Production optimisation of *Tisochrysis lutea* as a live feed for juvenile Sydney rock oysters, *Saccostrea glomerata*, using large-scale photobioreactors

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ABSTRACT

The aquaculture industry uses microalgae as a live feed for juvenile oysters in hatcheries to meet their nutritional requirements, including their need for several essential Poly Unsaturated Fatty Acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The mass culture of microalgae is not only a major bottleneck for the production of juvenile oysters, but also a significant cost, accounting for 20-50% of hatchery operating costs. Currently, low biomass concentrations, high production costs and poor cultivation systems limit the quantity and quality of microalgae feed. This study focused on *Tisochrysis lutea,* a microalgae species commonly used in aquaculture, and we assessed the potential of photobioreactors with an improved light source and CO_2 input to increase biomass production and improve biochemical composition of algal feed. Two photobioreactor systems were compared: the current industry set up (DPI) comprising fluorescent lighting and minimal CO_2 input versus an optimized system utilising LEDs and increased CO_2. Cultures of *T. lutea* were monitored over a 12-day growth period and harvested on day 14 for biochemical analysis. Final cell density was significantly higher in the optimized system relative to the conventional culture systems ($6.2x10^6$ cells / mL versus $3.7x10^6$ cells / mL, respectively). The biochemical profile of *T. lutea* was not significantly different between the two photobioreactors systems. The algal biomass produced during this comparative experiment was used in a feeding trial on oyster spat, *Saccostrea glomerata*. Spat fed with algae produced in optimized vs conventional photobioreactors showed no significant difference in growth, but oyster spat fed with *T. lutea* grown in optimized photobioreactors did show a significant increase in their EPA content. Overall, our results contribute to our understanding of how altered culture conditions affect microalgal production and biochemical composition, and subsequently of oyster spat. This study further supports the potential of LEDs to reduce

45 operating costs for oyster hatcheries but also to significantly improve microalgae yield with
46 no negative effect on the condition of oyster spats production.

47

48 **KEYWORDS:** microalgae, *Tisochrysis lutea Microalgae;* photosynthesis; oysters; *Saccostrea*
49 *glomerata*; hatchery

50

INTRODUCTION

Aquaculture production facilities utilise microalgae's rapid growth rates and balanced nutritional value as a live feed (Borowitzka, 1999) for juvenile stages of abalone, crustaceans, fish and bivalves (Brown, 2002; del Pilar Sánchez-Saavedra et al., 2016; Parwadani-Aji, 2011; Shields and Lupatsch, 2012). Bivalve hatcheries require amongst the largest quantities of microalgal feed in aquaculture, as bivalves are obligate filter-feeders throughout their life history (Shields and Lupatsch, 2012). The nutrients in microalgae are fundamental for successful rearing of bivalve larvae, and in turn improve survival and growth rates in many hatcheries (Coutinho et al., 2006; del Pilar Sánchez-Saavedra et al., 2016). Nutritional requirements of the Sydney rock oyster, *Saccostrea glomerata,* remain poorly known (Guedes and Malcata, 2012). However, feeding trials have found Poly-Unsaturated Fatty Acids (PUFAs) and carbohydrates are essential to support the metamorphosis of oysters during juvenile growth stages (Enright et al., 1986). PUFAs, comprising of eicosapentaenoic acid (EPA, 20:5n-3), arachidonic acid (AA) and docosahexaenoic acid (DHA, 22:6n-3), in particular, are vital in the metamorphosis of larval bivalves (Guedes and Malcata, 2012).

The main algae genera commercially used for oyster feed are *Chaetoceros, Pavlova, Skeletonema* and *Tisochrysis* due to their high nutritional value (Heimann, 2015). *T. lutea* has been identified as a more suitable feed due to its high content of EPA and DHA (Heimann, 2015). Numerous ecophysiological studies have noted its valuable lipid content, as well as large amounts of long-chain PUFAs (Bendif et al., 2013). Its small size, ranging between 3-7.5 µm, makes *T. lutea* a suitable feed during juvenile phase from early larvae to spat. Other favourable features of *T. lutea* include its tolerance across a wide range of physico-chemical

conditions and their fastgrowth rates (O'Shea et al., 2010). All these factors listed above make *T. lutea* an ideal algae feed source for oyster production in hatcheries.

As highlighted, microalgae cultivation is a fundamental practice in the commercial aquaculture industry (del Pilar Sánchez-Saavedra et al., 2016), with the mass production of microalgae accounting for 20-50% of hatchery operating costs, indicating it is a major financial consideration (Brown, 2002; Catarina and Xavier, 2012). Among the different factors playing a key role in microalgae cultivation, light is arguably one the most important asmicroalgae utilise light as the source of energy to achieve photosynthesis and synthesize nutrients (Hoshino et al., 1991; Wahidin et al., 2013a). The quality and quantity of light largely affect the amount of biomass and its biochemical composition in mass cultivation systems. When light is limited and below compensation point, growth conditions are classified as photo-limited, whereby growth rates decrease. However, with an increase in light intensity, a point of saturation can be reached whereby exposure to light is improved and growth rates increase. On the other hand, excess light above saturation can inhibit the growth of microalgae and can be detrimental to the photosynthetic apparatus (Wahidin et al., 2013b), as well as lead to the formation of reactive oxygen species (Cheng and He, 2014). Overall, the growth rate and lipid content of microalgae cultures is largely affected by two primary irradiance factors; the intensity of light and length of photoperiod (light : dark) cycles (Kitaya et al., 2008; Wahidin et al., 2013a).

Parmar et al. (2011) showed that light intensity is the key to optimising growth rates, and the intensity requirements vary greatly with culture density and depth. High-density microalgae cultures require increased light intensity in order to penetrate through the cell biomass

allowing a majority of cells access to light for photosynthesis (Parmar et al., 2011). Numerous studies have compared the photoperiod and intensity of light under laboratory conditions, in small-scale PhotoBioReactors (PBRs), across a variety of algae species (Parmar et al., 2011). However, industry based production systems and the range of microalgae species used in aquaculture have not changed for many years (Heffernan and Frater, 2007; Seiler et al., 2017; Wijgerde et al., 2012). Current industry PBRs are commonly equipped with tubular fluorescent lamps to artificially illuminate algae cultures for photosynthesis (Ugwu et al., 2008). These fluorescent lamps, are not energy or cost-effective systems, demanding 13 -15 W/unit light generated and a lifespan of 8, 000 hours. Light emitting diodes (LED) on the other hand utilise only 6 – 8 W/unit light generated with a lifespan of 50,000 h (Nardelli et al., 2017, Principi and Fioretti, 2014). Also, optimized thermal management using LEDs minimises the need for air conditioning to reduce room temperature for optimal algae growth. Therefore, LEDs provide a possible beneficial alternative to fluorescent lighting in conventional PBRs.

Previous research has highlighted the need to improve conventional large-scale PBRs, as inefficient lighting limits the optimum production of microalgae biomass (Edwards, 2015; Wijgerde et al., 2012). Several studies have attempted to improve production systems via adjusting the type and quantity of light; however, only at small scale (del Pilar Sánchez-Saavedra et al., 2016). De Pilar Sánchez-Saavedra *et al.* (2016) analysed the effects of various LED light spectra on the biochemical composition and growth rate of *T. lutea* in small-scale 1,000 mL Erlenmeyer flasks. Even though these studies contribute to the wider understanding of microalgae growth, they do not accurately reflect the needs of industry production systems and thereby they are not transferable to large-scale industry photobioreactors (Ippoliti et al., 2016).

Microalgae also require a steady supply of carbon to actively support active growth. Carbon can be utilised by some microalgae in either an inorganic form as carbon dioxide or in an organic form as glucose acetate. In most photoautotrophic microalgae species, much like *T. lutea,* CO_2 acts as a stable buffer system that assists in lowering pH and maintaining it at optimal levels for microalgal growth (pH 7.8- 8.2) (Lochlan de Beyer, Personal communication). A supply of CO_2 increases biomass over time along with the increased consumption of available macro and micronutrients. Carbon is often limiting, along with Nitrogen (N) and Phosphorous (P) in mass cultivation practices (Garnier et al., 2016). An oversupply of CO_2, however, can result in reduced growth and stress. Carbon dioxide in mass cultures affects the accumulation of carbohydrates, where a reduction in CO_2 coincides with an increase in carbohydrate concentration in biomass. This is due to carbon dioxide concentrating mechanisms (CCM), where the CCM is induced under low CO_2 so that microalgae are able to concentrate and obtain inorganic carbon (generally as bicarbonate) from the external environment (Huang and Su, 2014; Sun et al., 2016).

Several cultivation techniques can alter the composition of microalgae biomass (Markou et al., 2012). The regulation of carbon partitioning to favour carbohydrate and lipid synthesis could result from alterations in cultivation conditions including light intensity, photoperiod (light-dark cycle), nutrient starvation and CO_2 concentration (Cheng et al., 2017). Optimum light intensity favours the over accumulation and production of lipids in microalgae. This may be due to the optimal light benefiting the storage capacities of photoassimilates (Zhu et al., 2016). Normally, storage lipids accumulate under high light, whereas structural lipids (PUFAs) increase under low light conditions (Cheng and He, 2014). However, Brown et al.

147 (1993), found a higher content of certain fatty acids with increasing light intensity in *T. lutea*
148 cultures. The percentage of DHA (22:6(n-3)) increased with increasing light intensity, whilst
149 alpha-linolenic acid (ALA) (18:3(n-3)) and Linoleic acid (LA) (18:3(n-6)) decreased. With
150 regard to the effect of high lipid strains of *T. lutea* on juvenile oysters, EPA was found to be
151 one of the only fatty acids to associate positively with larval growth and survival, whilst
152 arachidonic acid (AA) negatively affected the growth of larval oysters (Da Costa et al.,
153 2016). Given that the content of saturated fats can be manipulated in microalgae cultures by
154 using different light conditions (Thompson et al., 1993; Marchetti et al., 2018), there is great
155 potential for optimising light in industrial-scale photobioreactors in order to improve not only
156 the quantity, but also the quality of microalgae feed for oysters spats.

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158 The modification of culture conditions, particularly alterations to light and CO_2 are expected
159 to affect growth rates and the biochemistry of microalgae cultures. Rapid assessment to
160 determine any biological and physiological parameters or 'sweet spots' in microalgae strains
161 is a vital tool to minimise costs, while increasing production to meet aquaculture demands. In
162 this study, we aimed to optimize the biomass production of microalgae cultures, *Tisochrysis*
163 *lutea,* by altering the existing light and CO_2 conditions on conventional large-scale
164 photobioreactors (PBRs) used by the aquaculture industry. We further assessed the nutritional
165 value of *Tisochrysis lutea* produced in optimized PBRs, by conducting feeding trials on
166 juvenile oysters, *Saccostrea glomerata*.

167

MATERIAL AND METHODS

Cell culture transfer process

Industry standard cell culture protocols were used to maintain and up-scale *T. lutea* (CS-177) cultures from 250 mL to a 500 L photobioreactor (DPI, Brandt Archer, pers com.). Stock cultures were kept in an incubator at 20 ˚C in 200 mL 0.22 µm filtered seawater, autoclaved f/2 media (from 1000x stock solution). Cultures were mixed daily via swirling to minimise the settling of cells and ensuring all cells have access to light and nutrients for photosynthesis. *T. lutea* stock cultures were sub-cultured every 7-10 days to ensure stable growth of cultures. After 10 days of growth in 250 mL, the flask was up-scaled to 2 L Schott bottle in 0.22 µm filtered seawater, f/2 media (f/2 media 1 mL/L) (Richmond, 2004) and incubated at 22 ˚C for 7-10 days under 183 µmol photon m^2/sec of white fluorescent light. The light:dark cycle was set at 12:12 during all incubation periods. The 2 L culture was then transferred to a 10 L Nalgene carboy and kept in an incubator at 23 ˚C for a further 7-10 days. Finally, the 10 L of culture was used to inoculate 500 L photobioreactor system at NSW DPI Port Stephens Fisheries Institute. All culture transfers except for 500 L inoculation were performed inside a laminar flow cabinet in a sterilised tissue culture room.

Experimental set-up

The comparative analysis of the two PBR systems was conducted at NSW DPI Port Stephens Fisheries Institute to obtain data collected under standard industry operational conditions. Current industry production systems use fluorescent lighting and air/CO_2 mix (2%) via a single airline port with 20:4 light/dark cycle (B. Archer, personal communication). 20:4 (L:D) light cycle was kept the same across both treatments in order to allow for a balance of dark respiration to light-saturated photosynthesis throughout the experiment.

The experimental treatments at DPI consisted of two varying PBR setups, with 3 replicates. Treatments were as follows:

I. UTS: Light Emitted Diode (LED) lighting, two airlines (A, B, C)
II. DPI: Fluorescent tube lighting, one airline (D, E, F)

Experiment ran for a period of 12 days and were monitored daily. Supplementary material Figure S1 illustrates the difference in light quantity/quality between the two treatments, both the position of light and light intensity at the centre of the PBR prior to inoculation.

Replicates – UTS (A, B, C)

DPI (D, E, F)

500 L Photobioreactor inoculation at DPI Fisheries

Within the facilities of NSW DPI Port Stephens Fisheries Institute, seawater is pumped from the nearby estuary of Fenningham's Island creek, passed through a 5 μm filter bag and left to settle in a 10-tonne tank. It then passes through pipes and undergoes UV sterilisation. Prior to inoculation, bags are inflated with 0.22 μm filtered air and then filled with 500 L of seawater. The 500L seawater is then chlorinated with 100 mL of sodium hypochlorite and left to settle for 24 h. 30 mL of sodium thiosulphate (4 mol) is then added to de-chlorinate and residual chlorine is checked using a 'DPD No4 Chlorine-testing tablet'. 500 mL of f/2 media 1000x is added to the 500 L of sterilised seawater. It is important to note that, because there was no dilution rate for any of the PBR systems (DPI and UTS), the algal culture in this study can be considered as static or batch culture system. Autoclaved glass pipette tips and airlines are then inserted into the base of the bag. Two airlines for each UTS replicate (A, B, C) and one airline per PBR in the DPI treatment (D, E, F).

CO_2 input

Throughout the comparative experiment at NSW DPI Port Stephens Fisheries Institute, continuous air/CO_2 (2%) mix was utilised via airlines (Nalgene 180 PVC – autoclaved) at the base of the PBRs.Double the CO_2 was provided to supplement the higher light intensity emitted by the LED lights. On day 6, CO_2 mix was increased slightly to 2.5% due to rapid increases in pH across all six PBR replicates.

Light intensity

The intensity of light was monitored every 12 h, using a 4π light sensor connected to a light meter (Li-Cor LI-250A, Nebraska USA) light meter. Prior to inoculation light intensity was recorded in each PBR and again at the time of inoculation (day 0). The sensor was placed in the centre of the bag approximately 30 cm in depth.

Dissolved Inorganic Carbon (DIC), Alkalinity (TA) and pH

PH was measured every 12 h using a pH-Electrode (Sentix 940-3) connected to a meter (multi 3620 IDS). DIC and TA samples were preserved daily in 100 mL airtight glass amber bottles fixed with 30 µL of (> 99% saturated) mercuric chloride $HgCl_2$ and stored at 4˚C. Fixed samples were later analysed using an 'Auto titrator (Metrohom 916 Ti-touch) equipped with a doser (Metrohom 800 Dosino 2 mL).

Cell density

Cell density was compared between two treatments; UTS and DPI throughout the 12-day experimental period. Nine hundred µL of cell suspension was chemically fixed in a solution

of 2.5% glutaraldehyde (final concentration) in 1.5 mL Eppendorf tubes from each PBR replicate every 12 h. Eppendorf tubes were stored in a refrigerator at 4˚C until further processing for automated cell counts.

After 12-day of experimental treatment, samples were analysed with an Automated Upright Fluorescence Microscope (Nikon) to conduct cell counts. Ten µL of culture sample was loaded onto both chambers on a dark-line haemocytometer and left to sit for 5 minutes. Using x20 objective, the microscope was set up with appropriate coordinates and used to take 25 consecutive images of the slide in both the upper and lower chambers of the haemocytometer. Fiji software 'Image J' was then used to perform cell counts and produce results of cells/mL, via using a cell count macro txt. and species-specific parameters.

Biochemical analysis

At the end of the exponential growth period, 5 L of culture from each PBR was collected. Cell samples were harvested by centrifugation at 3,500 RPM for 4 minutes at 4˚C. Supernatant was removed, and all the resulting pellets were rinsed three times in 5 mL of PBS solution (1M) and milliQ in order to eliminate salt residues and re-centrifuged twice prior to being stored at -80˚C for further biochemical analysis. Samples were then freeze-dried for 24 h and stored in -20˚C until further analysis.

Fatty Acid Methyl Esters extraction & analysis

Fatty acid methyl esters (FAMEs) were primed by saponification of crude lipids followed by transmethylation. Samples of algal cultures from different PBR replicates (n=3) were each

263 treated with 1 mL of 1% NaOH in MeOH and heated for a period of 15 min at 55˚C, followed
264 by a further 15 min at 55˚C after adding 2 mL of 5% methanolic HCl. For calibration
265 purposes the internal standard nonadecanoic (10 ppm) was used. FAMEs were then extracted
266 with hexane (3 x 1 mL) and evaporated until dry under nitrogen, redissolved in 300 µL of
267 hexane. Analysis of FAME was carried out on a Gas Chromatography-Mass Spectrometer
268 (GC-MS) instrument (Agilent 7890 series GC coupled to an Agilent quadrupole MS) using a
269 fused capillary column (5%-phenyl-methylpolysiloxane, 30 cm long, film thickness 0.25 µm,
270 Agilent Technologies). Briefly, helium was used as carrier gas (flow rate of 0.9 mL/min).
271 Five µL of samples were injected under a split less mode of injection and an inlet temperature
272 of 280 °C. The column temperature program was as follows: initial oven temperature 50 ˚C
273 (held for 2 min), followed by a 4 ˚C/min ramp up to 220 ˚C, and then to 300 ˚C at 60 ˚C/min
274 (held for 3 min). Data was analysed using proprietary software, Agilent GC Chemstation and
275 peaks were identified by comparison of their retention times and mass spectra with standards
276 for EPA, AA and DHA (Sigma Aldrich, NSW, Australia) (Laurens et al., 2012).

277

278 **Feeding trials**

279 ***Oyster spat feeding trial***

280 Seawater used throughout the feeding trial was obtained from estuarine waters in Port
281 Stephens, NSW. It passed through a 5µm filter and then underwent UV sterilisation before
282 going into the experimental tanks. Water was maintained at 20 ± 2 ˚C in 20 L aerated tanks
283 and circulated via a vertical air pump. All tanks were washed with disinfectant (Virkon S)
284 and freshwater every two days. Spat of Sydney rock oyster, *Saccostrea glomerata,* were
285 obtained from a December 2017 cohort in Cromarty Bay, Port Stephens, NSW and graded
286 with mesh sieves to attain spat with an approximate average size of spat 12.8 mm (O'Connor
287 et al., 1992). A total of 150 oyster spats (30 oyster spats were measured in each of the five

feeding regime replicate; N=5) were fed either *T. lutea* grown under LED lights (UTS Treatment) or *T. lutea* grown under fluorescent lights (DPI Treatment).

Spat were left unfed for 24 h before the commencement of the trial and then fed twice daily over a period of 9 days. Each feed involved adding sufficent *T. lutea* to each replicate tank to bring the cell concentration to 100,000 cells/mL. *T. lutea* was obtained from PBRs grown under UTS and DPI replicates from day 12 of growth until day 21, the final day for the feeding trial. Equal quantities of algae from the three independent replicates were combined for each treatment feed. Every three days spat were weighed (g) and measured (length, depth, width). On the final day, spat were measured for growth and shucked, and individual oysters were snap frozen in Eppendorf tubes in dry ice bath and stored in -80 ˚C freezer for further lipid extraction. Samples were then pooled into 50 mL Eppendorf tubes per replicate (n=5) and stored in -80 ˚C freezer until further processing for lipid and FAME analysis using the same methods as stated above in the microalgae FAMEs extraction.

Statistical analyses

Statistical analyses were performed using 'IBM SPSS statistics 19 software'. Assumptions for parametric tests (normality and homoscedasticity of data) were first tested by Kolmogorov-Smirnov and Levene's tests. When data did not meet these assumptions, non-parametric independent tests (Mann Whitney U) were applied to test significant difference between treatments per time point. Results were considered significant at $p < 0.05$.

RESULTS

Algal Biomass

Light intensity

The mean light intensity at the centre of the PBR prior to inoculation was 476.6 μmol photons m^{-2} s^{-1} in UTS treatment and 168.8 μmol photons m^{-2} s^{-1} in the DPI treatment. Following inoculation, UTS replicates where exposed to a mean irradiance, 2-fold higher than DPI light exposure 115 photons (Fig. 1a). Mann Whitney U test highlighted significant differences in light intensity from day 0 till day 9 ($p < 0.05$). Once the algal cultures had started, light intensity within the LED exposed PBR (UTS) then experienced a markedly more rapid decline, in comparison to fluorescent light PBR (DPI).

Both treatments experienced an increase in pH during light exposure and a decrease during dark, as presented in Figure 1b. Samples around day 4 hit a peak of 8.93 and 8.63, for UTS and DPI treatment respectively. From day 6 onwards pH in both treatments began to drop and stabilise with mild fluctuations in DPI replicates, whereas UTS replicates continued to drop to 8.21. No significant difference in pH was found between treatments ($p > 0.05$).

Total alkalinity (TA) and dissolved inorganic carbon (DIC) followed similar trends, with dips in TA corresponding with CO_2 limitation present (Fig. 1c & 1d). From day 0 to day 6, both UTS and DPI treatments saw a decrease in TA and DIC, from ~1730 to 1270 μmol and, ~1750 to 1100 μmol, respectively. A slight increase in CO_2 input on day 6, noted in the methodology, resulted in an increase in TA and DIC, which was steeper in UTS treatment due to the introduction of a second airline. The DPI treatment on the other hand demonstrated a stable buffer system after the slight increase in CO_2. On day 12, UTS replicates attained a

334 higher concentration of TA and DIC in comparison to DPI replicates, with a final TA and
335 DIC, 226 and 321 µmol higher, respectively. Statistical tests did however highlight time
336 points towards the end of the experiment where the difference between UTS and DPI
337 treatments were statistically significant ($p < 0.05$) (Fig. 1c and 1d).

338

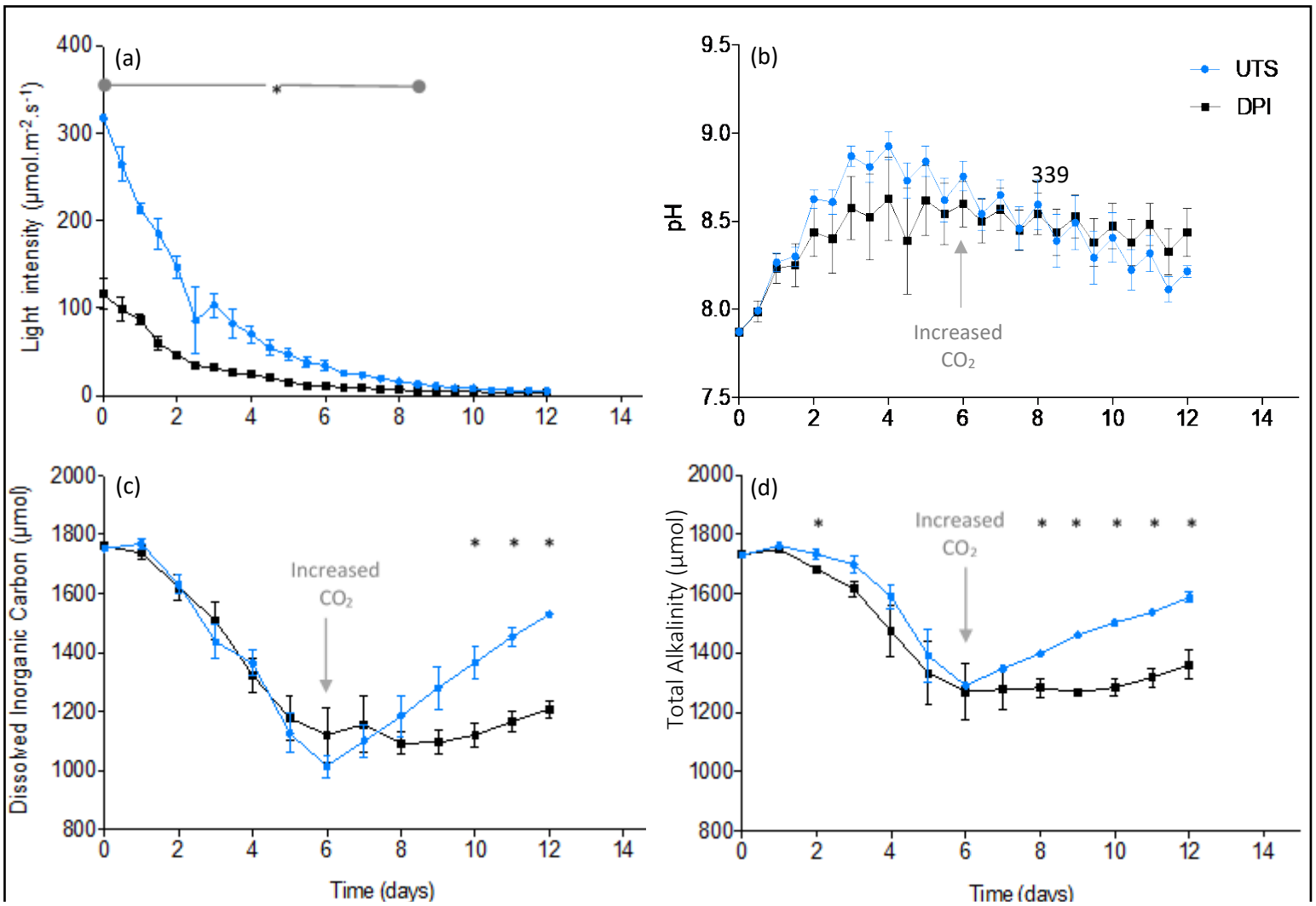

Figure 1. a) Mean light intensity expressed as µmol.m^{-2}.s^{-1} (± SEM, n=3) over 12-day growth period. Measurements taken from the centre of the PBR at 30 cm depth. b) Mean pH concentrations (± SE, n=3) over 12-day experimental period. c) Dissolved inorganic carbon samples expressed as µmol (± SEM, n=3). d) Total alkalinity samples expressed as µmol (± SEM, n=3).

(*) indicate significant results ($p < 0.05$). (Blue line – UTS treatment, Black line – DPI treatment)

Cell density

Cell density on day 12 indicated an average of $6.92x10^6 \pm 1.01x10^5$ and $4.40x10^6 \pm 3.54x10^5$ cells/mL in UTS and DPI treatments, respectively (Fig. 2). Cell density was significantly higher in UTS treatment relative to DPI treatment from Day 2 onward ($p < 0.05$).

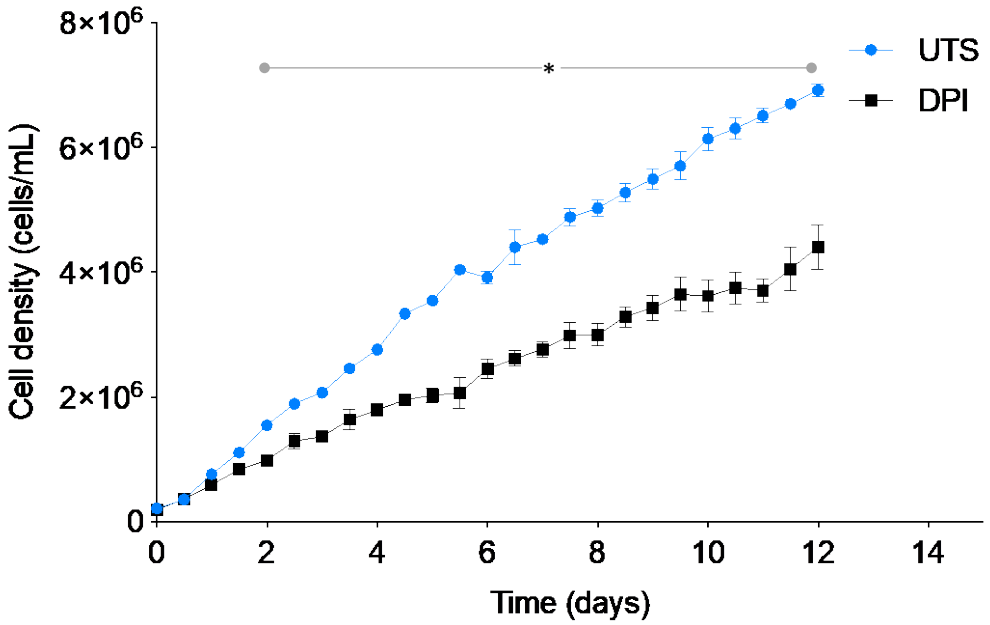

Figure 2. Mean values (±SE, n=3) of *T. lutea* cell density expressed as cells per mL under different light and CO_2 conditions. (*) indicate significant results ($p < 0.05$). (Blue line: UTS Treatment, Black line: DPI Treatment)

FAME content of algae

Lipid content was examined using FAMEs analysis, whereby fatty acids were identified. Analysis focused on the three fatty acids commonly identified as important for the aquaculture industry; Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and Arachidonic acid (AA) (Fig. 3). Among these 3 major fatty acids, DHA content was the most

abundant in *T. lutea* with ~4.8 µg/mg dry biomass. No significant difference was found in EPA, DHA and AA content between UTS and DPI treatment ($p > 0.05$).

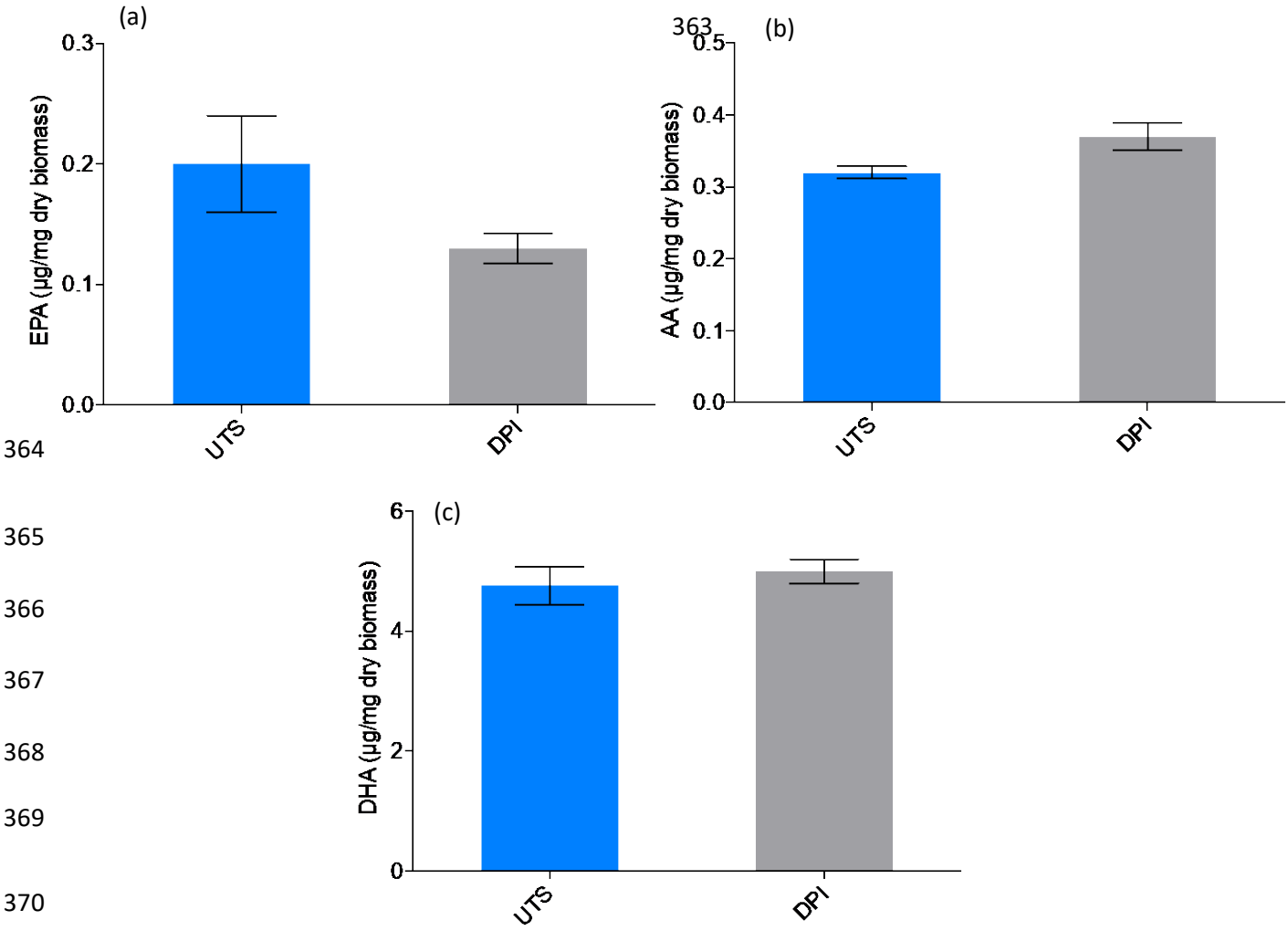

Figure 3. Measured FAMEs analysis from both treatments obtained day 12 of growth. Focusing on fatty acids, (a) Eicosapentaenoic acid (EPA), (b) Docosahexaenoic acid (DHA) and (c) Arachidonic acid (AA) expressed as µg/mg dry biomass (± SE, n= 3 biological replicates). Blue: UTS Treatment, Grey: DPI Treatment

Feeding trial

Feeding trials were continued until a significant difference in growth of oyster spat was found in both length and weight compared to day 0 (day 9). As seen in Figure 4 the difference in

both length and weight between treatments was not large and not found to be significant at the last time point of growth (Day 9, Mann Whitney U, $p > 0.05$). Although not significantly different, the increasing trend in length of spat from day 0 was higher in spat that were fed from UTS grown *T. lutea* in comparison to those fed from DPI grown *T. lutea*. The difference in weight also illustrates similar trends, with the weight of UTS *T. lutea* fed spat heavier than DPI treatment.

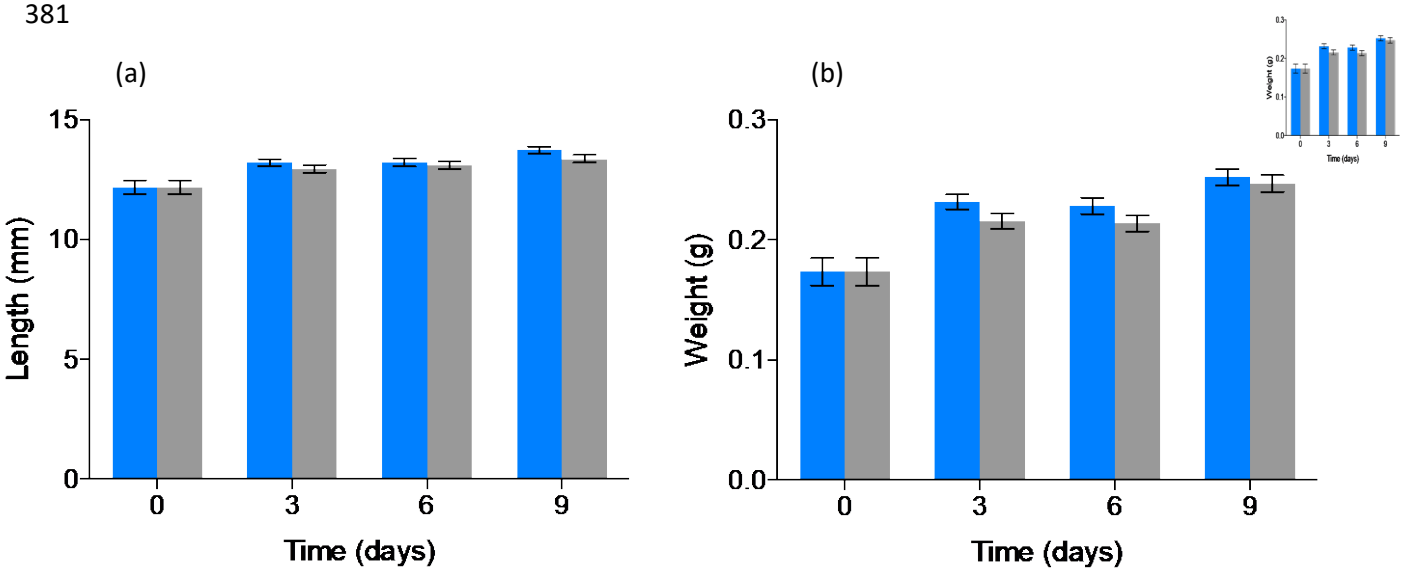

Figure 4. Growth of *Saccostrea glomerata* spat over 9 days, (a) growth examined by length (mm), and (b) weight (g), (±SE, n=5 biological replicates, a total of 30 oyster spats were measured for each replicate). Blue: UTS Treatment, Grey: DPI Treatment.

FAMEs

Total lipid content (%) of oyster spat was not significantly different between treatments; UTS fed and DPI fed *T. lutea* (Fig. 5). FAMEs analysis however highlighted some significant differences in PUFAs. EPA content was significantly higher in UTS treatment, whereas AA

was significantly higher in DPI treatment ($p < 0.05$). DHA showed no significant differences between treatments (Mann Whitney U test; $p > 0.05$) (Fig. 5).

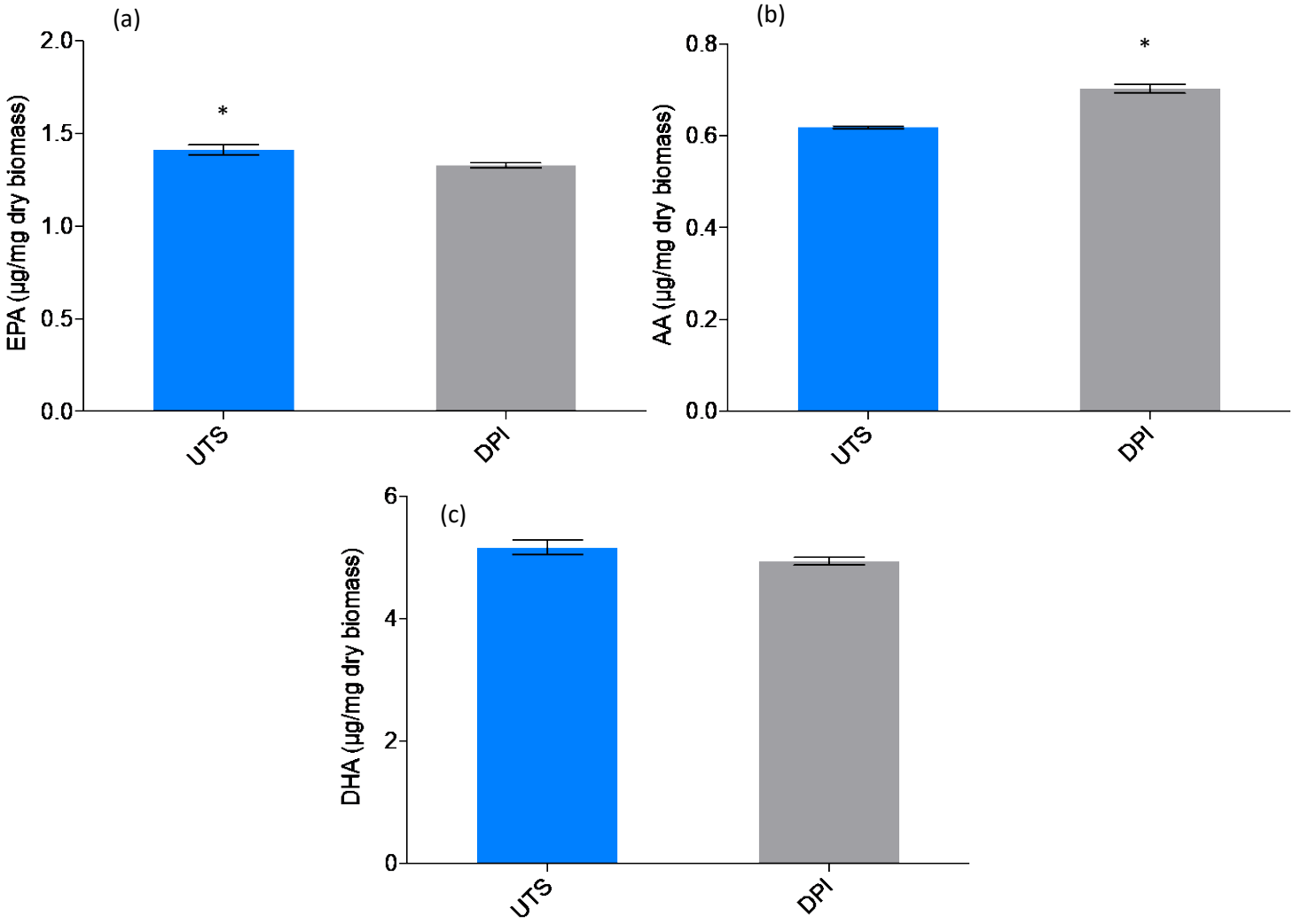

Figure 5. Measured FAMEs analysis from both treatments obtained from day 9 of *S. glomerata* growth. Focusing on fatty acids, (a) Eicosapentaenoic acid (EPA), (b) Docosahexaenoic acid (DHA) and (c) Arachidonic acid (AA) expressed as µg/mg dry biomass (± SE, n=5 biological replicates, a total of 30 oyster spats were measured for each replicate). (*) indicates significant difference. Blue: UTS Treatment, Grey: DPI Treatment

392 **DISCUSSION**

393 ***Effect of altered light & CO_2 parameters on T. lutea***

394 Light intensity is a driving factor in microalgal production, as it is linked to photosynthesis in
395 photoautotrophic cultures (Dubinsky et al., 1995). In this study, the higher light intensity
396 significantly increased cell density (Fig. 2). This is consistent with reports that showed
397 increased light enhances photosynthetic efficiency and, in turn, increases biomass (Hoshino
398 et al., 1991; Wahidin et al., 2013a). The final mean cell density on day 12 indicated
399 significantly higher density of $6.92x10^6$ cells/mL in UTS treatment compared to $4.40x10^6$
400 cells/mL in DPI treatment. This is likely due to the significantly higher light intensity emitted
401 by LEDs at the time of inoculation (Fig. 1a) where the intensity of light was 2-fold higher at
402 317 photons compared to fluorescent lights emitting 115 photons. Several reports have
403 demonstrated that increased growth rate corresponds with increased light intensity; however
404 only until saturation point is reached (Cheng and He, 2014; del Pilar Sánchez-Saavedra et al.,
405 2016; Li et al., 2008; Sforza et al., 2012; Wahidin et al., 2013b).

406

407 Another explanation for the increased growth rate of *T. lutea* in UTS treatment (Fig. 2)
408 compared to conventional DPI settings is the light distribution. As light radiates, it passes
409 through three zones within a PBR, strong illumination zone, weak illumination zone and dark
410 zone (Huang et al., 2017). The distribution of light varied greatly between the two treatments,
411 the spread of light in the UTS treatment allowed all regions within the PBR to be exposed to
412 light, instead of being limited to the back third demonstrated in current industry (DPI) PBR
413 systems. It is well known that the intensity of light decreases exponentially the further it is
414 from the light source. Hence, by increasing the illumination zone by multiple light sources,
415 we reduce the area of dark zone and increase cell growth rate.

It is important to note that heterogeneous light distribution is commonly associated with algal cultures grown in enclosed PBRs (Sforza et al., 2012). Particularly during linear growth and the stationary phases where cell density is high and the effect of shadowing and scattering of light is enhanced. However, in the presence of high light intensity, shading is significantly reduced and should result in beneficial effects in the linear and late exponential growth phase. In this case, cells exposed to the LEDs (UTS Treatment; Figure 1a) experienced faster growth relative to the cells exposed to fluorescent lighting (DPI Treatment). With the minimisation of shading potentially being one contributing factor to increased density of *T. lutea* cells obtained in UTS PBR system (Fig. 2). This could also be explained by the difference in light spectrum with the relative photon flux being significantly different for the two light systems (supplementary Figure S1), especially within the blue range (425–450 nm) and red range (600–700 nm), which are wavelengths of visible light most commonly used for photosynthesis (Wang et al., 2018).

Results also emphasised the significant interaction between light and carbon dioxide. Elevated pH occurs as a result of increased photosynthetic efficiency. This was observed in this study where elevated pH levels (Fig. 1b) were associated with decreased DIC (Fig. 1c) and high cell density (Fig. 2). Optimum cell growth has been shown to occur within pH 7.9-8.2, depending on the algal strain studied (Rolton et al, 2020). When the pH is above optimum levels, the transport of CO_2 to shift carbon dissolution in the water is heightened and the driving force in maintaining optimum growth medium (Scherholz and Curtis, 2013). In this study, the pH of *T. lutea* cultures was likely sub-optimal (>8.2) for most of the experimental period for both DPI and UTS PBR systems, as indicated by the lack of exponential growth. This was further evidenced on day 6, when additional CO_2 was added, DIC and TA (Fig. 1c & 1d) increased immediately and the pH steadily dropped (Fig. 1b).

441 Therefore, the increase in CO_2 supplemented the higher light intensity provided by the LEDs
442 (UTS treatment) to maintain a pH within similar levels to that of the DPI treatment. Hence, it
443 is likely that if only 1 airline was used, the pH would have exceeded optimum pH for cell
444 growth and would have negatively impacted cell growth. It is evident that DIC was being
445 taken up at a relatively similar rate between treatments as a result of the increased CO_2
446 provided by the second airline, in turn promoting enhanced photosynthetic conversion and
447 increasing the demand for CO_2 to photosynthesis and increase cell density. Microalgae
448 growth in mass cultures are known to be enhanced by optimum levels of CO_2, and these
449 results support this (Chen et al., 2011). In this study, because there was no dilution rate for
450 any of the PBR systems (DPI and UTS), the algal culture can be defined as static or batch
451 culture system. As the culture had unrestricted access to DIC after day 6 due to the increase
452 in CO_2 (Fig. 1c), the photosynthetic rate was relatively undisturbed and exponential growth
453 sustained, particularly in UTS treatment (Fig. 1c). The increase in DIC after the increase in
454 CO_2 (2% - 2.5% air mix) on day 6 highlighted that the initial few days of growth were CO_2
455 limited. The overall aeration of cultures with CO_2 assisted the control of pH by improving the
456 saturation of CO_2, increasing growth rate and supporting elevated cell density (Sun and
457 Wang, 2009).

458

459 ***Biochemical composition***

460 The accumulation of organic matter in relation to photosynthetic response are influenced by
461 the photochemical pigments, light intensity and carbon dioxide concentration (Muller-Feuga
462 et al., 2003). Kromkamp (1987) proposed that regulation of both abiotic factors should have a
463 positive feedback effect on the biochemical profile of microalgae.

464 Lipids account for 4-70% of total biomass, and of that PUFAs typically account for 30-50%
465 of the total lipid content (Jiang and Chen, 2000, Huerlimann et al., 2010)). *T. lutea* is known
466 to produce a high quantity of storage lipids and DHA (Garnier et al., 2016). In-depth
467 assessment of fatty acids through FAMEs profiling showed no significant difference between
468 PUFAs; DHA, EPA and AA in particular (Fig. 3). It has been found that EPA content was
469 highest in biomass during periods of high CO_2 concentrations with cultures of another
470 microalgae *Nannochloropsis oculata*, highlighting the potential for CO_2 regulation to
471 possibly further improve the EPA content within *T. lutea* cultures at a large scale (Shene et al.
472 2016).

473

474 Analysis of PUFAs was further assessed in *S. glomerata* spat fed with *T. lutea* grown under
475 the two culture conditions (DPI vs UTS treatment). Similarities were found in FAMEs
476 analysis between PUFAs omega-3 content in microalgae and oysters. EPA content was
477 significantly higher in oyster spat fed *T. lutea* from UTS treatment (Fig. 5). A similar trend
478 was also discovered in the AA levels, whereby significantly higher AA content was found in
479 oyster spat fed DPI *T. lutea* culture, further correlating with the slightly higher AA levels
480 found in *T. lutea* DPI culture (Fig. 3). Da Costa *et al.* (2016) found a positive correlation
481 between increased EPA levels in *T. lutea* and oyster growth, whist negative correlation with
482 increased AA levels (Da Costa et al., 2016). Clearly, there is a correlation between the
483 biochemical profile of *T. lutea*, and the biochemical content of *S. glomerata* fed with this
484 microalgal culture, highlighting the potential of improving oyster growth (Da Costa *et al.*
485 2016). Little research however has been done to support these results, in turn further
486 examination is required.

487

488 ***Response of oyster growth to altered growth of T. lutea***

489 A goal of this study was to determine if feeding oyster spat with *T. lutea* grown under the
490 optimized conditions would affect their overall growth. This study found no significant
491 difference in the growth or fatty acid content of oyster spat fed with the different *T. lutea*
492 cultures (DPI vs UTS). Previous research shows that the quantity and quality of lipids can be
493 critical, as it strongly influences bivalve larvae survival and growth (Delaunay et al., 1993;
494 Powell et al., 2002). The in-depth assessment of lipids, particularly PUFAs are known to be
495 essential in larvae (Brown, 2002) and numerous studies have recognised DHA as a greater
496 importance compared to EPA. This likely due to the integral role DHA plays in the structural
497 maintenance and functioning of cells (Delaunay et al., 1993; Marchetti et al., 2018), EPA
498 simply being an energy source. While the treatment used to grow *T. lutea* had a significant
499 effect on EPA and AA content in oyster spat (Fig. 5, $p < 0.05$), we did not observe any
500 significant difference for DHA content. This quite possibly coincided with the non-significant
501 difference observed in spat growth (Fig. 4). This is an encouraging result, given the UTS
502 treatment almost doubled algal cell density without an impact on the health and growth of
503 spat. While similar algal feeding trials have been conducted on oyster spat, this is the first
504 study to combine feeding trials with the manipulation of cultivation processes at large-scale
505 to alter *T. lutea* biochemical profile.

506

507 ***Fluorescent lights vs LEDs***

508 For many years, fluorescent lights were integral to many conventional microalgae cultivation
509 systems, but this is changing as LEDs are being more widely adopted, frequently on the basis
510 of their compartive energy efficiency. This study has shown an optimized and more efficient
511 LED system can also increase the quantity and quality of the *T. lutea* yield.

512

513 Although LEDs have a higher acquisition cost (Nardelli et al., 2017), they also have a longer
514 lifespan of approximately 40, 000 operational hours, over twice that of fluorescent lights with
515 18, 000 operational hours (Heffernan & Frater, 2007), which over the long term would
516 account for any cost disparity. Specifically, in our study the LED lights used in the UTS
517 system had a lifespan of 50,000 operational hours compared to 20,000 operational hours in
518 the case of the conventional fluorescent lights used for the DPI system. Further, life cycle
519 assessments have also highlighted a reduction in greenhouse gas emission and cumulative
520 energy requirements by 41-50%, through the use of LEDs instead of fluorescent lights
521 (Principi and Fioretti, 2014). In this study, the wattage of the two systems (36 watts for each
522 light of the DPI system compared to 24 watts for each lights of the UTS system) indicates an
523 energy saving of approximately 50,000 watts per year for the UTS PBR system compared to
524 DPI PBR system. In addition, LEDs are readily adjusted for optimum light intensity
525 throughout all stages of algal growth and their energy efficiency reduces the cost required for
526 temperature maintenance arising from conventional fluorescent lights. Collectively, these
527 advantages can significantly reduce the operational costs in oyster hatcheries.

528

529 **CONCLUSION**

530 Overall this study found that culture conditions can be manipulated in large-scale
531 photobioreactors to optimize the quality and quantity of microalgae yield, in turn producing a
532 cost- and energy-efficient algal feed for juvenile oysters. The difference between the response
533 of *T. lutea* to increased light and CO_2 conditions in UTS treatment (i) resulted in significant
534 differences in algal growth and to a smaller extent in their biochemical composition and (ii)
535 had no negative side effects on the production of *S. glomerata* spat fed with the same

536 microalgal culture. These results highlight the potential of LEDs not only to increase algal
537 yield, but also to provide the oyster industry with reduced hatchery operating costs.

538

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737 **Figure Legends**

738 **Fig. 1** Mean light intensity expressed as μmol.m^{-2}.s^{-1} (± SE)(n=3) over 12-day growth
739 period. Measurements taken from the centre of the PBR at 30 cm depth. (*) indicate
740 significant results ($p < 0.05$). (Blue line – UTS treatment, Black line – DPI treatment)

741

742 **Fig. 2** Mean pH concentrations (± SE) over 12-day experimental period (n=3). (Blue line:
743 UTS treatment, Black line: DPI treatment)

744

745 **Fig. 3** Dissolved inorganic carbon samples expressed as μmol (± SE, n=3) from DPI (Black
746 line) and UTS (Blue line) treatments over 12-day growth period. On day 6 CO_2 change from
747 2 to 2.5%. (*) indicate significant results ($p < 0.05$).

748

749 **Fig. 4** Total alkalinity samples expressed as μmol (± SE, n=3) from DPI (Black line) and
750 UTS (Blue line) treatments over 12-day growth period. (*) indicate significant results ($p <$
751 0.05). On day 6 CO_2 input was changed from 2 to 2.5%.

752

753 **Fig. 5** Mean values (±SE) of T. lutea cell density expressed as cells per mL under different
754 light and CO_2 conditions, UTS (n=3), DPI (n=3). (*) indicate significant results ($p < 0.05$).
755 (Blue line: UTS Treatment, Black line: DPI Treatment)

756

757 **Fig. 6** Measured FAMEs analysis from both treatments obtained from day 12 of growth.
758 Focusing on fatty acids, Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and

759 Arachidonic acid (AA) expressed as µg/mg dry biomass (± SE, n= 9). Blue: UTS Treatment,
760 Grey: DPI Treatment

761

762

763 **Fig. 7** Growth of Saccostrea glomerata spat over 9 days, growth examined by length (mm)
764 LEFT, and weight (g) RIGHT, (±SE, n=150). Blue: UTS Treatment, Grey: DPI Treatment.

765

766 **Fig. 8** Measured FAMEs analysis from both treatments obtained from day 9 of S. glomerata
767 growth. Focusing on fatty acids, Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA)
768 and Arachidonic acid (AA) expressed as µg/mg dry biomass (± SE, n= 5). (*) indicates
769 significant difference. Blue: UTS Treatment, Grey: DPI Treatment