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Towards a *Phaeodactylum tricornutum* biorefinery in an outdoor UK environment

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Abstract

A series of commercial powdered media (Cell-Hi F2P, JWP and WP) and a hydroponics medium (FloraMicroBloom) were investigated for the cultivation of *P. tricornutum*, and compared with f/2 (a commonly employed laboratory cultivation medium; costlier to scale). Cell-Hi JWP showed good performance characteristics including cost-effectiveness. Outdoor cultivation of *P. tricornutum* in an airlift photobioreactor, using Cell-Hi JWP in the United Kingdom (UK) during September and October (average daily temperature ranging between 8-18°C and natural sunlight) was comparable to cultivation indoors under controlled temperature and lighting. A strong positive correlation between fucoxanthin and chlorophyll *a* content, and a weak inverse correlation between eicosapentaenoic (EPA)

content and temperature were observed. Commensal bacterial counts revealed a sinusoidal growth profile with a change in community dominance from *Halomonas sp.* to *Marinobacter* sp. This investigation reveals for the first time that a multi-product approach can be adopted with *P. tricornutum* in a UK outdoor environment using commercially viable powdered media.

Keywords: microalgae; *Phaeodactylum*, outdoor cultivation, airlift, photobioreactor, biorefinery

1.1 Introduction

The five most important microalgae in terms of annual biomass production (>29 tons per annum valued at >US $650 million) are Spirulina *(Arthrospira), Chlorella, Dunaliella, Haematococcus* and *Nannochloropsis* with biomass selling prices of US $13-120 kg^{-1} (Olaizola & Grewe, 2019). It has previously been shown that biomass productivities up to 146 tons dry cell weight (dcw) ha^{-1} y^{-1} in small scale cultivations and 60–75 tons dcw ha^{-1} y^{-1} in mass cultivations are possible (Sethi et al., 2020). It is important in the Microalgae Biotechnology sector to develop novel species/strains and production platforms.

Phaeodactylum tricornutum is a model diatom cultivated industrially for high value products; fucoxanthin (AlgaTechnologies, Israel) and eicosapenatenoic acid (EPA) (Simris, Sweden). *P. tricornutum* is a potential biorefinery chassis which can be exploited for a range of natural

products with market potential including high value products such as fucoxanthin (US $175 kg^{-1} for biomass containing 1 % fucoxanthin, US $0.20-0.74 in capsular/softgel form) and lower value products; EPA (US $200-500 kg^{-1})/chrysolaminarin) for nutraceuticals and protein for animal and aquaculture feed, via sequential extraction (Butler et al., 2020; Butler, 2021). It is a commercially viable species, and is cultivated industrially by at least eight companies in Europe (eicosapentaenoic acid, whole cell biomass for aquafeed, and extracts for cosmetics) with an estimated annual production of four tonnes of dry biomass (Araújo & García-tasende, 2021). *P. tricornutum* dominates and often outcompetes other microalgal species in mixed cultures and is able to tolerate high pH/light intensities and can also grow under low light (Huete-Ortega et al., 2018; Butler et al., 2020). It is robust at laboratory, pilot and demonstration scale, and can be cultivated in a range of cultivation media (Walnes, f/2 and COMBO), enriched seawater and fertiliser media without the requirement for silica (Branco-Vieira et al., 2020; Sethi et al., 2020).

P. tricornutum can be cultivated indoors and outdoors in a range of photobioreactors (PBRs), including tubular, flat-plate, and bubble-columns, and open (raceway) ponds with biomass and product yields described in Butler et al. (2020). The biomass productivity of *P. tricornutum* has been reported to range from 0.03-1.7 g L^{-1} d^{-1} (Fernández et al., 1998; Veronesi et

al., 2015). To date the highest biomass concentration (25.4 g L^{-1}), productivity (1.7 g L^{-1} d^{-1}) and EPA productivity (56 mg L^{-1} d^{-1}) were obtained in an outdoor split-cylinder airlift PBR (60 L) in Almeria, Spain utilising mixotrophic cultivation with 0.1 M glycerol using *P. tricornutum* UTEX 640 (Fernández Sevilla et al., 2004). Comparatively, the highest oil (TAG) yield (58.5 mg L^{-1} d^{-1}) in the same strain was attained in flat-panel PBRs (Green Wall Panel III) using photoautotrophic conditions (up to 45 % TAG dry weight) (Rodolfi et al., 2017). The highest fucoxanthin productivity (4.7 mg L^{-1} d^{-1}) was also obtained in a flat-plate system using photoautotrophic conditions (Baoyan et al., 2017). Flat-panel configurations have been shown to be optimal for biomass, EPA and fucoxanthin productivities attributable to their low shear stress and effective illumination (Guler et al., 2019), but are nevertheless constrained by high rates of biofouling and the resultant difficulty to clean (Lizzul, 2016). To date the majority of studies on outdoor productivity have been performed in temperate countries such as Spain and Italy, with high irradiances resulting in high growth. Only limited studies are available from higher latitudes where microalgae are grown in cold climates with lower irradiances and photoperiods, for example from Norway (Steinrücken et al., 2018). Interestingly, low light appears to favour the accumulation of fucoxanthin and EPA (Gómez-Loredo et al., 2016; McClure et al., 2018), whereas higher

light intensities favour storage compounds (carbohydrate and TAG) with the degradation of the carbohydrate chrysolaminarin in the dark under N-limitation (Wagner et al., 2016).

PBRs result in higher productivities and more hygienic processes than raceway cultivation and can provide (to some degree) a physical barrier against contamination and grazers (Chiaramonti et al., 2013), but they are cost prohibitive for most microalgal production facilities. The cost of a hypothetical large scale microalgal production is estimated to be in the region of €3-10 kg^{-1} DW in PBRs (biorefinery at 100 hectare scale in the south of Spain) compared with €0.3-1.8 kg^{-1} in open raceway systems (Slade & Bauen, 2013; Ruiz et al., 2016). However, no such facility exists. The cost of production in aquaculture hatcheries is €329 kg^{-1} (25 m^2 scale) in a greenhouse setting and decreases to €43 kg^{-1} through the use of artificial illumination at 1500 m^2 scale (Oostlander et al., 2020) which is a more realistic cost of microalgal cultivation.

High productivities of microalgal cells require the formulation of suitable culture media, since standard media typically result in low productivities. The development of a standardised, optimal cell medium is of paramount importance because deviations can induce alterations in cell growth and product formation. The preparation of cell culture media is typically complex (requiring chemical compatibility), expensive (labour,

106 sterilisation, water purification, mixing, chemical storage), requires
107 refrigeration, and is time consuming. In industrial scale operations, the
108 large number of process steps and the numerous components required for
109 media formulation can lead to reduced efficiency and increased costs in
110 conjunction with the potential introduction of variability which can impact
111 the growth of the cells; care has to be taken when adding components to
112 enable dissolution and avoid precipitation Butler, 2021).

113 Dry media powder formulation is desirable to ensure a standardised
114 product which does not vary from batch to batch and does not affect cell
115 growth or composition. These can be cost-effective as they decrease
116 preparation time, simplify the workflow, and reduce the complexity of the
117 media preparation, all of which are important in a commercial setting.

118 Dry powdered media have traditionally been manufactured using
119 ball-milling technology and the constituents are simultaneously crushed and
120 mixed under controlled temperatures and humidity whilst avoiding
121 contaminating dust (Jayme et al., 2002). The Varicon range of Cell-Hi dry
122 powder preparations (F2P, WP, and JWP) are available as cost-effective
123 solutions. To date Cell-Hi F2P has been used in at least 24 studies for
124 cultivation of microalgae in the published literature with concentrations
125 ranging from 0.1 to 0.5 g L^{-1} (Butler, 2021). Cell-HI WP has been used in
126 at least 8 publications with a concentration ranging from 0.1 to 0.67 L^{-1}

(Butler, 2021). The JWP range has not been tested for microalgal cultivation in the published literature and to date no study has evaluated the performance of these powdered media formulations with standardised liquid media commonly used for microalgae in terms of growth and biochemical analysis. It has been found that media can have a dramatic effect on the growth and biochemical composition of microalgae (Butler et al., 2017; Praba et al., 2016) and this warrants investigation.

In the current study the aim was to investigate parameters important for the development of a *P. tricornutum* biorefinery. The first aim of this work was to determine the optimal cost-effective medium for obtaining high biomass and product (fucoxanthin, EPA, protein, carbohydrate and total fatty acid - TFA) productivities towards a biorefinery approach for the model strain *P. tricornutum* CCAP 1055/1. The next step was to cultivate *P. tricornutum* CCAP 1055/1 outdoors (under natural light and temperatures) in a prototype airlift photobioreactor, comparing it with cultivation indoors (under controlled lighting and temperature) to investigate the effect of fluctuations in temperature and light on biochemical composition and evaluate the potential of multiple products outdoors for potential industrial exploitation. The effect of cultivation time, light, and temperature on biochemical composition was investigated. The commensal

147 bacterial population was monitored and compared in the airlift PBR indoors
148 and outdoors.

149 **1.2 Materials and methods**

150 *1.2.1 Phaeodactylum tricornutum culture and routine maintenance*

151 *P. tricornutum* CCAP 1055/1 stock cultures were routinely
152 maintained as detailed within the literature (Butler et al., 2021) but with
153 white lights ca. 150 µmol photons m^{-2} s^{-1} surface irradiance (2700 k Hansa
154 ECO Star silver lamps) with cultivation in f/2 medium (0.882 mM nitrate
155 and 0.036 mM phosphate).

156 *1.2.2 Shake flask experimental approach for powdered media performance*
157 *testing*

158 Powdered media formulations Cell-Hi F2P, WP and JWP were
159 prepared from stock solutions to a final concentration of 0.1, 0.15, and 0.1 g
160 L^{-1} as recommended by the manufacturer (Butler, 2021). A pre-optimised
161 FloraSeries Hydroponic fertiliser medium (GHE, Fleurance, France) [(2 mL
162 L^{-1} FloraMicro (M) and 1 mL L^{-1} FloraBloom (B)] (M2B1) was also tested
163 for comparison, along with f/2 medium (sterile and non-sterile) which have
164 both been adopted for microalgae cultivation (Gómez-Loredo et al., 2016;
165 Butler et al., 2017; Song et al., 2020; Butler, 2021). Each culture was pre-
166 acclimatised in the respective medium for one week before experimentation
167 (preliminary experimentation). The cultures (150 mL in 250 mL flasks)

were incubated at 21°C (Series 4, LMS incubator, UK) and agitated at 120 rpm using a Stuart reciprocating table shaker (SSL2, UK) under continuous light at 142 ± 33 µmol photons m^{-2} s^{-1} (2700 k Hansa ECO Star silver lamps) for 7 days. Growth was monitored daily by measuring cell count and biomass concentration (determined by dry weight) (Butler, 2021). The maximum growth rate (µ) was calculated according to Butler et al. (2017). Except for the control (f/2 medium) all media formulations were made up with tap water, under non-sterile conditions to compare the microbial community profile.

1.2.3 Indoor/outdoor cultivation in a prototype airlift photobioreactor

A proprietary demonstration scale 10 L airlift glass tubular photobioreactor (ALR) PhycoLift (8 L working volume) similar to that previously described (Lizzul, 2016; Butler, 2021) was supplied by Varicon Aqua Solutions Ltd. and setup at The University of Sheffield for outdoor cultivation in a greenhouse without temperature control and utilising only natural sunlight for illumination. Details about the reactor configuration can be found in Butler (2021).

For the indoor trial the PBR was illuminated with dimmed light emitting diode (LED) lamps (4000K, 10 W, BD03, RoHS) at 143 µmol photons m^{-2} s^{-1} for the first 7 days and increased to 221 µmol photons m^{-2} s^{-1} (12 h light:dark) (representative of the outdoor PBR) until day 15. The

189 temperature was controlled at 22 ± 1°C. The PBR was aerated at 5 L min^{-1}
190 (0.625 vvm) using a 20 W ACO-308 air compressor (Hailea, China) and
191 controlled through flow gauges and connected using 8 mm polypropylene
192 tubing. The pH was controlled on demand at pH 7.8 which ensured >95 %
193 of the carbonic species were in the form of bicarbonate. The average DIC
194 concentration after 1 d was 3327 and 2847 µmol kg^{-1} for the indoor and
195 outdoor PBR respectively (Butler, 2021).

196 *P. tricornutum* cells were obtained from an inoculum pre-
197 acclimatised in Cell-Hi JWP (Butler, 2021). Cell-Hi JWP (0.4 g L^{-1}) was
198 added at two time points: 0 and 7 d to avoid nutrient limitation. On day 15,
199 the reactor was harvested (estimated biomass concentration of 0.1-0.2 g L^{-1})
200 for the repeated batch run and supplied with fresh JWP medium.

201 Light impinging on the reactor surface was monitored using a digital
202 lux meter MM-LM01 (Max Measure, UK) and logged every 5 mins
203 (LuxMeter Communication Tool). The daily total irradiance (mol photons
204 m^{-2} d^{-1}) was obtained from the sum of the recorded light intensity (µmol
205 photons m^{-2} s^{-1}). PBR temperature was monitored every 30 mins using an
206 RC-4 temperature logger (Ellitech, UK).

207 A sample was taken daily at the same time (11 am) for OD_{750}, cell
208 count, DW, dissolved inorganic nitrate (DIN) and dissolved inorganic
209 phosphate (DIP). Samples for bacterial enumeration (1 mL) were taken

every other day. Samples for biochemical composition determination (5 mL triplicate aliquots) were taken every 2 days for the indoor and outdoor system and lyophilised. All methods were previously described in full in Butler (2021).

1.2.4 Commensal bacterial isolation, identification, enumeration, and growth experiments

Commensal, cultivable bacteria from the Cell-Hi media screen experimental cultures, and the indoor/outdoor PBRs were isolated by streaking out 100 µL culture to form single colonies on a 'modified marine agar medium' with the growth medium supplemented with 33 g L^{-1} Instant Ocean, 5 g L^{-1} peptone (Sigma, UK), 1 g L^{-1} yeast extract (Sigma, UK) and 15 g L^{-1} agar. Isolated cultures were identified by 16S rRNA molecular typing (Butler, 2021).

Growth (OD_{600}) experiments with bacterial isolates in monocultures were performed in 24-well microtiter plates (Corning® Costar) with f/2 marine broth (modified marine agar without agar) (Butler, 2021). Fatty acid methyl ester (FAME) analysis of the bacteria was conducted using a modified version of the protocol described elsewhere (Kapoore et al., 2019). Briefly, 5 mL of the frozen (-20ºC) wet biomass culture was directly transesterified with methanolic-HCl (7%) replacing BF3 as the acid catalyst. In addition, a bacterial biofilm and the *P. tricornutum* culture obtained from

231 the outside PBR run 2 after 15 d were analysed to determine the FAME
232 profiles.

233 *1.2.5 Analytical methods*

234 Dissolved inorganic nitrogen (DIN) in the media was determined at
235 OD_{220} nm and dissolved inorganic phosphate (DIP) in the media was
236 determined at OD_{885} (Kapoore et al., 2019; Butler, 2021). Combined
237 extraction of chlorophyll *a*, carbohydrate, and protein (biochemical
238 composition) was carried out according to Chen & Vaidyanathan (2013) and
239 Butler (2021) using lyophilised biomass.

240 FAME analysis was conducted as above using direct
241 transesterification but using dry biomass (Butler, 2021). Fucoxanthin
242 content was determined using the spectrophotometric method of Wang et al.
243 (2018) ensuring the biomass was washed with MilliQ water before analysis
244 for maximum fucoxanthin recovery.

245 *1.2.6 Scanning electron micrograph imaging of biofilm*

246 The biofilm was spread evenly on a glass slide and allowed to dry in
247 a laminar flow. Fixed cells were examined using a JSM-6010LA
248 InTouchScope™ Multiple Touch Scanning Electron Microscope (JEOL
249 Ltd., Japan) at an accelerating voltage of 15Kv (Butler, 2021).

1.2.7 Statistical analysis

All statistics were conducted as detailed elsewhere (Butler et al., 2021), unless stated otherwise. Statistical analysis of the experimental data was conducted using SPSS statistical software (SPSS Statistics 28, IBM). The data was tested for normality using a Shapiro-Wilk test and if these data were normally distributed ($P > 0.05$) they were subsequently tested for equal variance using Levene's test. A one-way/two-way ANOVA and a post-hoc Tukey's test was utilised to understand where the differences were. If samples were not normally distributed ($P < 0.05$) or equal variance was not observed ($P < 0.05$), then a Kruskal-Wallis and post-hoc Dunn's non-parametric comparison was undertaken to understand the differences.

1.3 Results and discussion

1.3.1 Evaluation of powdered Cell-Hi range culture media

A maximum cell density (1.69×10^7 cells mL^{-1}) and biomass concentration (0.45 g L^{-1} DW) was observed with Cell-Hi JWP after 7 d cultivation. The maximum specific growth rate ($\mu = 1.27$) was highest in Cell-Hi JWP medium (1.9-fold higher than f/2) but was not significantly higher with Cell Hi-JWP. Comparatively, the biomass productivity (0.08 g L^{-1} d^{-1}) was significantly higher (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, H=11.87, df=5, $P<0.05$) for Cell-Hi JWP compared to the other media, with a 1.3-fold increase compared with f/2 (Figure 1A,

B). The biomass concentration attained after 7 days with f/2 medium (0.34 g L^{-1} DW) was similar to that reported elsewhere (Penhaul Smith et al., 2020). The EPA (3.31 % DW) and fucoxanthin (1.33 % DW) contents obtained with Cell-Hi JWP were significantly higher than with f/2 (2.10 and 0.74 % DW respectively) (ANOVA and *post hoc* Tukey's test, F=3.57, $P<0.05$, df= 12) (ANOVA and *post hoc* Tukey's test, F=22.77, $P<0.001$, df= 12) (Figure 1D). The protein content (26.87 % DW) was 1.71-fold higher than f/2 medium (Figure 1E).

The effectiveness of the nitrate and phosphate uptake per unit biomass were compared, as their content in the media differed (see supplementary material). The nitrate and phosphate uptake per unit biomass (both average and maximum) for nitrate (2.16 and 2.56 mM g^{-1} respectively) and phosphate (0.49 and 0.57 mM g^{-1} respectively) were the lowest for the JWP medium (Figure 1B and C). The highest product yields and productivities were also observed with Cell-Hi JWP which were significantly higher than the other media ($P<0.05$) (Table 1). It is possible that the higher phosphate (0.1 mM), magnesium (138.2 mM), sulphur (138.5 mM), calcium (70.0 mM), manganese (12.6 µM), and molybdenum (2.9 µM) in Cell-Hi JWP contributed to its better performance (see supplementary material). Since the interest was mainly on the production of high value products (fucoxanthin and EPA) and protein from *P.*

292 *tricornutum*, it was thus decided that Cell-Hi JWP was the optimal medium
293 for scale-up.

294 In a commercial setting, agricultural fertilisers typically replace pure
295 chemicals (Acién et al., 2012). When evaluating the cost of a medium two
296 key factors need to be taken into account, the cost of the medium itself and
297 the time taken for preparation. Varicon Aqua's Cell-Hi powders were found
298 to be more economical than the hydroponics medium (FloraMicroBloom)
299 and laboratory media with Cell-Hi F2P being the most economical (£2.56
300 per m^3), followed by Cell-Hi JWP (£2.69 per m^3), both > 2-fold cheaper
301 than laboratory f/2 in terms of media cost alone (Butler, 2021). Overall, due
302 to the medium cost and biomass concentrations attained, the most
303 economical medium for *P. tricornutum* was Cell-Hi JWP (£6.03 per kg dry
304 biomass) compared with £16.88 per kg dry biomass for laboratory f/2.

305 At industrial scale, axenic conditions are nearly impossible to attain,
306 especially in open cultivation systems (Croft et al., 2005; Kazamia et al.,
307 2012). Microalgae live in close association with heterotrophic bacteria
308 which can have synergistic influences (Buhmann et al., 2016; Vuong et al.,
309 2019). Commensal bacterial populations isolated from the culture media
310 employed in the study were therefore characterised to observe dominant
311 associations with *P. tricornutum*. Three bacterial species were identified
312 with *Marinobacter* sp. and *Halomonas* sp. detected in all media but

313 *Algoriphagus* sp. was only detected in f/2 (both sterile and non-sterile) and
314 Cell-Hi F2P. After 7 days of cultivation in each medium, the highest
315 bacterial content was observed in *P. tricornutum* cultivated with M2B1
316 (1.52×10^5 cells mL^{-1}) and the lowest content was observed with Cell-Hi
317 F2P (2.67×10^4 cells mL^{-1}) (see supplementary material). *Halomonas* sp.
318 was dominant (>94 %) on day 1, in all media compositions but on day 4 the
319 composition decreased to <79 % and on day 7 to <50 %, with a subsequent
320 increase in *Marinobacter* sp.. Comparatively, *Algoriphagus* sp. was only
321 observed in f/2 and Cell-Hi F2P throughout the cultivation period but was
322 <2 % of the bacterial composition. The bacteria detected here are similar to
323 those reported in the literature, where in addition to these species other
324 species have been reported; *Muricauda sp.*, *Devosia sp.*, Alcanivorax spp.,
325 *Stappia sp.*and *Isomarina sp.* (Chorazyczewski et al., 2021; Vuong et al.,
326 2019).

327 As Cell-Hi JWP is a cost-effective medium that showed higher
328 biomass productivities and yields/productivities of fucoxanthin, EPA, and
329 protein, and a relatively low bacteria content it was further investigated for
330 comparing growth and biochemical composition in an indoor and outdoor
331 prototype airlift PBR.

332 *1.3.2 Outdoor cultivation in a prototype airlift PBR*

333 *1.3.2.1. Biomass and product yields in outdoor cultivations*

334 During the one-month cultivation there was no relationship observed
335 between irradiance and temperature (Butler, 2021). This was surprising as
336 irradiance and temperature were strongly correlated in Bergen, Norway
337 (Steinrücken et al., 2018). The minimum temperature (daily average)
338 observed during run 1 in September was 8°C, the maximum was 18°C and
339 the overall cultivation period average and median were 13°C (Figure 2,
340 Table 1). There was significant variation in light and temperature during the
341 outdoor cultivation run and also within a given day (14-21°C during the day
342 and as low as 1°C to 14°C at night) (Figure 2). The greatest fluctuation in a
343 24 h period was between 10°C at night and 21°C during the day (Figure 2).
344 In contrast, the indoor temperature and light intensity were controlled (Table
345 1).

346 In the current study *P. tricornutum* CCAP 1055/1 always
347 predominated as the fusiform morphotype (>97 %) with the remainder being
348 a mixture of oval and triradiate (data not shown). CCAP 1055/1 has
349 previously been observed to dominate in the fusiform morphotype (De
350 Martino et al., 2007).

351 The final biomass concentration attained indoors was 1.6 g L^{-1} DW
352 after 15 days, whilst it was lower for the outdoor cultivations (1.13 g L^{-1} and
353 0.93 g L^{-1} for runs 1 and 2, respectively) (Figure 3A). The lower biomass
354 concentration in the second run was likely due to lower light and colder

355 temperatures towards the end of the run. A similar biomass concentration
356 (1.3 g L^{-1}) was achieved in indoor cultivation using a 20 L hanging bag PBR
357 at a similar light intensity and temperature to the current study (120 µmol
358 photons m^{-2} s^{-1} and 23°C) with continuous CO_2 supply at 1 % (Wang et al.,
359 2018).

360 The volumetric biomass productivity indoors (0.10 g L^{-1} d^{-1}) was
361 significantly higher than the final volumetric productivities outdoors for run
362 1 (0.07 g L^{-1} d^{-1}) and run 2 (0.05 g L^{-1} d^{-1}) (Table 1). However, the
363 maximum biomass yield on light was higher for outdoor runs (Table 1).
364 This could be attributed to the higher average light intensity and temperature
365 indoors compared with fluctuating conditions outdoors.

366 Typically, higher biomass productivities have been achieved in
367 southern latitudes, (1.4 g L^{-1} d^{-1} in Spain) (Acién Fernández et al., 2003) and
368 0.43 g L^{-1} d^{-1} in Italy (Rodolfi et al., 2017). However, data in Western
369 Europe is lacking. In The Netherlands, the biomass productivity has been
370 found to range from 0.02-0.27 g L^{-1} d^{-1} cultivated in outdoor flat panel PBRs
371 in October (average of 9.58 mol photons m^{-2} d^{-2}, 200 µmol photons m^{-2} d^{-1},
372 temperature control at 20-22°C), with a higher biomass productivity attained
373 at lower biomass densities (0.4 g L^{-1} DW compared with 1.1 g L^{-1}) (Gao et
374 al., 2020).

375 The lower biomass productivities in our study were most likely
376 attributable to the limited irradiance (average 82-117 µmol photons m^{-2} s^{-1},
377 3-5 mol photons m^{-2} d^{-1}) and temperature (average 12-13 °C) as higher
378 productivities have been observed in spring and summer in The Netherlands
379 and Norway (Steinrücken et al., 2018; Gao et al., 2020). Nitrate and
380 phosphate were not found to be limiting throughout growth (data not shown)
381 but light limitation and photorespiration at night could also have accounted
382 for the lower biomass productivity (Kroth et al., 2008).
383 EPA yield was higher in the outdoor cultivations (3.42 and 3.74 %
384 DW) for run 1 and run 2 respectively compared with indoor cultivation
385 (2.57 % DW), and the same was true for fucoxanthin (1.18 and 1.21 % DW
386 indoors vs 1.15 % DW indoors) but these were not significantly different
387 (Figure 3G, Table 1). The biochemical composition was observed to be
388 more stable in indoor cultivations than in outdoor runs (Butler, 2021).
389 There was no statistically significant difference in fatty acid composition
390 between the indoor and outdoor runs (Butler, 2021).
391 A weak inverse correlation between temperature and EPA was
392 observed (Figure 4A). EPA is known to be accumulated under low
393 temperature and a reduction in temperature to 10°C from 25°C for 12 h has
394 been shown to result in a 120 % increase in EPA yield (Jiang & Gao, 2004).
395 The rapid accumulation of EPA in colder conditions appears to be a

response to maintain membrane fluidity, allowing acclimation to low temperature stress (Jiang & Gao, 2004).

The lower temperatures observed outdoors likely resulted in an increase in EPA. Another interesting finding was that fucoxanthin and chlorophyll *a* were positively correlated (Figure 5B) and this appears to indicate that chlorophyll *a* could be a good indicator and a proxy for cellular fucoxanthin content in the airlift PBR.

1.3.2.2 Commensal bacterial population dynamics

During cultivation no eukaryotic contaminants, such as protozoa or rotifers, were observed outdoors or indoors. The *P. tricornutum* cultures remained unialgal. Unsurprisingly, commensal bacteria were found to be present and the genera present were in agreement with the flask experiments, *Halomonas sp.* and *Marinobacter sp.* being the predominant species (Figure 5). The bacteria numbers were higher in outdoor cultivation compared with indoors and the bacterial populations both indoors and outdoors were less numerous than *P. tricornutum* (Figure 5). Interestingly, the bacteria showed a sinusoidal profile with a population shift occurring between *Halomonas sp.* and *Marinobacter sp.*, both indoors and outdoors.

Further work is required to understand the interactions between bacteria and *P. tricornutum* through whole-transcriptome and metabolome analyses and future work should determine under which conditions bacterial

levels are elevated and suppressed. Further work should also be conducted on determining which bacteria are beneficial for growth and product formation, for example, *Stappia* sp. K01 has been revealed to increase growth, chlorophyll, and fucoxanthin content in *P. tricornutum* (Vuong et al., 2019).

1.3.3 Growth and characterisation of commensal bacteria identified during cultivation

Only dominant and cultivable bacteria were recovered from *P. tricornutum* cultures after conducting spread plates using a modified marine agar medium. The bacteria were all found to grow in co-culture with *P. tricornutum* (without any added organic carbon source) implicating that *Halomonas*, *Marinobacter* and *Algoriphagus* solely utilised diatom-derived carbon. The commensal bacteria also grew on f/2 modified marine agar and in liquid f/2 marine medium when supplemented with peptone and yeast extract but could not grow in f/2 medium or seawater nutrient agar alone. The bacteria were osmotolerant and could be grown on freshwater and seawater modified marine agar (Butler, 2021). As the bacterial strains developed in microalgal cultures without organic carbon supplementation, it is suspected that they were able to grow on organic carbon released by the microalgal cells, indicating interactions between the bacteria and *P. tricornutum*.

Interestingly *Algoriphagus marincola* (red colony) was only observed in f/2 and Cell-Hi F2P in flask studies (albeit low in number) but was not detected indoors or outdoors when using JWP medium. *Halomonas* sp. was a large white colony and *Marinobacter* was a small white colony.

When the growth rates of all three bacterial species were compared, *Halomonas sp.* had the highest growth rate (lowest doubling time) (Figure 6B). The bacteria were all found to have a unique fatty acid profile which could be used as biomarkers for their presence (Figure 6D). The predominant fatty acids for *Halomonas* sp. and *Algoriphagus* sp. were C18:1 (57 and 51 % TFA respectively) which has also been found in the literature (Sánchez-Porro et al., 2010) and C16:0 was dominant in *Marinobacter* sp. In comparison, *P. tricornutum* had only <2 % C18:1. It was also confirmed that only *P. tricornutum* was capable of the synthesis of the long-chain polyunsaturated fatty acids (LC-PUFAs) EPA and DHA.

During the outdoor cultivation a bacterial biofilm was observed within areas of low flow in the PBR. This was surprising, as the total run outside was only 45 days, and this finding had not been reported in earlier pilot scale trials (Borowitzka, 1999; Steinrücken et al., 2018). The biofilm appeared to primarily be composed of *P. tricornutum* with *Halomonas sp.* and *Marinobacter sp.* also being present, which was confirmed by 16 S sequencing, SEM, and fatty acid analysis. The bacteria were observed to

adhere to *P. tricornutum* cells (Figure 6C). The planktonic *P. tricornutum* in suspension culture predominated as the fusiform morphotype but the benthic form predominated as the oval morphotype. Interestingly, both cell types were revealed to have a similar fatty acid profile, however, the fusiform morphotype appeared to have a higher EPA composition of TFAs (Figure 6B, C). This is similar to reported by Desbois et al. (2010) who found that fusiform cells had a higher EPA content than the oval morphotype cells. The transition to the oval morphotype in the benthic stage is likely because only oval morphotypes can adhere strongly to surfaces (Buhmann et al., 2016).

Further work should be conducted on determining the conditions which result in biofouling in PBRs, to prevent production downtime. Further work should also be conducted on understanding the microalgal-bacterial relationships in PBRs to determine which bacteria are 'friend' and 'foe' and if they can be exploited for improved biomanufacturing for the implementation of a biorefinery chassis (Padmaperuma et al., 2018).

1.4 Conclusion

Cell-Hi JWP was the optimal cost-effective medium for cultivating *P. tricornutum* CCAP 1055/1 for a multi-product approach. Outdoor UK cultivation was possible utilising a prototype PhycoLift PBR under natural light and temperature fluctuations comparing favourably with controlled indoor cultivation. A weak inverse correlation between temperature and

EPA content was observed, and a higher EPA content was observed in the outdoor cultivations. Commensal bacteria showed a sinusoidal growth profile. *Halomonas* sp. was dominant at low algal densities but *Marinobacter* sp. was more dominant at higher algal densities. This investigation reveals potential for developing the biorefinery concept towards realisation in an outdoor UK setting.

E-supplementary data for this work can be found in the e-version of this paper online.

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Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the writing of the manuscript. ML and JM from Varicon Aqua Solutions Ltd. supplied the powders and airlift PBR.

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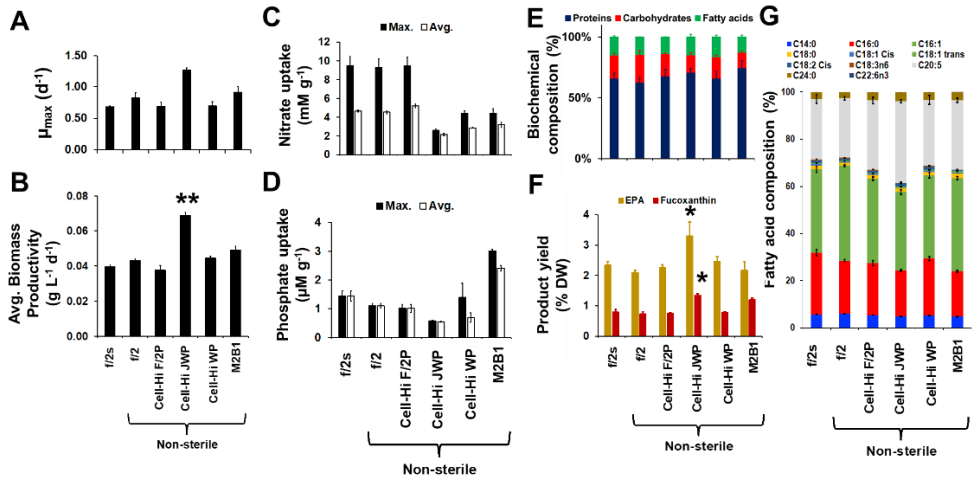

Fig 1. Performance of Varicon Cell-Hi range powders compared with f/2 control (sterile), f/2 (non-sterile) and optimal FloraMicroBloom formulation (M2B1): A) maximum specific growth rate, B) average biomass productivity, C) nitrate uptake, D) phosphate uptake, E) normalised biomass biochemical composition (proteins, carbohydrates and fatty acids), F) product yield (eicosapentaenoic acid (EPA) and fucoxanthin content), and G) fatty acid composition (%). * indicates that the component is significantly greater than f/2 medium and ** significantly greater than all media

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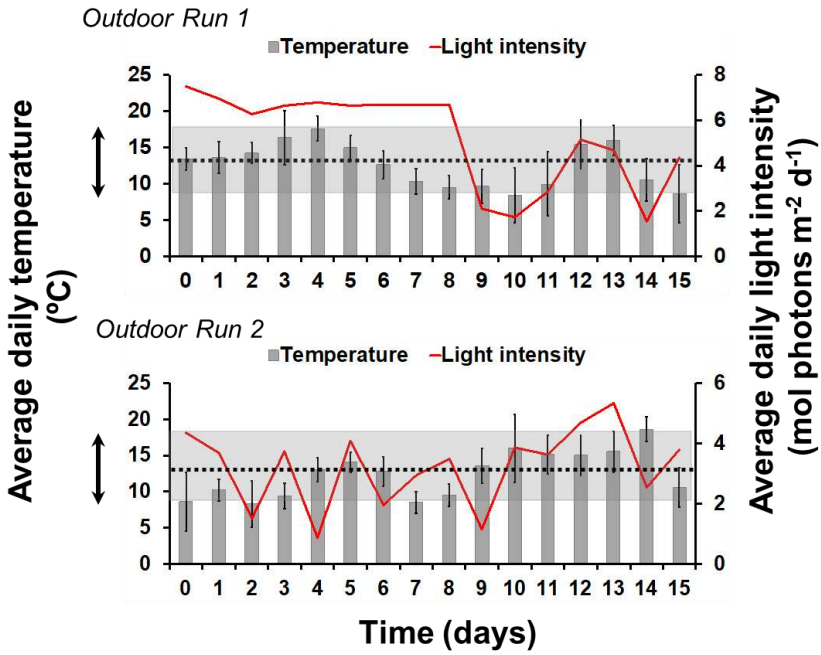

Fig. 2. Outdoor cultivation conditions in 8L PhycoLift PBR. Daily average temperature (ºC) and light intensity (mol photons m^{-2} d^{-1}) profiles in the outdoor cultivation run 1 (September) and run 2 (late-September/October) (the median temperature over the period is indicated for both the runs as a black dotted line with the grey shaded area as well as the arrows on the left of the y-axis indicating the operational temperature range)

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633 **Table 1. Climatic conditions, biomass (specific growth rate and productivity) and biochemical composition of *P. tricornutum* after 15**

634 **d cultivation indoors and outdoors using a fed-batch approach**

			Indoor	Outdoor run 1	Outdoor run 2
Median temperature (°C)			22	13	12
Minimum temperature (°C)			21	2	1
Maximum temperature (°C)			23	21	22
Photoperiod (L:D)			12:12	12:12	12.5:11.5
Mean total daily light (mol photons m^{-2} d^{-1})			6.22 (first 7 days), 9.61 (8-15 d)	5.19 ± 2.07	3.22 ± 1.29
Mean light intensity (µmol photons m^{-2} s^{-1})			143 (first 7 days), 221 (8-15 d)	117 ± 45	82 ± 33
Maximum light intensity (µmol photons m^{-2} s^{-1})			221	298	384
Biomass	**Specific growth rate (d^{-1})**	Average	0.16 ± 0.00	0.14 ± 0.00	0.12 ± 0.00
		Maximum			
	Biomass concentration (g L^{-1} d^{-1})	Final	1.57 ± 0.01	1.13 ± 0.01	0.93 ± 0.01
	Volumetric productivity (g L^{-1} d^{-1})	Final	0.10 ± 0.00	0.07 ± 0.00	0.05 ± 0.00
	Areal productivity (g m^{-2} d^{-1})	Final	4.26 ± 0.01	2.90 ± 0.01	2.34 ± 0.02
	Yield on light (g mol^{-1})	Final	0.54 ± 0.00	0.43 ± 0.01	0.82 ± 0.03

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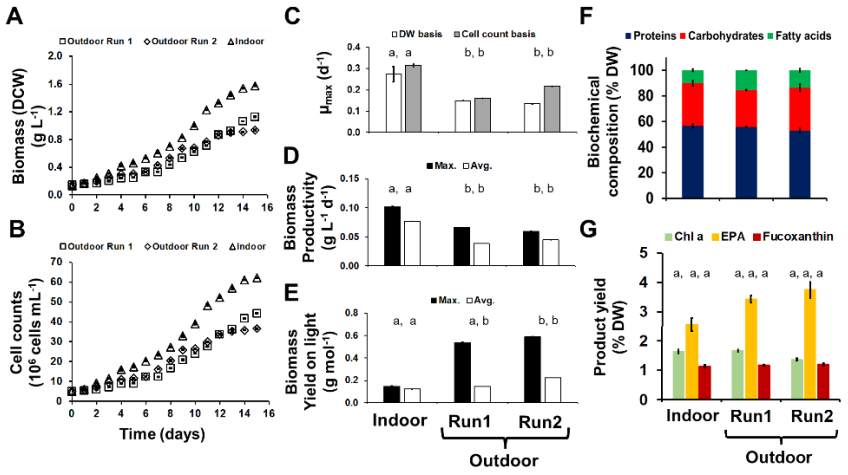

Fig. 3 Performance in outdoor compared to indoor cultivation in 8L PhycoLift bioreactor. Biomass concentration over the cultivation period, as DCW (A) and cell counts (B), the maximum specific growth rate (C), on DW and cell count basis, the maximum and average volumetric biomass productivity over the cultivation period (D), as well as the biomass yield on light supplied (E) are plotted alongside the normalised biomass biochemical composition (proteins, carbohydrates, fatty acids) (F) and product yield on biomass for chlorophyll a, EPA and fucoxanthin (G) for the outdoor, compared to the indoor cultivations. Note: The same letter on each bar indicates that the difference is not significant (P<0.05), and different letters indicate a significant difference (P<0.05)

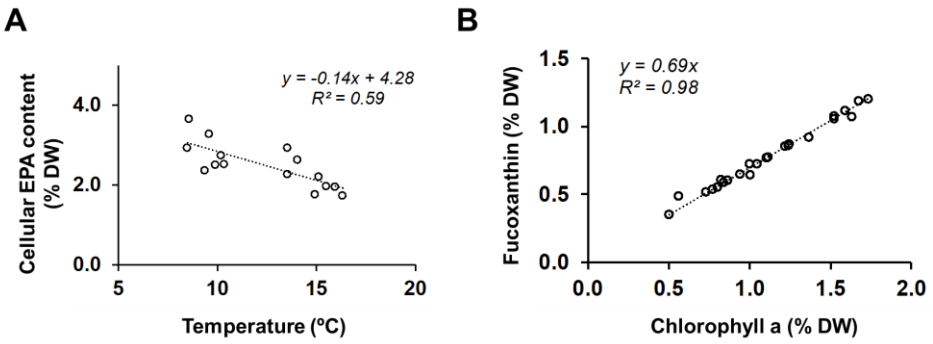

Fig. 4. Relationship between temperature and EPA content (A) in outdoor cultivations, and chlorophyll *a* and fucoxanthin (B), in indoor and outdoor cultivations, over the course of the one month

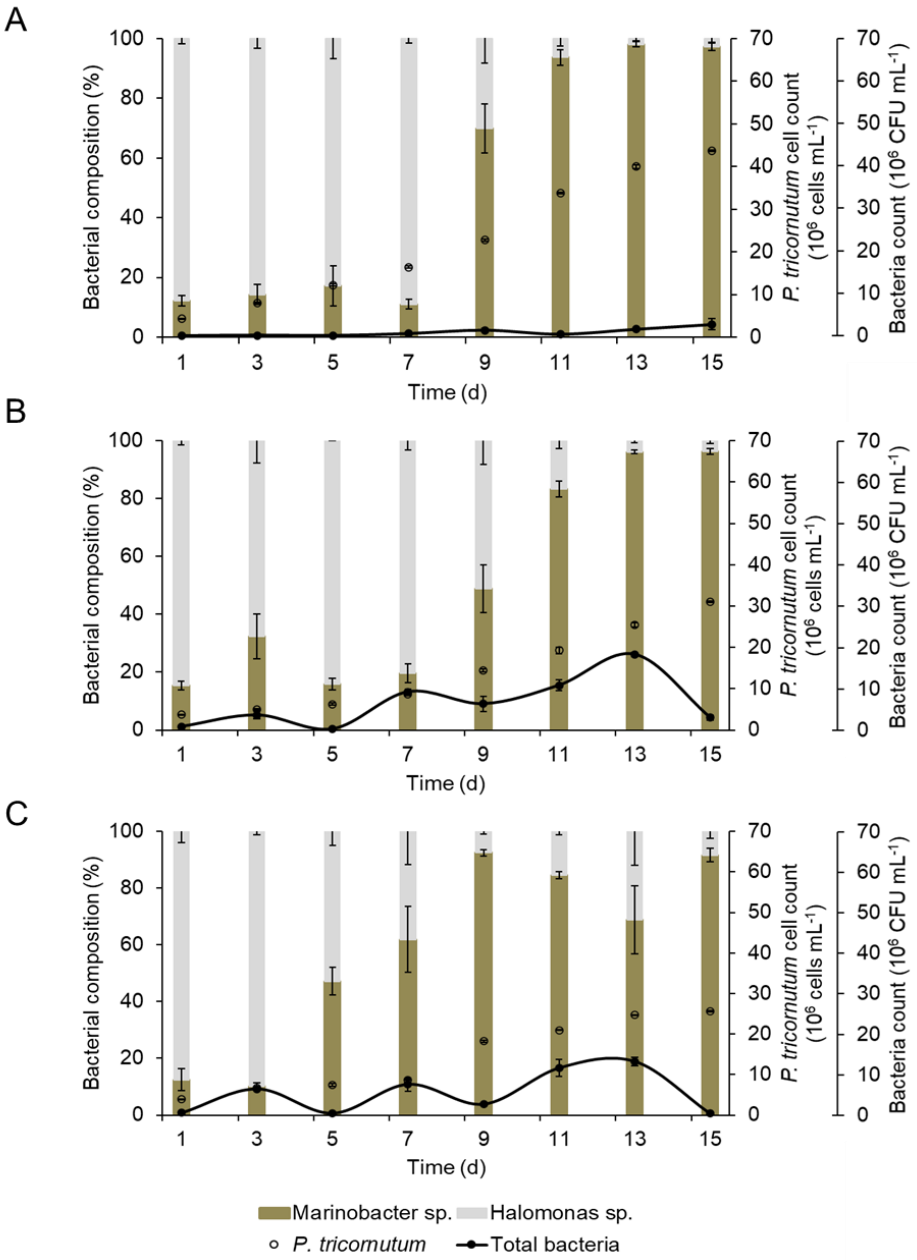

Fig. 5. *P. tricornutum* and bacteria profile variation over 15 d cultivation; a) indoor, b) outdoor run 1 and c) outdoor run 2

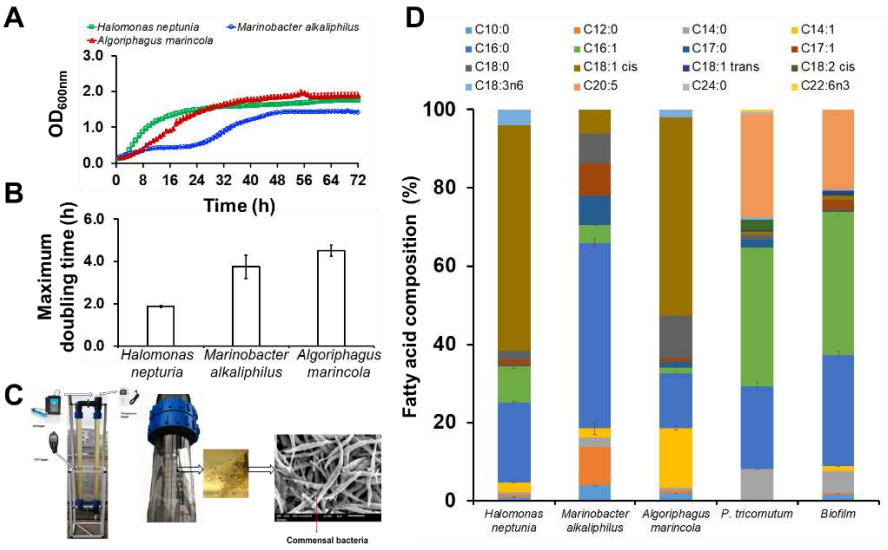

Fig. 6. Characterisation of bacteria detected in outdoor cultivation A) growth of bacteria, B) average doubling time of bacteria, C) fouling biofilm formation on the airlift photobioreactor (ALR) at low flow zones, with scanning electron micrograph (SEM) sample from biofilm with bacteria clearly observed adhering to cells, and D) fatty acid analysis of bacteria, *P. tricornutum* (after 15 d growth outdoor run 2) and biofilm obtained after 15 d growth for outdoor run 2 showcasing commensal bacteria attached to *P. tricornutum*