of algal biomass, much focus has been placed on selecting algal strains, as well as downstream biomass processing. However, major innovations in algae cultivation techniques have remained elusive. To cultivate algae for large-scale biofuel production, the areal productivity (grams biomass m^{-2}) and harvest concentration (grams biomass L^{-1}) must be sufficient so that the chemical energy output exceeds the energy that is required for cultivation, harvesting, mass transfer and downstream chemical transformation.¹ Additionally, for a sustainable system to be able to produce biomass in an energetically positive manner, it is essential that the system does not require unsustainable quantities of raw materials (e.g., water, nutrients).¹

Open Pond (OP) and Photobioreactor Systems (PBR)

Current platforms for cultivating algae are quite diversified but can be predominantly categorized as OPs or PBR systems. An OP system is comprised of a race track shaped shallow canal that is typically circulated by a paddle wheel and thus OP systems are often referred to as *raceway ponds*.² In contrast, the PBR system is a closed translucent system comprised of flasks, bags, tubes or other translucent containment vessels.² These systems are similar in that they both are typically maintained at an alkaline pH to accommodate elevated CO_2 concentrations, but have various advantages and disadvantages in comparison to one another.

The OP system is very simple, requires minimal maintenance to operate, and has low investment costs, yet has many disadvantages in relation to performance.^{2,3} OP systems have lower productivities ($\text{g biomass m}^{-2} \text{d}^{-1}$) than PBR systems and have comparatively dilute harvest concentrations (g biomass L^{-1}) necessitating significant mass transfers to transport and

subsequently dewater algae.³ OPs are open to the environment, allowing for significant contamination and experience a typical evaporative loss of approximately 1 liter of water per gram of biomass produced.⁴

The PBR offers a closed system that minimizes loss from evaporation and contamination, but the closed system requires the use of temperature control and constant maintenance to remove algae agglomeration.³ Algae have a tendency to accumulate on surfaces and this accumulation eventually leads to the reduced productivity of PBRs without constant maintenance. While the PBR system has significantly greater areal productivity compared to OPs ($10 \text{ g m}^{-2} \text{ d}^{-1}$ for OPs, $20 \text{ g m}^{-2} \text{ d}^{-1}$ for PBRs) and harvest concentrations (0.5 g L^{-1} for OPs and 3.0 g L^{-1} for PBRs), the PBR system requires significant energy input to mix the algae suspension and to remove oxygen gradients.^{4,5,6} For these reasons, neither of these systems is ideal, and both have significant challenges to overcome before they can be developed into commercially viable systems for large scale biomass production.

Aeroponic Substrate Based Cultivation Strategy

Because of the inabilities of OPs and PBRs to produce financially viable biofuel at scale, AL-G Technologies Inc. designed a novel cultivation system which is able to increase algal productivity as well as harvest concentration in comparison to OP and PBR systems (US Patent Application number: 20140127776). The AL-G system is based on the principle of attached growth that utilizes specific species of algae that have a tendency to adhere to substrates and create dense biofilms.²³ This system is comprised of multiple vertical aeroponic substrates which are suspended from a scaffolding system such that at commercial scale 8-10 one-meter squared substrates would be placed within a meter squared of floor space. The substrates are suspended from a scaffolding system using simple loops or hooks so that the substrates can be easily

removed. At commercial scale, the substrates would be spaced 10 – 12.5 cm apart from one another and would be mounted within a greenhouse in long rows of hundreds of substrates. The substrates themselves are composed of a simple 50/50 (%) cotton and polyester weave, are approximately 5 mm thick and readily retain water. Using this approach, algae are grown up to a density of approximately 5g/L in a PBR system and then the substrates are either removed from the scaffolding system and soaked in the inoculum or remain on the scaffolding system and are sprayed with the inoculum. Following inoculation, the substrates on the scaffolding system are continuously irrigated with a nutrient solution (water and nutrients) and the algae begin to grow on the substrates.

The spent nutrient solution is collected from the bottom of the substrates and can be replenished with nutrients and reused or disposed of. Over a period of a few weeks the inoculum takes hold on the substrates and forms a dense biofilm that can subsequently be harvested. The algae-covered substrates are typically harvested every 3-4 days using a simple mechanical roller press. The substrates can be removed from the scaffolding system and pressed elsewhere or can remain attached to the scaffolding system and be pressed in-place. The moist substrates are simply passed through a mechanical roller press that removes approximately 50-70% of the algae from the substrates and leaves the remaining algae on the substrate for regrowth. Because the substrates contain minimal water, the effluent concentration from this process is high (as high as 4.94 g/L) which minimizes downstream dewatering. The substrates can maintain this harvesting and regrowth process for long periods of time and can produce significant areal biomass yields with a dense harvest concentration. This entire process can be described and illustrated in four major steps: 1) Stock Solution (inoculum production), 2) Inoculate Substrate, 3) Algae Growth on Substrate and 4) Harvest (**Figure 1**). Following steps 1 and 2, steps 3 and 4 are conducted as a

continuously repeated process until the substrates are damaged from mechanical harvesting or removed from the system for other reasons.

For the experiments described here, the commercial scale AL-G system was scaled down to allow for sufficient replicates within the budgetary and space constraints. This scaled down system is illustrated in **Figure 2** and shows 15 by 25 cm growth substrates housed within a controlled environment chamber and flanked by two dummy substrates to provide more equal lighting distribution. **Figure 2** also shows the LED lighting system as well as the individual irrigation outlets at the top of every substrate and the collection bin for the excess irrigation water dripping from the bottom of the substrates. In this paper, we report on the evaluation of this substrate system for the growth and development of the marine alga *Tetraselmis chuii* and the freshwater alga *Parachlorella kessleri*. *T. chuii* and *P. kessleri* both create resilient biofilms, allowing them to grow continuously on the substrates.

Tetraselmis chuii was chosen since it has significant economic benefit due to its ability to provide food cultures for use in shellfish hatcheries that produce shellfish for human consumption and for its nutritional profile, that is dense in essential fatty acids.⁷ In contrast, *P. kessleri* does not have a favorable composition for use in hatcheries or as biofuels since it contains minimal lipids by weight (0-29%).^{8,9} Rather, *P. kessleri* was selected because of its rapid growth rate and ability to grow under varying conditions, as well as its similarity to *Chlorella vulgaris*, a commercially relevant algal strain.⁹ While both of these algae grow successfully on the substrates, the objective of this research was to compare the impact of aeroponic culturing of these different algal species on biomass productivity and fatty-acid composition using the aeroponic substrate production system.

MATERIALS AND METHODS

Evaluating algae production for aquaculture applications

For our studies, *T. chuii* was obtained from the Institute of Marine and Coastal Sciences (Rutgers University, New Brunswick, NJ) and *P. kessleri* was obtained from AL-G Technologies Inc (Quebec, Canada). Both species were initially transferred to 2 L Erlenmeyer flasks containing their respective nutrient media and placed 20 cm below an LED lighting array with a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (16 hours light, 8 hours dark). The media used for *P. kessleri* was as follows: KNO_3 200 mg L^{-1} , NH_4Cl 106 mg L^{-1} , $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ 25 mg L^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 25 mg L^{-1} , K_2HPO_4 25 mg L^{-1} , $\text{Na}_2\text{EDTA} \cdot 2 \text{H}_2\text{O}$ 1.1 mg L^{-1} , $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ 1 mg L^{-1} and the media used for *T. chuii* was f/2 media.^{10,20} The media for *P. kessleri* had been optimized by AL-G Technologies Inc. for growth on the substrate system. The *P. kessleri* nutrient media was made using New Brunswick NJ tap water and reduced to a pH of 5.5 using 0.8 M HCl while the *T. chuii* media was made using 35 parts per thousand (ppt) seawater collected from Corson's Inlet (Cape May, NJ) and autoclaved prior to use. The inoculation flasks were aerated at a rate of 2 liters per minute (2:1 v/v) with ambient air and following 10 days of growth, the biomass concentration was sufficient to begin experimentation.

To model traditional non-aeroponic growth, the controls for both *T. chuii* and *P. kessleri* were placed into three 1000 ml Erlenmeyer flasks in which 50 ml of inoculum was added to 950 ml of their respective media. The flasks were capped with a one inch Styrofoam plug and covered with aluminum foil to minimize loss due to evaporation. Throughout the experiment, the appropriate media was added to the flasks to compensate for media lost to evaporation and the temperature was maintained between 24-27°C. These control flasks were aerated at a rate of 2 liters per minute (2:1 v/v) with ambient air and were placed 20 cm below an LED lighting array

with a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (16 hours light, 8 hours dark). The 7.5 mm diameter aeration tubing was placed at the bottom and center of the flasks to provide thorough mixing.

In parallel to the control flasks being inoculated, 1000 ml of the *T. chuii* inoculum and the *P. kessleri* inoculum was used to inoculate ten proprietary cotton-like aeroponic substrates (15 by 25 by 0.75 cm) that were made of a proprietary material (AL-G Technologies, Inc.). To inoculate the substrates with algae, they were soaked in 200 ml of the inoculum for 20 seconds and then rolled through a mechanical roller press (ATLAS, AT01, Winnipeg, Canada) to allow for infiltration of the inoculum throughout the substrate structure. The effluent from the mechanical press was captured in a bin and reused so that approximately 100 ml of inoculum was used for each substrate. These substrates were then hung from a stainless steel scaffolding system using plastic loops such that the substrates were placed 1 cm below a 1/16" irrigation dripper and spaced 5 cm apart from one another. In addition to the ten substrates that were inoculated, an additional substrate was placed at either side of the ten substrates so that the ten substrates experienced equal lighting conditions. The substrates were illuminated with an LED array consisting of seven 60 cm LED strips (Mouser Electronics, 901-SB-6500-TR) spread 3 cm apart and placed 8 cm above the substrates. The LED strips provided a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured at the top of the substrates using a 16 hours on 8 hours off light cycle. The set of 10 *T. chuii* substrates and 10 *P. kessleri* substrates are placed on separate shelves within a 75 by 75 by 40 cm growth chamber with an air exchange rate of 1 volume h^{-1} , while the temperature and relative humidity were maintained at 24-27°C and 96-99%, respectively. The two sets of ten substrates were irrigated with 5.76 liters of their respective media per day using a peristaltic pump (Masterflex, HV-07522-20). The delivered media volume to the sets of ten substrates was

4 ml min⁻¹, which was dispensed intermittently (12 seconds on, 168 seconds off) using a pump setting of 60 ml min⁻¹.

Following inoculation, the substrates were harvested using the mechanical roller press every 3-4 days and the effluent was collected for subsequent evaluation. For this experiment the harvest data used from each substrate system was from the 34th day since inoculation for the *T. chuii* and from the 50th day since inoculation for the *P. kessleri*. These days were selected because the systems reached a plateau in biomass productivity based on data collected during previous experiments conducted under the same experimental conditions (results not reported here). For the control treatments of *T. chuii* and *P. kessleri*, both samples were taken on day 10 when the systems both reached their growth plateau. All harvested algae was immediately centrifuged (4000 rpm, 5 min, 5°C) and transferred to a freezer (-80°C). These samples were then freeze dried, and stored at -20°C for subsequent analysis. In addition to the biomass being harvested every 3-4 days, 5 ml of effluent was collected from each set of substrates twice a week to determine the quantity of algae falling off of the substrates (non-adherent biomass) and the amount of exopolysaccharides being produced.

Carbon Sequestration

In order to determine the total productivity of an algae production system, the total mass of carbon sequestered needs to be determined. Carbon sequestration is the measure of how much carbon is fixed by algae from the air and describes overall productivity. In the case of our experiments, they were all conducted at an ambient CO₂ concentration of approximately 400 µmol mol⁻¹ (ppm) and the CO₂ that was sequestered came from the air within the growth chambers.

The productivity of the substrate system cannot be determined as is typically done for traditional algae systems using optical density (OD). Instead, the total productivity of the aeroponic system needs to be determined based on three separate components: 1) Biomass harvest, 2) Non-adherent biomass and 3) Polysaccharides. The biomass harvest and non-adherent biomass components can be determined using OD₆₈₀ in accordance with **Equation 1**:^{13,14}

$$\text{Yield}_B (\text{g biomass L}^{-1}) = 0.379 \times \text{OD}_{680} \quad (R^2 = 0.9652) \quad (1)$$

Using two volumes, the harvest volume and total volume of media used per day, the biomass per day from each one of these components can be determined. To determine the amount of polysaccharides produced, the effluent used to determine non-adherent biomass was filtered through a 0.20 µm filter and diluted 2:1 with deionized water. The samples were then analyzed using the phenol-sulfuric acid assay with a standard curve constructed using a serial dilution of glucose as depicted in **Equation 2**:¹¹

$$\text{Yield}_C (\text{mg of carbon L}^{-1}) = 29.468 \times \text{OD}_{490} + 1.827 \quad (R^2 = 0.9818) \quad (2)$$

To determine the total mass of carbon sequestered per day, the biomass harvest and non-adherent biomass need to be converted into mg carbon using the percentage of carbon in each type of biomass for each algae species. The percent carbon was determined from three replicate samples of freeze-dried biomass of *P. kessleri* and *T. chuii*, using a Carlo Erba NA1500 series 2 Elemental Analyzer. The total percent carbon of *P. kessleri* and *T. chuii* were 49.8% and 23.6%, respectively.

Extraction and Derivatization of Fatty Acids

Heptadecanoic acid (3.884 mg mL^{-1} dissolved in toluene) was selected for use as an internal standard (this fatty acid was not detected in any algae samples) in order to validate experimental recovery and instrumental precision. Supelco 37 component fatty acid methyl esters (FAME's) mix (10.0 mg mL^{-1} in CH_2Cl_2) was used as a reference material for the identification and quantization of derivatized fatty acids within the algal samples. The only FAME not contained in the 37 component mix, but was detected in the algal samples, was stearidonic acid (18:4). To quantify this fatty acid, the response factor for alpha-linolenic acid (18:3) was multiplied by the ratio of molecular weight of stearidonic to alpha-linolenic.

Fresh HCl in methanol was prepared as follows: 20 mL anhydrous methanol (fresh from SIGMA ALDRICH®) was added to a dry Erlenmeyer flask; 2.00 mL Acetyl-chloride was added drop-wise. Culture tubes (2x20 cm) and a 25 mL volumetric flask, and a 50 mL Erlenmeyer flask were rinsed with methanol and dried in an oven at 90°C overnight. The glassware was removed from the oven and placed in a CaCl_2 desiccator and cooled down to room temperature before use. 97.1 mg heptadecanoic acid (17:0) was diluted to 25.00 mL with toluene (3.884 mg mL^{-1}).

Fatty acid methyl esters (FAMES) were prepared as follows. Approximately 50 mg of freeze-dried algal sample was added quickly to each culture tube, followed by 1.00 mL heptadecanoic acid internal standard solution (3.884 mg mL⁻¹ in toluene), and 1.00 mL toluene. For the marine alga *T. chuii*, the freeze dried algal mass took into account the mass of the 35 ppt salinity that was present in the media. Next, 3.00 mL freshly prepared methanolic HCl was added, followed by a magnetic stirbar. The samples were very gently vortexed to avoid splashing sample on the side of the vials. The culture tubes were flushed with N₂ (atmospheric pressure) and sealed tightly. The sealed culture tubes were placed in a water bath at 80°C for 3 hours with magnetic stirring. Periodically the reaction vials were visually checked for leaks. Tubes which leaked were not included in subsequent analyses. The reaction tubes were removed from heat and conditioned to room temperature. Next, 5.00 mL Na₂CO₃ solution (6% m/v) was added, followed by 2.00 mL toluene. The resultant biphasic mixture was thoroughly vortexed (~30 seconds). The tubes were centrifuged to settle the emulsion. The organic phase was separated and diluted to 5.00 mL volume. Approximately 2 mL of the clear brown/yellow upper layer (toluene layer) was aliquoted and dried over Na₂SO₄. The dried solutions were placed in amber autosampler vials and analyzed immediately by Gas Chromatograph with a Flame Ionization Detector (GC-FID). All samples were repeated in triplicate.

Gas Chromatography Flame Ionization Detector Analysis for Fatty Acid Methyl Esters

A volume of 0.5 µL toluene solution was injected on an HP1125 GC-FID, using a 1:25 split injection. The column used was an Alltech EC-WAX, 30 m, 0.25 mm I.D., 0.25 µm film. The inlet temperature was set at 220°C, Helium carrier gas flow was set at 71.8 kPa, 23.7 mL min⁻¹. The following temperature gradient was employed which provided adequate separation of all

fatty acids: Initial temperature, 60°C, hold 1 min; 10 °C/min to 220°C, hold 23 min, 5°C/min to 240°C, hold for 16 minutes.

RESULTS AND DISCUSSION

Biomass Productivity

It is challenging to make a biomass productivity comparison between the control flask and the substrate based system since the substrate system is not a traditional aqueous system. However, a practical approach to use when comparing these two types of systems is to compare them in terms of areal productivity ($\text{g biomass m}^{-2} \text{ floor space day}^{-1}$) and in volumetric productivity ($\text{g biomass L}^{-1} \text{ day}^{-1}$). The substrate based systems used for this experimentation covered a floor space of 450 cm^2 (10 by 45 cm), whereas the flask based control systems covered 201 cm^2 (8 cm radius), thus making their conversion factors to 1 m^2 of floor space 22.2 and 49.8, respectively. However, such a relationship ignores scaling factors associated with larger scale systems, but can be used as a means for comparison. To compare the two systems volumetrically, the amount of media retained within the substrate based system was used, which was 21 ml per substrate. This equates to a volume of 4.7 liters per meter squared of floor space and thus the areal productivity for the substrate based system can be converted into a volumetric productivity as presented in **Table 1**.

Following inoculation, the control *P. kessleri* grew initially at a quicker rate than the substrate based system while the *T. chuui* grew more quickly on the substrate based system than the control (**Figure 3**). However, following a 34 day acclimation period the substrate based *P. kessleri* achieved a quicker growth rate than the *P. kessleri* control. When comparing the *T. chuui* and *P. kessleri* growth on the substrates, we observed that both require an acclimation period

before a resilient biofilm forms.¹⁵ Following this acclimation period (11 days for *T. chuii*, 34 days for *P. kessleri*), both *T. chuii* and *P. kessleri* maintained linear biomass production of $2.78 \text{ g m}^{-2} \text{ d}^{-1}$ ($R^2 = 0.9958$) and $7.95 \text{ g m}^{-2} \text{ d}^{-1}$ ($R^2 = 0.9937$), respectively (**Table 1**). As well as increasing biomass production, cultivating *P. kessleri* and *T. chuii* on the substrates increased their harvest concentration in comparison to the control flasks. The reason that *P. kessleri* grows better than *T. chuii* on the substrates may be in part because marine algae are more prone to desiccation on the substrates compared to freshwater algae and salinity gradients reduce overall growth rates. Regardless, both species were able to increase their productivity on the substrates, including increased biomass concentrations since the substrates contain minimal quantities of water and yet provide improved irradiance and gas exchange. This ability to increase algal concentration can reduce energetic and financial expenditures associated with the dewatering and transportation of algae.

Because of the challenges of comparing the productivity of the substrate based system to the control system, it is important to compare these results to the productivities reported in the literature for similar systems. Ozkan *et al.*²⁴ conducted research on a novel algae biofilm photobioreactor (using *B. braunii*) that was oriented horizontally and achieved an areal productivity of $0.71 \text{ g m}^{-2} \text{ day}^{-1}$. For *B. braunii* grown in raceway ponds, flat-plate PBRs and tubular PBRs, the areal growth rates were 11, 27 and 25 g m^{-2} , respectively.²⁴ While depending highly on the system, conditions and algal species, generally the areal productivity of an OP system is described in the literature to be approximately $10 \text{ g m}^{-2} \text{ d}^{-1}$ and the areal productivity of a PBR system is approximately $25 \text{ g m}^{-2} \text{ d}^{-1}$.^{24,26} These productivities vary in the literature, but the PBR system consistently outperforms the OP system by 100-200%. When comparing the AL-G system to these values as reported in the literature, it appears that the AL-G system does

not have a higher productivity than the PBR systems and only slightly outperforms the OP system. However, it is inherently difficult to convert small-scale results to larger-scale systems and at this point in time the AL-G system has not yet been tested in a larger system, nor has it been optimized.

While the AL-G system is patented and is unique in many aspects, there are other attached growth systems such as the system evaluated by Ozkan *et al.*²⁴ A more common type of attached growth system is the rotating algal biofilm (RAB) system that has been shown to have a biomass productivity of between 3-31 g m⁻² d⁻¹.^{25,26} These RAB systems achieve similar biomass productivities to the AL-G system and have the same major benefit of greatly increasing harvest concentration.²⁶ The major difference between the two systems resides in their ability to scale up. While both systems have similar productivities, the infrastructure required to hang substrates is simpler and more inexpensive than the infrastructure required to have large spinning discs or wheels. Overall, we conclude that the AL-G system is able to achieve significant productivity, but that the system must be further evaluated with respect to productivity and optimization in order to compare it to current production systems.

Carbon Sequestration

The total cumulative biomass for the control system is displayed in **Figure 3** and is representative of carbon sequestration; this data does not represent the total carbon sequestration for the substrate based systems. Compared to the control system, the substrate based system is not a closed system because significant quantities of sequestered carbon are lost from the system in the form of non-adherent biomass and exopolysaccharides. In **Figure 4**, the biomass harvested from the substrate based system is shown to be only 46% for the *P. kessleri* and 14% for the *T. chuii*. For the *P. kessleri* 12% of the sequestered carbon was in the form of exopolysaccharides

and 42% was in the form of non-adherent biomass while for *T. chuui* 70% was in the form of exopolysaccharides and 16% was in the form of non-adherent biomass. This partitioning of the carbon can be explained based on the growth data of the two algal species and their respective environments. The overall carbon sequestration of the *P. kessleri* is greater since it has a faster growth rate than *T. chuui*, but the amount of exopolysaccharides produced by the *T. chuui* is greater since the *T. chuui* media is maintained at 35 ppt salinity and the *T. chuui* requires this additional polysaccharide matrix to prevent desiccation.^{16,17} Since the substrates are suspended in air they are vulnerable to gradients of increasing salinity due to evaporation. Although the relative humidity of the growth chambers was maintained between 96-99%, there was an exchange rate and air was lost from the controlled growth environment to the outside air that has a lower humidity. The media used for *T. chuui* had a salinity of 35 ppt and the loss of water from the system resulted in salinity gradients. For example, the periphery of the substrate was measured to have a salinity of up to 47 ppt. These results indicate that for both *T. chuui* and *P. kessleri* the non-adherent biomass is proportional to the amount of biomass produced on the substrates. This correlates with the *P. kessleri* having a faster growth rate and greater overall production.

In comparison to the amount of polysaccharides being produced by algae on the substrate based system, most algae species produce minimal quantities of exopolysaccharides under normal conditions in traditional aqueous systems.¹⁷ For this reason algae from the substrates were investigated under the microscope and it was observed that in addition to the exopolysaccharides produced by the algae, there were also fungi producing exopolysaccharides. These fungi appear to live in tandem with the algae and aid in producing an exopolysaccharide biofilm which helps prevent desiccation. Spontaneous symbiotic relationships between fungi and

algae have been demonstrated in the literature and it is possible both the fungi and algae are contributing to the total exopolysaccharide matrix.¹⁸ Such a relationship can be mutualistic, but requires further study to investigate the complex interplay between the autotroph (algae) and heterotroph (fungi).¹⁸ The loss of carbon from the substrate based system represents a major deficiency that needs to be rectified as this greatly reduces the total usable biomass production of the system. Future research on the system needs to investigate the capture and placement of exopolysaccharides and non-adherent biomass back onto the substrates. This type of carbon recycling loop could increase the frequency by which the substrates can be harvested and would increase the total biomass collected from the system.

Fatty Acid Composition

The absolute content of fatty acids was determined as a percentage of freeze dried biomass, allowing for a quantitative comparison of fatty acid (FA) content and composition between experiments. A comparison between the fatty acid content of *T. chuii* and *P. kessleri* grown using substrates and in flask is presented in detail in **Table 2**; the same data are represented graphically in **Figure 5**. Additionally, the statistical significance of the results was determined using a two-tailed, unpaired t-test; p values are depicted in **Table 2**. For results deemed significant ($P < 0.01$), the difference between the average FA content of substrate biomass and flask biomass was calculated.

On the surface, it appears that for *T. chuii*, there was little impact on fatty acid content due to growth on the substrate, as there was no significant difference in the total content of omega-3, polyunsaturated, monounsaturated, or saturated fats. However, on closer scrutiny, there was a significant difference in 6 fatty acids: an increase of oleic acid (18:1n9), linolenic acid (18:2n6), and eicosapentaenoic acid (EPA, 20:5n3) on the substrate biomass, and a decrease

in myristoleic (16:1n7), stearidonic (18:4n3) and eicosenoic acid (20:1n9). Importantly, there was an increase in 20:5n3, one of the most important FAs in nutrition and aquaculture.

Interestingly, the 0.111% increase in content of 20:5n3 in substrate biomass was balanced by a corresponding 0.150% decrease in 18:4n3. The changes in composition were slight, and cumulatively affected no significant change in the total compositions of any major class of FAs. As such, this data confirms that the growth of *T. chuii* on substrates provided a slight overall benefit to fatty acid profile, and most importantly, provided no measurable disadvantage with respect to FA composition. This illustrates the utility of aeroponic substrate growth for use in aquaculture of *T. chuii*.

In contrast, the impact of substrate growth on the FA content of *P. kessleri* was dramatic. There was a highly significant difference determined for nearly every fatty acid detected in substrate-grown *P. kessleri* biomass compared to aqueous-grown. There was a substantial increase in omega-3, polyunsaturated, and mono-unsaturated FAs. Accordingly, there was a 2-3x increase in every major fatty acid detected in substrate grown *P. kessleri* biomass except for palmitic acid (16:0). Total fatty acid content was $4.830 \pm 0.533\%$ in aqueous *P. kessleri* biomass, which increased to $10.395 \pm 0.173\%$ on the substrate, whereas polyunsaturated FAs nearly tripled from $0.914 \pm 0.143\%$ to $2.269 \pm 0.037\%$.

While these results demonstrated a favorable impact of substrate-grown *P. kessleri* biomass with respect to FA content, the underlying reasoning behind the substantial differences demands more thorough explanation. A comparison of the conditions used to grow *P. kessleri* to the conditions used to grow *T. chuii* is illustrative since substrate grown *T. chuii* showed minimal change in FA composition. The critical variable is pH: the pH of the media used to grow *P. kessleri* was maintained at 5.5, whereas for *T. chuii*, the pH was maintained at 8.2. This media

has been historically used for *P. kessleri* growth since it minimized contamination and *P. kessleri* appears to thrive at this pH. From the acid-base equilibrium equation for carbonate species, it can be shown that concentration of carbonate species in aqueous media is dependent on pH. At pH less than 5, total CO₂ content is constant and at a minimum—the predominant species is aqueous CO₂ (in the form of carbonic acid) and is limited by the diffusion of CO₂ into media by Henry's law.²⁷ A graph representing the total CO₂ solubility in water at 25°C at various pH values is shown in **Figure 6**. As can be seen in the figure, there is a substantial increase in the solubility of CO₂ in water as the pH increases.

In order to better understand the difference in FA productivity in *P. kessleri* and the absence of such a difference in *T. chuii*, it is important to examine the solubility of CO₂ at the pH of each media used. Using the equation to calculate the CO₂ solubility at a pH of 5.5, the media used for growing *P. kessleri*, a solubility of 0.013 mM CO₂ was calculated, just slightly above the Henry's law imposed minimum of 0.012 mM CO₂ content. The media used to grow *T. chuii* had a pH of 8.2, resulting in a solubility of 0.865 mM CO₂. This indicates that the *T. chuii* was exposed to a much higher content (>60-fold increase) of dissolved CO₂ than the *P. kessleri*. The difference in solubility of CO₂ in the media can be used to explain the differences in FA content between the growing systems and species. It is known that FA content increases with increased CO₂ content and decreased nitrogen content.²² It appears that *T. chuii* was exposed to saturating CO₂ content in the substrate system as well as the flask, since the FA content remained relatively consistent. Since there is a high content of dissolved CO₂ available at a pH of 8.2, CO₂ was therefore not a limiting factor in lipid synthesis, and at atmospheric levels, the substrate offered only a slight benefit in FA production due to increased exposure of *T. chuii* to CO₂. It therefore follows that since *P. kessleri* was exposed to low levels of CO₂ in the flask system, that CO₂ was

a limiting nutrient, and therefore lipid synthesis was reduced in flask-grown *P. kessleri*. In substrate-grown *P. kessleri*, the exponentially greater surface area of algae exposed to CO₂, coupled with the effective increase in concentration of algae on the substrate, leads to a compensation for the decreased solubility of CO₂ in acidic media, resulting in a substantial increase in FA synthesis in substrate grown *P. kessleri*.

CONCLUSIONS

Because of increased oxidative stress, it was originally hypothesized that an aeroponic system would allow for increased biomass productivities while positively effecting FA content and increasing harvest biomass concentrations. Our results indicate that *P. kessleri* and *T. chuii* appear to have a greater productivity on the substrate based system than traditional systems and that both *P. kessleri* and *T. chuii* showed increases in biomass harvest concentration. This was surprising since it was originally thought that marine species would grow poorly on the system due to salinity gradients. It appears that the overall FA content of algae grown on the substrates would not necessarily change and that the only reason the FA content of *P. kessleri* differed from the control was the growth conditions were fundamentally changed between the two systems. It is suspected that even though the substrate system is contained in air that the algae on the system are essentially within an aqueous environment as water is contained within the pores of the substrates and exopolysaccharides prevent significant desiccation. Therefore, the FA content of most species on the substrate system is suspected not to differ from an aqueous system. In regards to carbon sequestration, substrate based systems are highly amenable for this task as they can be placed in greenhouses where CO₂ is passively provided instead of needing to be pressurized and pumped into an aqueous medium. The growth of algae on the system is also very unique as the substrate based system does not have the traditional log, exponential, stationary

and death phase of growth as do PBRs or OPs. With routine mechanical harvesting, substrate based systems are able to maintain a stationary phase for extended periods of time (> 6 months) without requiring cleaning or maintenance. From these results, we conclude that the substrate based system can produce significant quantities of biomass and that cultivation on the substrate does not substantially alter the nutritional profile of algae. Based upon the volumetric and areal productivity of the substrate based system, this system is capable of productivities in the same range as the OP and PBR traditional systems.

While the substrate system does have benefits in comparison to traditional systems, it must be noted that there are some considerable downsides to the substrate based approach. Since the substrates are suspended in air within a humid and hot environment, contamination by heterotrophs and other algae is a major concern. Growing extremophiles on the system can avoid some of this contamination, but this represents a major drawback for the system and makes it difficult for the system to grow a highly pure product. This could potentially limit the system in its ability to be used as a source of feed for fisheries as marine fish larvae are very vulnerable to contamination and it greatly reduces their productivity.¹⁹ Additionally, if ten 1 m^2 substrates are grown within 1 m^2 of floor space, then the effective surface area per 1 m^2 of floor space is 20 m^2 . Even though the substrates are housed within controlled environments (e.g., greenhouses) this surface area is 20 times greater than the surface area of an open pond and thus these systems lose considerable amounts of water in comparison to traditional systems. While the substrate based system does have disadvantages, it can still serve as a useful cultivation process even if it is not more successful than OPs or PBRs for all applications as it could be used in tandem with traditional systems to provide a concentrated inoculant. Regardless of its future commercial

application, the substrate based system does show value in its ability to produce dense effluent concentrations and to grow unique algae species and as such warrants future study.

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Table 1. Maximum growth rate and maximum biomass concentration achieved during the experiments. The Control in the table represents the flask photobioreactor system that was used for testing, and the biomass represented in grams is dry biomass. The maximum growth rates were determined based on the maximum growth rates achieved during the experiments and the area units represent square meters of floor space.

	Max. Growth Rate (g day ⁻¹ m ⁻²)	Max. Growth Rate (g L ⁻¹ day ⁻¹)	Standard Error (n=3)	Max. Concentration (g/l)	Standard Error (n=3)
<i>P. kessleri</i> Control	9.92	0.20	± 2.53	0.68	± 0.02
<i>P. kessleri</i> Substrate	15.04	3.23	± 3.10	4.94	± 1.31
<i>T. chuui</i> Control	0.78	0.02	± 0.22	0.06	± 0.01
<i>T. chuui</i> Substrate	3.43	0.74	± 0.33	2.10	± 0.43

Table 2. Fatty acid content^A of *T. chuii* and *P. kessleri* grown on a vertical aeroponic substrate or in a flask (control). Bold p-values are statically significant (p < 0.05).

Fatty Acid	<i>T. chuii</i> – substrate	<i>T. chuii</i> – control	p-value (n=6)	Absolute Change (substrate – control)
14:0	0.147% ± 0.003%	0.148% ± 0.010%	0.8203	N.S.
14:1n7	0.074% ± 0.001%	0.075% ± 0.006%	0.8099	N.S.
16:0	1.434% ± 0.041%	1.365% ± 0.071%	0.2217	N.S.
16:1n7	0.055% ± 0.005%	0.074% ± 0.004%	0.0070	-0.018%
16:2n4	1.158% ± 0.048%	1.152% ± 0.070%	0.9098	N.S.
18:0	0.032% ± 0.031%	N.D.	0.1484	N.S.
18:1n9	0.365% ± 0.026%	0.227% ± 0.012%	0.0011	0.138%
18:2n6	0.710% ± 0.027%	0.583% ± 0.030%	0.0056	0.126%
18:3n3	1.383% ± 0.056%	1.358% ± 0.078%	0.6701	N.S.
18:4n3	0.528% ± 0.019%	0.677% ± 0.039%	0.0038	-0.150%
20:1n9	0.070% ± 0.005%	0.121% ± 0.007%	0.0004	-0.052%
20:3n3	0.187% ± 0.015%	0.161% ± 0.009%	0.0533	N.S.
20:5n3	0.300% ± 0.016%	0.189% ± 0.010%	0.0006	0.111%
Total	6.442% ± 0.228%	6.130% ± 0.344%	0.2601	N.S.
Omega-3	2.398% ± 0.100%	2.384% ± 0.136%	0.8965	N.S.
Polyunsaturated	3.966% ± 0.163%	3.931% ± 0.226%	0.8399	N.S.
Monounsaturated	0.564% ± 0.024%	0.497% ± 0.027%	0.0330	N.S.
Saturated	1.612% ± 0.031%	1.513% ± 0.081%	0.1182	N.S.
Fatty Acid	<i>P. kessleri</i> – substrate	<i>P. kessleri</i> – control	p-value (n=6)	Absolute Change (substrate – control)
14:0	0.285% ± 0.013%	0.213% ± 0.038%	0.0365	N.S.
14:1n7	0.220% ± 0.013%	0.049% ± 0.005%	< 0.0001	0.171%
16:0	1.690% ± 0.021%	1.609% ± 0.093%	0.2168	N.S.
16:1n7	0.857% ± 0.006%	0.209% ± 0.253%	0.0114	0.648%
18:0	0.276% ± 0.023%	0.307% ± 0.134%	0.7094	N.S.
18:1n9	0.812% ± 0.031%	N.D.	< 0.0001	0.812%
18:2n6	3.986% ± 0.069%	1.459% ± 0.169%	< 0.0001	2.527%
18:3n6	N.D.	0.070% ± 0.003%	< 0.0001	-0.070%
18:3n3	2.269% ± 0.037%	0.914% ± 0.143%	0.0001	1.354%
Total	10.395% ± 0.173%	4.830% ± 0.533%	0.0001	5.565%
Omega 3	2.269% ± 0.037%	0.914% ± 0.143%	0.0001	1.354%
Polyunsaturated	6.255% ± 0.106%	2.374% ± 0.311%	< 0.0001	3.881%
Monounsaturated	1.890% ± 0.050%	0.258% ± 0.258%	0.0004	1.632%
Saturated	2.251% ± 0.057%	2.129% ± 0.265%	0.3823	N.S.

^A Fatty acid content expressed as percentage of freeze dried biomass.

N.D. = not detected, N.S. = not significant.

Figure 1. Aeroponic substrate based cultivation system overview. The process from growing inoculum through harvesting is comprised of four distinct steps: 1) Stock Solution, 2) Inoculate Substrate, 3) Algae Growth on Substrate and 4) Harvest.

Figure 2. Small scale aeroponic substrate based system that was inoculated with *P. kessleri* and irrigated for three weeks. The left most and right most substrates were not inoculated or irrigated and served to maintain consistent light environment for all ten growth substrates. Each substrate measured 15 cm wide by 25 cm long. At the top of each substrate a small 1/8" irrigation dripper can be seen and the media that drips off the bottom of the substrates is collected and used for analysis. The substrates were contained within a closed chamber and were illuminated with an array of LED lights on a 16 hour on and 8 hour off light cycle.

Figure 3. Cumulative grams of biomass produced from *T. chuii* and *P. kessleri* using substrate and control photobioreactor flask system. The experiments were terminated at a point in time when growth rates peaked based upon findings from preliminary data. The inflection point seen for *P. kessleri* Substrate growth represents the point in time when a dense biofilm rapidly forms on the membranes. The vertical bars represent the standard error of the data from three replicate trials.

Figure 4. Carbon partitioning within *P. kessleri* and *T. chuii* cultivated on the substrate based system. The non-adherent biomass is defined as the algae that did not adhere to substrate and fell of the substrate with the effluent drip from the bottom of the substrate. Non-adherent biomass was separated from polysaccharides in substrate effluent for analysis using centrifugation and harvest biomass was determined directly following mechanical harvesting. The percentages seen at the top of both bar graphs represent the percentage of carbon sequestered in the harvested biomass for *P. kessleri* and *T. chuii*.

Figure 5. Fatty acid content (as % of freeze dried mass) for *Parachlorella kessleri* and *Tetraselmis chuii* grown on the substrate based system compared to grown in the control photobioreactor system. Fatty acid description: 18:4n3 – “18” is the number of carbons, “4” is the number of double bonds, “3” is location of double bond. The vertical bars represent the standard error and the three boxes represent where the fatty acid components are significantly ($p < 0.01$) different from one another.

Figure 6. Total CO₂ content in water at atmospheric pressure and background CO₂ levels (0.0400% atmospheric content), showing non-linear pH dependency. This relationship is based on aquatic CO₂ speciation as described by Morel and Hering.²⁷ mM CO₂ can be converted to mg CO₂ per liter by multiplying by 44.