

Cultivation of the polyextremophile *Cyanidioschyzon merolae* 10D during summer conditions on the coast of the Red Sea and its adaptation to hypersaline sea water

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15

16 Abstract

17 The west coast of Saudi Arabia borders the Red Sea, which maintains high average temperatures and
18 increased salinity compared to other seas or oceans. Summer conditions in the Arabian Peninsula
19 may exceed the temperature tolerance of most currently cultivated microalgae. The Cyanidiales are
20 polyextremophilic red algae whose native habitats are at the edges of acidic hot springs.
21 *Cyanidioschyzon merolae* 10D has recently emerged as an interesting model organism capable of
22 high-cell density cultivation on pure CO₂ with optimal growth at 42 °C and low pH between 0.5-2. *C.*
23 *merolae* biomass has an interesting macromolecular composition, is protein rich, and contains
24 valuable bio-products like heat-stable phycocyanin, carotenoids, β-glucan, and starch. Here,
25 photobioreactors were used to model *C. merolae* 10D growth performance in simulated
26 environmental conditions of the mid-Red Sea coast across four seasons, it was then grown at various
27 scales outdoors in Thuwal, Saudi Arabia during the Summer of 2022. We show that *C. merolae* 10D is
28 amenable to cultivation with industrial-grade nutrient and CO₂ inputs outdoors in this location and
29 that its biomass is relatively constant in biochemical composition across culture conditions. We also
30 show the adaptation of *C. merolae* 10D to high salinity levels of those found in Red Sea waters and
31 conducted further modeled cultivations in nutrient enriched local sea water. It was determined that
32 salt-water adapted *C. merolae* 10D could be cultivated with reduced nutrient inputs in local
33 conditions. The results presented here indicate this may be a promising alternative species for algal
34 bioprocesses in outdoor conditions in extreme desert summer environments.

35

36 **1 Introduction**

37 Currently, bioprocesses that use microalgae are being implemented with increasing intensity in nearly
38 all countries due to their broad potential for low input waste-revalorization. Algae can convert the
39 nutrients and trace elements from waste streams into their biomass using light energy via
40 photosynthesis and CO₂ as a carbon source (Fuentes-Grünewald et al., 2021). There are numerous
41 market opportunities for different products made from algal biomass such as animal feed, food,
42 pigments, bioplastics, speciality chemicals, and biostimulant fertilizers (Rumin et al., 2020). The
43 worldwide production of microalgae biomass is currently low, ~56,000 tons per annum compared to
44 production of marine seaweeds, ~34.6M tons per annum (Ferdouse et al., 2018; Cai et al., 2021).
45 There is currently an opportunity, especially in desert countries, for increased algal production
46 processes aimed at bio-resource circularity, especially connected to urban and industrial areas.

47 Algae can be cultivated in photobioreactors built on non-arable land and directly connected to
48 industrial side-streams to derive value from effluents produced in otherwise overlooked geographical
49 areas such as desert regions or other extreme environments. The implementation of algal
50 bioprocesses in growing contexts will further promote algal biomass and bioproduct integration into
51 common use. Microalgal bioprocesses are most strategically suited to geographies with high solar
52 irradiation and stable temperatures, having local CO₂ emissions sources, non-arable land, access to
53 sea water, and inexpensive energy sources. It was recently reported that the geographical areas with
54 the highest potential for onshore marine microalgae biomass production were between 30°N and
55 30°S in the so-called “hot belt countries” (Greene et al., 2022). This area is dominated by countries
56 with enormous desert regions such as southern US (Arizona), Northern Chile (Atacama), Northern
57 Africa, Northern Australia, and the Middle East. It is predicted that in these environments, microalgal
58 biomass production from local inputs can be maximized. To capture the value of resources in waste-
59 streams, the development and integration of algal bioprocesses can be parts of circular reuse
60 concepts in these environments. New target microalgal species with high biotechnological potential,
61 that can tolerate extreme conditions must be identified, characterized, and developed for these
62 contexts.

63 The unicellular red microalgae *Cyanidioschyzon merolae* is a poly-extremophile that thrives in low pH
64 and high temperatures. Its biomass contains interesting amounts of valuable metabolites such as
65 thermostable phycocyanin (PC) in addition to starch, β-glucan, β-carotene and zeaxanthin carotenoid
66 pigments. This extremophile exhibits its best growth at temperatures from 42-50 °C and low pH (0.5-
67 2.5) (Miyagishima and Tanaka, 2021). Tolerance to acidic conditions can allow cultivation with
68 continuous injection rates of high concentration CO₂ and ammonia while also minimizing
69 contamination. This species may be a promising candidate for algal bioprocesses in extreme
70 environments. It is an excellent candidate for waste circularization concepts which seek to take high
71 concentration feed streams, especially in summertime conditions in desert environments, and
72 convert them into valuable biomass.

73 Here, we show the performance characteristics of *C. merolae* 10D in lab-scale photobioreactors
74 modelling different seasons on the mid Red Sea coast in Saudi Arabia, successful scaling of these
75 cultures to outdoor pilot scale (1 m³) cultures in summer desert conditions, biomass characterization
76 of the strain grown outdoors, and the adaptation of this species to the high saline conditions to allow
77 cultivation with medium made using Red Sea water. This is the first report that *C. merolae* 10D has
78 been grown at a pilot scale to identify its potential use for industrial applications in desert contexts.
79 Our results indicate that *C. merolae* 10D, and maybe other Cyanidiophyceae, will be valuable for
80 bioproduction or waste-stream bio-conversion in summer conditions in extreme desert environments
81 where other common commercial algal species may reach temperature threshold limitations.

83 **2 Materials & Methods**

84 **2.1 Lab-scale cultivation of *C. merolae* 10D and bioreactor growth tests**

85 *C. merolae* 10D (Toda et al., 1995; Matsuzaki et al., 2004) was received from the lab of Prof. Peter
86 Lammers (Arizona Center for Algae Technology Innovation (AzCATI), Arizona State University (ASU))
87 which we routinely maintained in MA2 liquid medium (Minoda et al., 2004): pH 2.3, adjusted with
88 H₂SO₄ in 125 mL Erlenmeyer flasks shaken at 100 rpm under 90 μ mol photon m⁻² s⁻¹ (hereafter, μ E)
89 continuous white light and 42 °C in a Percival algae incubator supplemented with 4% CO₂ in air
90 mixtures. Preculturing prior to growth analysis was performed by inoculation of cells to shake flasks
91 with MA2 medium for 4 days, then cells were resuspended according to desired densities in 400 mL
92 flasks prior to growth analysis in Algem photobioreactors (Algenuity®, UK). Cultures in biological
93 duplicates with starting cell concentrations of 3x10⁶ cells mL⁻¹ were cultivated in environmental
94 simulations of the mid Red Sea coast conditions of Thuwal, Saudi Arabia (22.3046N, 39.1022E) as
95 recently reported (de Freitas et al., 2023) and compared with control cultures using 12:12 h light:dark
96 or 24 h constant 1500 μ E illumination at 42 °C. All cultures received continuous 7% CO₂ in air at a 25
97 mL min⁻¹ flow rate. Modeled environmental conditions were from each season with local historical
98 weather data used to build profiles of temperature and light for February (Winter), May (Spring),
99 August (Summer), and November (Autumn). Daily, 15 mL of algae culture from each replicate flask
100 was taken for cell concentration (100 μ L), and biomass quantification (3x 4 mL). Cell densities were
101 measured using an Invitrogen Attune NxT flow cytometer (Thermo Fisher Scientific, UK) equipped
102 with a Cytkick microtiter plate autosampler unit. Each *C. merolae* 10D biological replicate was diluted
103 1:100 with 0.9% NaCl solution and loaded into a 96-well microtiter plate in technical duplicates prior
104 to injection into the flow cytometer using the autosampler. Samples were mixed three times
105 immediately before analysis, and the first 25 μ L of the sample was discarded to ensure a stable cell
106 flow rate during measurement. For data acquisition, 50 μ L from each well was analyzed. Optical
107 densities were measured as absorbance at 740 nm (OD_{740nm}) by the Algem photobioreactors every
108 10 min. Cell dry mass was measured by vacuum filtration of 3x 4 mL of each biological replicate on
109 pre-weighted filters (0.45 μ m). The algal cell masses were dried at 60 °C for 24h prior to weighing.

110 To adapt *C. merolae* 10D to saline conditions and enable its cultivation on a medium made from
111 acidified seawater, MA2 medium was prepared with different dilutions of filtered and autoclaved Red
112 Sea water (RSW-MA2) as 100, 80, 60, 40 and 20% mixtures with fresh water plus MA2 nutrients. In a
113 6-well plate, 200 μ L *C. merolae* 10D late logarithmic phase preculture culture was inoculated into
114 wells each containing the different RSW-MA2 concentrations. The cells were cultivated in the algal
115 incubator for 7 days as above. Cultures which reached reasonable cell densities were re-inoculated
116 in new media across the salinity dilution range and grown for another 7 days. This process was
117 repeated another two times and the culture adapted to 100% RSW-MA2 was chosen for further
118 cultivation in emulated local weather conditions in Algem photobioreactors as described above.

119 **2.2 Cultivation of *C. merolae* outdoors at increasing culture volumes**

120 Precultures of *C. merolae* 10D grown in Algem photobioreactors were pooled and transported to the
121 Development of Algal Biotechnology Kingdom of Saudi Arabia (DAB-KSA) pilot plant (KAUST, Thuwal,
122 Saudi Arabia), and cultivated outdoors in various photobioreactors from June-August 2022. Cultures
123 were started in 8 L south-facing glass columns at outdoor conditions with 39 ± 4.0°C and mid-day
124 irradiance 1503 ± 241 μ E, for two weeks. The culture medium was prepared using Altakamul

125 industrial/agricultural-grade salts rather than analytical grade as in lab cultivations. The medium
126 contained 5.3 g $(\text{NH}_4)_2\text{SO}_4$, 1.09 g KH_2PO_4 and 0.481 g $\text{MgSO}_4 \text{ L}^{-1}$ with F/2 medium trace elements
127 added to 1 mL L^{-1} (Guillard, 1975). The pH was adjusted to 2.5 with H_2SO_4 . Additionally, an
128 ammonium-based booster solution (10 mL L^{-1} , with 50% of the original initial concentration of NH_4^+)
129 was added every two days during the whole cultivation period. Once enough inoculum was produced,
130 the culture was transferred to a 60 L column photobioreactor (Varicon Aqua® Phyco-Bubble model)
131 with constant CO_2 sparging at a rate of 0.2 L min^{-1} and the unit was exposed to outdoor conditions of
132 $32.4 \pm 2^\circ\text{C}$, $1351 \pm 110 \mu\text{E}$. This culture was used as an inoculum for a 600 L raceway (Varicon Aqua®
133 Phyco-Pond model) for 1 week, and then it was transferred to a 1,000 L tubular photobioreactor (PBR,
134 Varicon Aqua® Phyco-Flow model) and the cultivation run for 24 days. Finally, this culture was used
135 as inoculum for two other 1,000 L PBRs started at 30% dilution from the first reactor. Two reactors
136 were run with 100% CO_2 sparging - PBR-1: "Commercial" pure CO_2 Alpha Gaz®, 99.995% (CG) and PBR-
137 2: "Green" CO_2 from flue gas, Gulf Cryo® (GC), while a third was not given CO_2 - PBR-3: "Atmospheric"
138 0.04% CO_2 (AC). In all treatments, gassing was supplied constantly (0.2 L min^{-1}) from 8 am to 5 pm.

139 $\text{OD}_{750\text{nm}}$ was recorded daily with a UV-Vis Spectrophotometer (Thermo Scientific® Genesys™ 50, UK).
140 Cell dry weight measurement (in duplicates) was performed every other day using a gravimetric
141 method, where aliquots were rinsed with acidified fresh water (pH 2.5) and filtered through glass
142 microfiber filters (Whatman GF/F) and weighed after drying. Nutrient uptake was measured as N (as
143 ammonium) and P (as phosphate), using Spectroquant® assay kits following manufacturer's
144 protocols. Temperature and pH were monitored continuously during the experiment (10 d) with the
145 built-in probes of the PBRs. Light intensity was measured as irradiance (quantum flux) with a quantum
146 meter probe (Biospherical instruments Inc® Model QSL2000). For biochemical characterizations, at
147 the incubation site, the cell suspension was harvested and washed twice with acidified freshwater
148 (pH 2.5). The washed microalgal pellets were freeze-dried overnight and stored in dark at -20°C until
149 further analysis.

150 2.3 Biochemical characterization of *C. merolae* 10D biomass

151 All chemicals and analytical reagents were HPLC grade (Fisher Scientific, U.K.) unless stated otherwise.
152 A multi-assay procedure (Chen and Vaidyanathan, 2012, 2013; Kapoore et al., 2019) was modified for
153 the quantification of total protein, carbohydrate, chlorophyll and carotenoids as follows. Briefly,
154 freeze-dried microalgal pellets were weighed (1.5 - 2 mg) in 2 mL Safe-Lock microcentrifuge tubes.
155 The dry pellets were resuspended in 24.3 μL of phosphate Buffer (pH 7.4) and 1.8 mL of 25% (v/v)
156 methanol in 1M NaOH along with an equal volume of glass beads (425-600 μm i.d., acid washed).
157 Cells were disrupted using Retsch MM 400 Mixer Mill (Retsch, GmbH) for 3 cycles (10-min bead
158 beating and 2-min stand). For carbohydrate analysis, 2x 0.2 mL extract was transferred to 2 mL PTFE
159 capped glass vials: for the control by adding 1.2 mL 75% H_2SO_4 ; for the experimental sample by adding
160 0.4 mL 75% H_2SO_4 and 0.8 mL anthrone reagent; completing the assay as described (Chen and
161 Vaidyanathan, 2012, 2013; Kapoore et al., 2019; Fuentes-Grünewald et al., 2021) by incubating at
162 100 °C for 15 minutes followed by measurement in polystyrene cuvettes (ΔA_{578}). The remainder
163 extract after cell disruption was stored at -80 °C in 4 mL PTFE capped glass vials and later saponified
164 as described (Chen and Vaidyanathan, 2012, 2013; Kapoore et al., 2019; Fuentes-Grünewald et al.,
165 2021) by incubating at 100°C for 30 min. For the pigment assay, saponified extracts of 0.7 mL were
166 transferred after vortexing to 2 mL Safe-Lock microcentrifuge tubes containing 1.05 mL 2:1 mixture
167 of chloroform with methanol. After centrifugation and phase separation, chlorophyll and carotenoid

168 concentrations were determined as previously described (Chen and Vaidyanathan, 2012, 2013;
169 Kapoor et al., 2019). The remainder of the saponified extract was stored at -80 °C for subsequent
170 protein assay where saponified extracts of 25 μ L were first placed directly into 96-well assay plates
171 with the following additions: controls, 0.2 mL BCA reagent alone (Thermo Scientific); experimental,
172 0.2 mL BCA/Cu mix (Thermo Scientific) and incubated at 37°C for 30 minutes, measuring ΔA_{562} .

173 A previously reported procedure for phycocyanin extraction was modified for the assessment of this
174 pigment in *C. merolae* 10D (Coward et al., 2016). Briefly, 1 mL of microalgal cell suspension was
175 harvested and then centrifuged for 5 min at 2,500xg at 4 °C supernatant was decanted and the
176 biomass was washed X3 with 1 mL of acidified water (pH 2.5). After washing, 1 mL of water was added
177 to the wet biomass followed by four consecutive freezing (-20°C) and thawing (4°C) cycles for cell
178 disruption. The extract was vortexed and then centrifuged at 10,000xg for 10 min to remove cell
179 debris. The clear blue supernatant containing extracted phycocyanin was quantified using
180 spectrophotometry with OD at 455, 564, 592, 618 and 645 nm using GENESYS 50 UV-Vis
181 Spectrophotometer (Thermo Fisher Scientific). The concentrations of phycocyanin were quantified
182 using the previously reported equation (Coward et al., 2016).

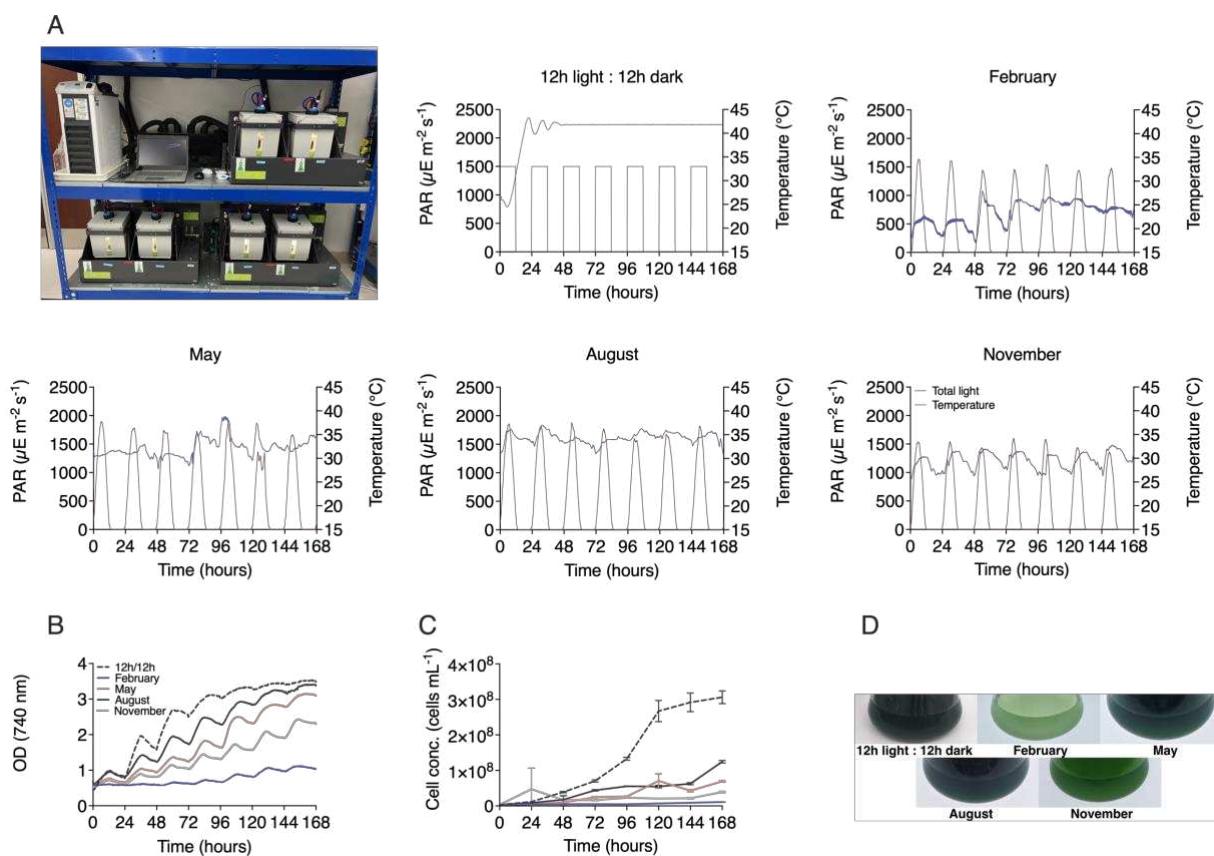
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185 **3 Results**

186 **3.1 Modeled growth of *C. merolae* 10D in lab-scale photobioreactors**

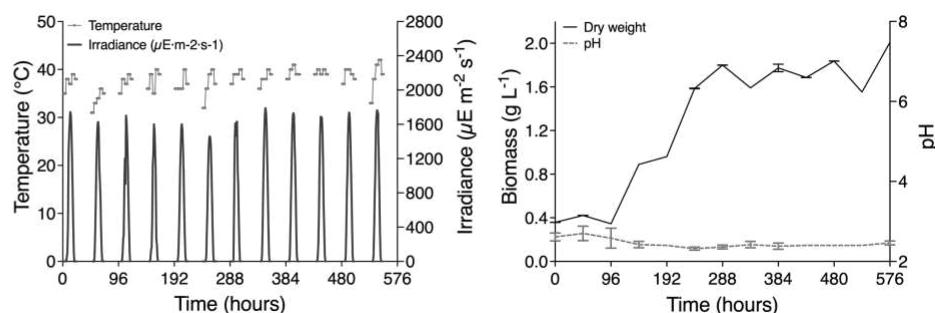
187 The Cyanidiophyceae tolerate extreme conditions in their native environments on the edges of acidic
188 hot springs. Here, we modeled whether the extreme conditions *C. merolae* 10D has evolved to thrive
189 in would enable its cultivation in conditions which are found at the mid-Red Sea Saudi Arabian coast
190 and by extension to similar desert countries in different seasons. Photobioreactor programs were
191 designed using local weather-station data to set light and temperature profiles in lab-scale
192 photobioreactors (de Freitas et al., 2023) and cultures were grown with standard laboratory MA2
193 medium (Minoda et al., 2004). The individual seasonal conditions, Winter - February, Spring - May,
194 Summer - August, Autumn - November, were compared to a control culture grown with a set light
195 cycle like the photoperiod found in Saudi Arabia (12:12 day:night), and constant temperature of 42
196 °C (Figure 1A). Culture performance was determined by optical (Figure 1B) and cell densities (Figure
197 1C) throughout the 8-day cultivation. Photographs of the cultures are also shown to indicate culture
198 health at the end of cultivation using the different modeled seasons (Figure 1D). The best culture
199 performance was observed in the control culture with constant temperature and OD_{740nm} above 2
200 were achieved fastest in Summer, Spring and Autumn reactor programs, respectively (Figure 1B).



201 **Figure 1.** Modeled seasonal productivity variation of *C. merolae* 10D in photobioreactors. **A** temperature and light profiles
202 in Algem photobioreactors (pictured) which are based on historical weather data from Thuwal, Saudi Arabia were used
203 to cultivate *C. merolae* 10D in MA2 medium and assess performance across different months in this locale. A control
204 culture was set to 42 °C with 12:12 hour day:night illumination cycling, while February, May, August, and November
205 profiles were used to model winter, spring, summer, and autumn, respectively. Growth performance was assessed by
206 optical density (**B**) recorded every 10 min in the bioreactors and cell densities (**C**) which were recorded daily by flow
207 cytometry. **D** Culture health and densities were also assessed photographically at the end of cultivations.

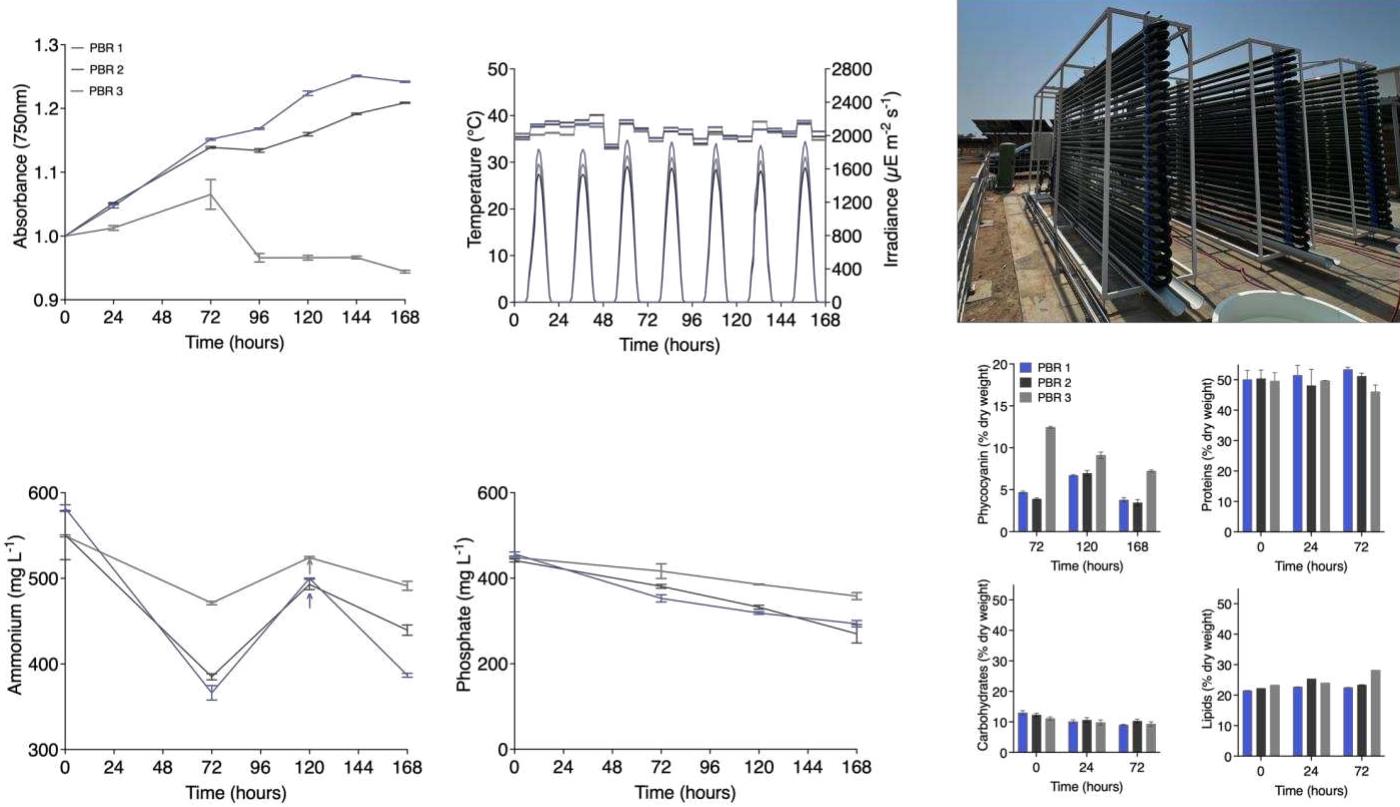
208 3.2 Scaled cultivation of *C. merolae* 10D outdoors on the mid-Red Sea coast

209 From May until August 2022, scaled cultivations of *C. merolae* 10D were performed outdoors using
210 natural light irradiance and temperature at the DAB-KSA algal pilot facility at KAUST. Cultures were
211 taken from lab-scale cultivations above, inoculated in 8-L columns and grown in medium prepared
212 with industrial grade fertilizers as described in the Material & Methods section. From 8-L columns (6-
213 L working volume), *C. merolae* 10D inoculum was transferred to a 60 L vertical column with pure CO₂
214 injection during daylight hours and subsequently to a 600 L raceway pond over several weeks
215 (Figure 2). In August, the raceway pond culture was used as inoculum for starting a 1000 L tubular
216 photobioreactor (at 0.38 g L⁻¹) culture, which was maintained for 28 days of operation in batch mode
217 (Figure 2). The culture experienced temperatures between 32-42 °C (average 37.7 °C) and daily peak
218 irradiance of ~1800 µE (Figure 2, recorded every other day). After the 5-day lag phase, the culture
219 exhibited a steady increase in biomass for 7 days reaching a maximum biomass production of 1.8 g L⁻¹
220 on the 12th day of cultivation (Figure 2). The maximal productivity achieved was ~300 mg L⁻¹ d⁻¹
221 between 96-288 h of the cultivation.



222 **Figure 2.** Scaled cultivation of *C. merolae* 10D in various culture set-ups in Thuwal, Saudi Arabia in July and August 2023.
223 Cultures in a 60 L column photobioreactor were used as inoculum for a 600 L raceway pond, which was consequently
224 used to inoculate a 1 m³ tubular photobioreactor (picture left). Temperature and irradiance at the tubular reactor were
225 recorded every other day for 24 days during cultivation (middle). Culture performance was measured by cell dry weight
226 throughout the cultivation and pH was also recorded (right).

227 The biomass obtained from this 1000 L tubular reactor was used as inoculum for two other reactors
228 set beside the first (Figure 3). The inoculum allowed each reactor to be started with OD 1.0 and each
229 reactor was cultivated for an additional 7 days (Figure 3). The reactor position determined maximal
230 light irradiance, with the middle reactor receiving less light than the external reactors (Figure 3).
231 Culture temperatures were relatively consistent across the three units, between 34-40 °C (average
232 36.5 °C) (Figure 3). One outer (east facing) and inner reactor were cultivated with pure CO₂ injections
233 while one outer (west facing) reactor was only sparged with air. As anticipated, the air cultivated
234 culture (PBR 3) did not proliferate beyond inoculum density, while those given CO₂ injections (PBR
235 1&2) continued to increase in their optical densities (Figure 3). These behaviors mirrored ammonium
236 and phosphate uptake rates, which consistently was taken up by *C. merolae* cells during the
237 cultivation period. At ~100 hours, supplemental ammonium was added to the cultures to determine
238 if the extremophile could continue to consume this in excess, and a second boost in terms of growth
239 (OD) was recorded. All cultures consumed the excess ammonium added (Figure 3). Regardless of CO₂
240 source (beverage grade of industrially reclaimed CO₂), the cultures given these gasses in excess were
241 able to proliferate (Figure 3, PBR 1&2). The culture grown with air (PBR 3), exhibited higher
242 phycocyanin content when biomass was sampled. Yet the biochemical composition of other
243 components was consistent in samples from all reactors (Figure 3).

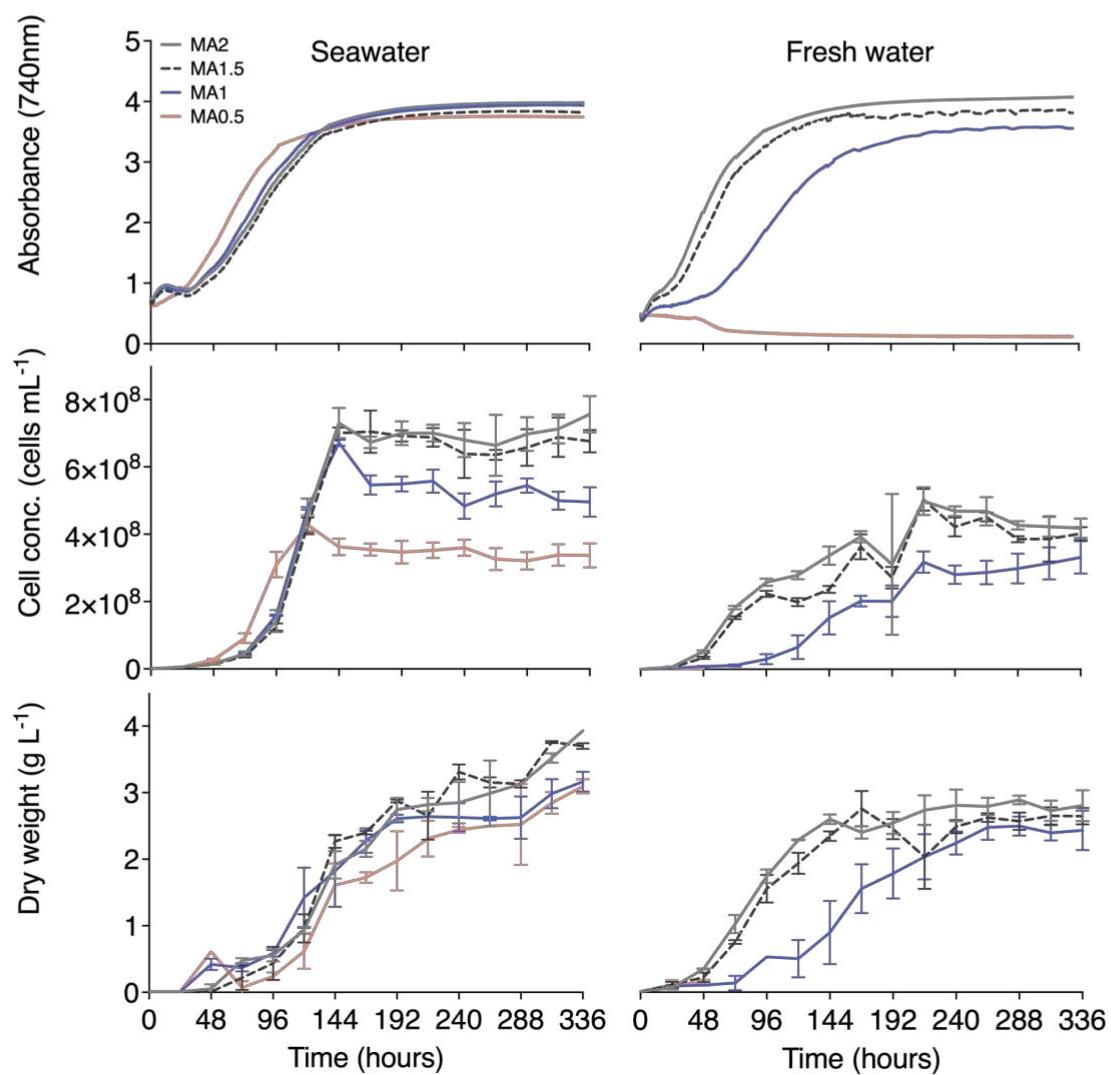
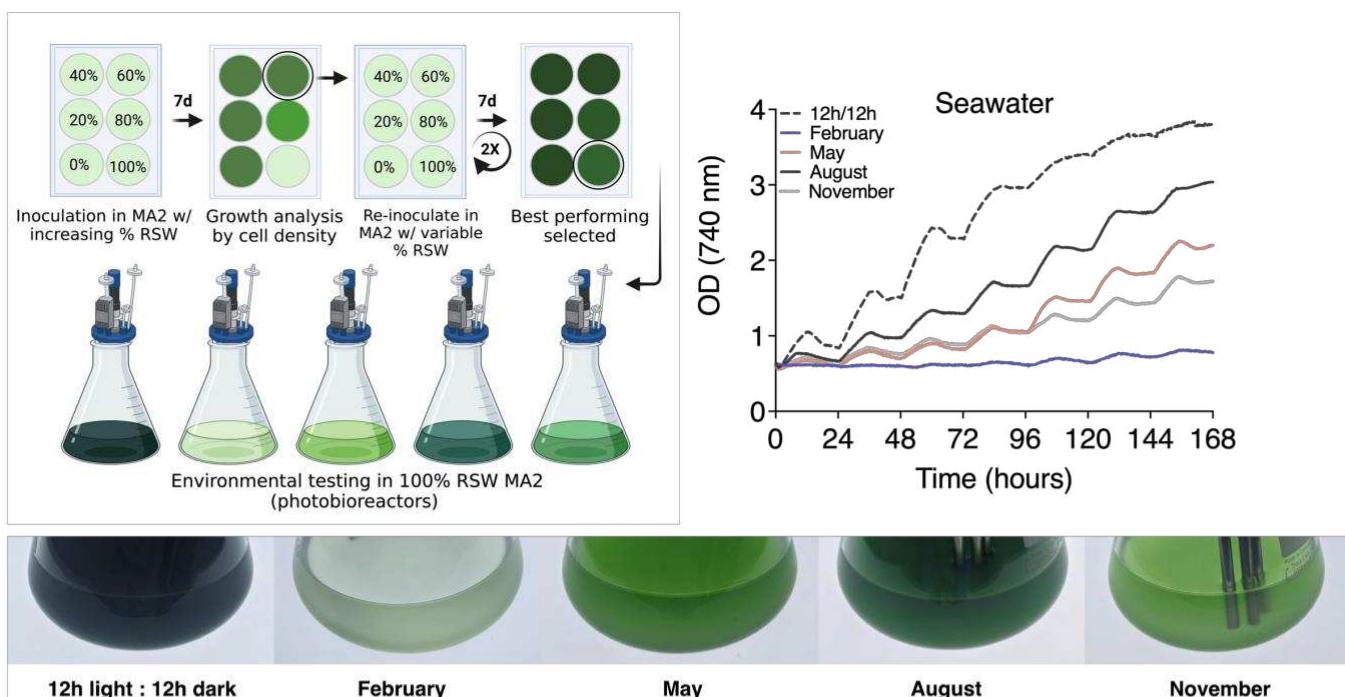


244 **Figure 3.** Three 1 m³ tubular photobioreactors were inoculated with the stationary phase culture of Figure 2. OD_{750nm},
245 temperature, and irradiance were measured throughout the cultivation. The three reactors (pictured upper right)
246 were given beverage grade (PBR 1), Industrially reclaimed “green” CO₂ (PBR 2), or atmospheric air (PBR 3). The reactors each
247 experienced slightly different irradiances based on their position to one another, with the outermost having higher
248 incident light intensities. Ammonium and phosphate levels were recorded at different intervals, and supplemental
249 ammonium solution was added to all reactors after 120 hours. Culture biomass was analyzed at different time-points for
250 phycocyanin, protein, carbohydrate, and lipids content (lower right).

251 3.3 Adaptation of *C. merolae* 10D to high saline culture medium using Red Sea water

252 Cultivation of an extremophile in a desert environment can have the advantage of its
253 thermotolerance, especially in summer conditions, however, the use of fresh-water resources for
254 algal cultivation is only appropriate in these environments as part of waste-water treatment and
255 reuse strategies. The use of seawater improves the sustainability of large scale algal cultivation
256 concepts, especially in coastal-desert regions. It was important to determine if we could adapt the
257 osmo-tolerance of *C. merolae* 10D to the high saline conditions found in Red Sea water as an
258 alternative source of cultivation medium for this organism. In lab-scale photoincubators, *C. merolae*
259 10D was cultivated in increasing mixtures of Red Sea water supplemented with MA2 nutrients (Figure
260 4). Inoculum was cultivated in microtiter plates and after 7 days of light and CO₂ conditions, cells in
261 the best growing well were used to inoculate a fresh set of dilutions and the process repeated over
262 several weeks (Figure 4). After 4 total passes, cells proliferating in MA2 medium made with 100 %
263 Red Sea water were obtained (Figure 4). This adapted strain was then subjected to the same
264 environmental modeling in photobioreactors as performed for its freshwater progenitor (Figure 1) to
265 determine if salinity tolerance had affected its growth behavior (Figure 4). Based on optical density,
266 the strain exhibited similar growth behaviors in saline conditions. The salt-water adapted *C. merolae*
267 10D performed best in constant 42 °C and 12:12 d:n control culture conditions with Summer (August)
268 the best seasonal performance, followed by spring (Figure 4).

269



270 **Figure 4.** Adaptation of *C. merolae* 10D to the hypersalinity of Red Sea water. Inoculum cultures were grown for 7 days in
271 a range of autoclaved Red Sea water mixtures with fresh water and MA2 nutrient supplementation. At the stationary
272 phase, the salt content condition that resulted in the highest cell density was used as inoculum for the same conditions
273 in a new culture plate. This process was repeated for a total of 4 passes until reasonable growth was observed in 100%
274 Red Sea water MA2 medium. This culture was used as inoculum for photobioreactor modeled environmental testing of
275 *C. merolae* 10D performance in temperature and light conditions for different seasons on the mid-Red Sea coast (as
276 illustrated, upper left). Optical density ($OD_{740\text{ nm}}$) was recorded continuously for the salt-water adapted *C. merolae* for a
277 control culture (42 °C with 12:12 hour day:night illumination), modeled seasonal programs for February, May, August,
278 and November were used to simulate winter, spring, summer, and autumn, respectively (upper right). Culture
279 performance was also observed photographically at the end of cultivation (pictured). Saltwater adapted and its
280 freshwater progenitor *C. merolae* 10D were subjected to continuous illumination and 42 °C temperature conditions with
281 culture media made using different ratios of MA2 nutrient solutions to determine the possibility of reducing nutrient
282 inputs for its growth. Culture performance was assessed by continuous optical density monitoring in the
283 photobioreactors, daily cell density, and biomass (dry weight) measurements over 14 days (bottom panels). Figure
284 partially created with Biorender.

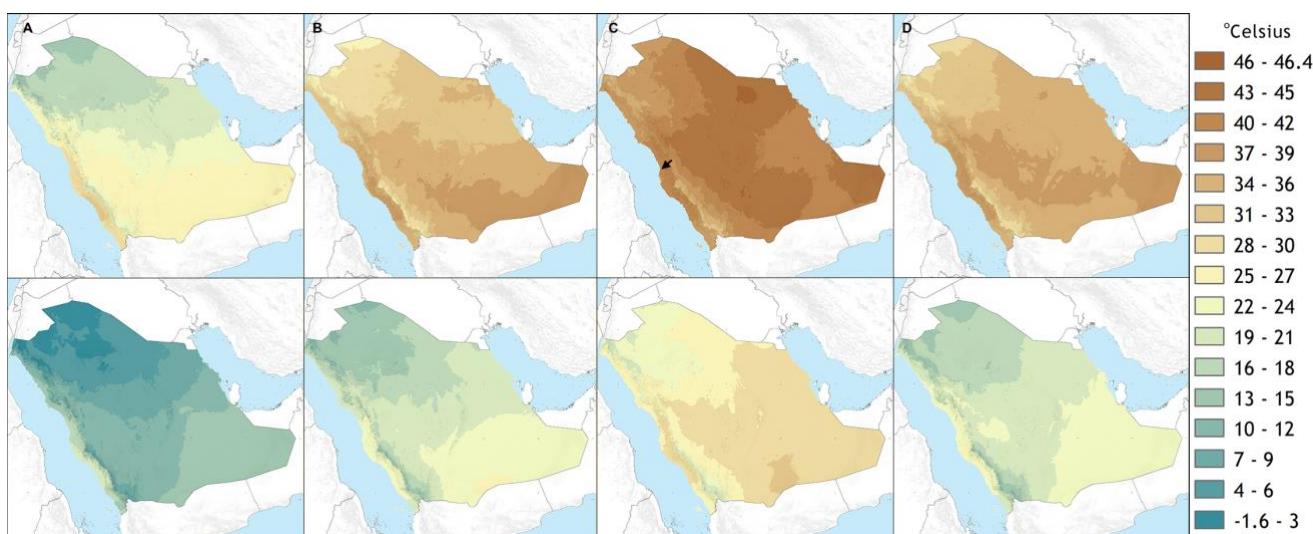
285 As nutrients (fertilizers) can be an expensive input to algal culture concepts at large scale, and MA2
286 has very high concentrations of ammonium and phosphate compared to other algal cultivation media
287 (Minoda et al., 2004), we sought to determine if it was possible to reduce the nutrient load and still
288 achieve reasonable growth performance from *C. merolae* 10D. Here, both fresh-water and the saline-
289 adapted *C. merolae* 10D were grown in lab-scale photobioreactors with a constant light and
290 temperature program, but the culture medium was prepared using full, 1.5, half, and 0.5
291 concentrations of the nutrient solutions used in MA2 medium. Cultures were grown for 2 weeks in
292 photobioreactors with constant light and CO_2 gassing. Culture performance was assessed by optical
293 and cell densities, as well as dry weights. It was determined that it is possible to dilute the nutrient
294 composition of MA2 to MA0.5 in saline conditions and achieve a comparable performance of the alga
295 in full medium. Red Sea water adapted *C. merolae* 10D achieved culture dry weights of 3 g L^{-1} in 14 d
296 even when diluted to MA0.5 (Figure 4). Saline-adapted cultures grown with MA0.5 exhibited less cells
297 per volume culture, but comparable biomass, suggesting heavier cells, this could be due to
298 accumulation of lipids or increased starch/beta-glucan inside the cells. The optical density and
299 biomass reached in these cultures were comparable, although slightly lower than MA1.5 and MA2
300 counterparts (Figure 4). However, fresh-water prepared MA2 could only be diluted to MA1 (Allen,
301 1959) where reduced culture performance was observed (Figure 4). Freshwater MA2 cultivated *C.*
302 *merolae* 10D exhibited lower final cell densities and dry weights compared to their salt-water
303 counterparts, although reaching comparable $OD_{740\text{nm}}$ in a replete medium.

304

305 **4 Discussion**

306 **4.1 Polyextremophilic algae for regional bioresource reuse concepts**

307 The Arabian Peninsula is one of the more extreme environments in which human settlements have
308 been established. Temperatures in these desert environments are consistently high, with strong
309 irradiance and minimal precipitation (Almazroui et al., 2012; AlSarmi and Washington, 2014). These
310 conditions are similar in most of desert countries found in the so called “hot belt” (see introduction).
311 Despite extreme conditions, the urban population in these regions is steadily increasing, generating
312 waste streams which require treatment and often contain nitrogen and phosphorous concentrations
313 that can eutrophicate aquatic environments if not properly treated. This is already an issue in dense
314 urban areas in Europe and other countries, having direct environmental impact in water bodies that
315 cause harmful algae bloom proliferations due to nutrient emission eutrophication process. Controlled
316 microalgal cultivation has been identified as one of the technologies to bioremediate eutrophied
317 industrial side-streams from aquaculture, wastewater treatment plants, and anaerobic digestion
318 facilities (Mayhead et al., 2018; Silkina et al., 2019; Fuentes-Grünewald et al., 2021). In addition, Saudi
319 Arabia has many local sources of CO₂ rich emissions, especially from industrial activities, that can be
320 readily sourced with minimal transport distances. In this locale, the combination of the high
321 irradiance, local waste-water streams, flat non-arable land and CO₂ sources can be synergistically
322 combined to support algal bio-processes in Saudi Arabia and neighboring countries in the Gulf
323 Cooperation Countries (GCC) area.



324 **Figure 5.** Mean maximum (upper) and minimum (lower) average monthly temperature for January (A), April (B), August (C), and October (D) at ground level from 1970-2000 in Saudi Arabia (Fick and Hijmans, 2017). Location of the KAUST DAB-KSA pilot facility site of cultivation is indicated with a black arrow.
325
326

327 The mean maximal and minimal temperatures experienced across the Arabian Peninsula are higher
328 than the current temperate zones where microalgal cultivation is currently conducted (Figure 5). The
329 maximal summertime temperatures exceed 45 °C, with regional variability depending on the site.
330 These temperatures are above the threshold of heat stress and growth cessation of many currently
331 cultivated algal species (Ras et al., 2013). In order to implement algal bioprocesses as part of broader
332 resource circularity bio-economy drives, thermotolerant species are required for installations that
333 will operate during the summer months in some specific countries, or throughout the year in desert
334 countries.

335 The temperature modeling in lab-scale photobioreactors used here was designed from atmospheric
336 weather station data at sea level, the temperatures of which would be lower than that experienced
337 inside a photobioreactor which would warm from the solar and infrared radiation. Growth in these
338 tests was possible in modeled August conditions but not at maximal rates of productivity observed in
339 control cultures, and much lower when other seasons were investigated (Figure 1). *C. merolae* 10D
340 was able to proliferate in 1000 L photobioreactors on the mid-Red Sea coast and achieve up to 2 g L⁻¹
341 biomass at a maximal rate of 300 mg L⁻¹ d⁻¹ in batch mode. There, it experienced temperatures ~5
342 °C warmer than those modeled (Figure 2), much closer to its optimum at 42 °C. In coastal regions of
343 this area, high humidity moderates temperature extremes compared to inland regions. It is likely that
344 at in-land sites in urban areas, cultures would experience significantly higher temperatures than those
345 experienced at our study site. *C. merolae* would be an ideal candidate to proliferate in such situations
346 and is a promising strain for phased seasonal growth concepts in such an extreme environment.

347 4.2 Acidophiles in high-strength waste-stream valorization

348 Outdoor cultivations conducted in this study were performed with culture medium using agricultural
349 grade chemical fertilizers. These were found to be sufficient to enable growth of the alga outdoors at
350 various scales. Nutrients are one of the key expenses in large scale microalgae production facilities.
351 Using agricultural grade fertilizers instead of analytical grade nutrients, the reduction in production
352 cost, and the sustainability of microalgae production has a significant effect in the reduction of
353 operational expenditure (Singh and Das, 2014). When the culture was grown in 3-parallel 1 m³
354 tubular reactors, the cultures tolerated addition of extra ammonium, which would cause acidic pH
355 shifts in other algal species which require neutral pH. The cultures also tolerated either commercial
356 beverage-grade or industrial emission reclaimed “green” CO₂ gas sources (Figure 3). Cells grown with
357 either CO₂ source did not differ in biomass composition containing equivalent phycocyanin (PC),
358 protein, carbohydrate, and lipid contents (PBR 1+2, Figure 3). The culture which was starved from
359 carbon, only atmospheric CO₂ levels, exhibited higher PC concentrations at 72 hours (PBR 3, Figure 3).
360 This could be a means of increasing PC content prior to harvest, but needs further investigation. The
361 PC of *C. merolae* is considered more thermostable than that of other currently harvest algal species
362 and could be a valuable co-product from the biomass (Rahman et al., 2017). These behaviors suggest
363 *C. merolae* 10D as an acidophile is highly suited to industrial waste re-valorization processes which
364 could convert high-strength ammonia containing wastewaters and industrial CO₂ sources into
365 valuable biomass. We suggest this promising extremophile as a unique strain to be used in large scale
366 production facilities for CO₂ reuse applications.

367 4.3 Opportunities for Cyanidiophyceae biotechnology in a regional context

368 Extremes of heat in summer are balanced by moderate and even low temperatures in winter and
369 spring months in the Arabian Peninsula (Figure 5). Photobioreactor modeling of *C. merolae*'s
370 performance in these months indicated that the polyextremophile did not perform optimally under
371 lower temperature regimes (Figure 1 and 4). From a bioprocess standpoint, engineering in-culture
372 heating in these off-months is technically straightforward as heating processes require less energy
373 than cooling which can be achieved, for example, with industrial heat waste (Ekendahl et al., 2018).
374 It should be possible, therefore, to cultivate *C. merolae* 10D year-round in this environment, so long
375 as bio-processes are designed with relevant parameters and approaches in mind.

376 The cultivation in acidic conditions requires some considerations in the source of inputs for scaled *C.*
377 *merolae* 10D cultivations. CO₂ injections into the culture medium (effluent inputs) could be used to
378 reduce pH of solutions depending on alkalinity. High-strength ammonia containing waste-waters will
379 also be appropriate for *C. merolae* 10D cultivation as the consumption of ammonium can will further
380 drive the culture to acidic conditions (Henkanatte-Gedera et al., 2015; Selvaratnam et al., 2016;
381 Nirmalakhandan et al., 2019). This additional acidification of input waters by CO₂ may enhance the
382 potential of *C. merolae* as a vehicle for carbon reuse, valorization, and circularity. *C. merolae*
383 cultivation could be best performed on already acidic waste streams, like those of the dairy industry.
384 Process designs with this organism will have to determine the best ways to incorporated its acidic
385 cultivation conditions.

386 The ability to adapt *C. merolae* to saline cultivation conditions (Figure 4), also opens the possibility
387 for cultivation in sea waters sourced along the coastlines of desert countries, improving the
388 sustainability of commercial large scale microalgae facilities. Here, high-strength aquaculture
389 effluents with CO₂ injections may be used as inputs for culture of saline adapted *C. merolae* 10D. The
390 species could be highly valuable for nitrogen and phosphorous removal from on-land marine
391 aquaculture concepts, while generating a protein-rich biomass that can be added to feeds, generating
392 a truly circular economy. As *C. merolae* is a cell-wall deficient species, its rupture and incorporation
393 into feed as a protein biomass is straightforward and requires little energy inputs during downstream
394 processing. This property also allows simple extraction of its thermostable phycocyanin or its biomass
395 could be used to make bio-stimulant fertilizers due to its high protein content, for emerging contained
396 environment agriculture concepts suitable for this region (Lefers et al., 2020).

397 **5 Conclusions**

398 Here, we demonstrate that the polyextremophile *C. merolae* 10D is a promising candidate for algal-
399 based resource circularity in hot desert environments. Its thermotolerance allows its cultivation even
400 in the extremes of desert summers and its acidic preferences can be used to minimize contamination
401 and maximize ammonia removal from liquid and CO₂ waste streams. The work reports scaled
402 cultivation of *C. merolae* in the summer months in Saudi Arabia and shows that it can be adapted to
403 salinities at least as high as those observed in Red Sea waters. *C. merolae* could be an interesting
404 candidate for on-land marine aquaculture wastewater treatment and revalorization in addition to the
405 reuse of high CO₂ concentration emissions. This is the first report of the scaled cultivation of *C.*
406 *merolae* 10D and the first demonstration of its growth in the Middle Eastern context. Our report sets
407 a foundation for increasing investigations into the use of *C. merolae* and its biomass for bioresource
408 circularity and applications like feed, fertilizer, and other high value bio-products such as
409 thermostable phycocyanin. This report indicates that *C. merolae* 10D may hold unique promise for
410 biotechnological application using the resources found in abundance in the Arabian Peninsula and
411 other desert regions with urban and industrial development.

412

413

414 **6 Conflict of Interest**

415 The authors declare that the research was conducted in the absence of any commercial or financial
416 relationships that could be construed as a potential conflict of interest.

417 **7 Author Contributions**

418 MVV was responsible for cultivation of *C. merolae* 10D in lab-scale, adaptation to sea water, lab-scale
419 photobioreactor operation, sampling, data analysis, and figure preparation. BBdF was responsible for
420 lab-scale bioreactor operation, sampling, data analysis, and figure preparation. REGP and GIRV were
421 responsible for outdoor cultivation of *C. merolae* from 5L-1000L. RVK and RM were responsible for
422 biochemical characterization, data interpretation, data reporting and analysis. CFG and KJL were
423 responsible for project design, funding acquisition, data analysis and manuscript writing. All authors
424 contributed to the writing of this manuscript and figure layout decisions.

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435 **10 References**

436 Allen, M. B. (1959). Studies with cyanidium caldarium, an anomalously pigmented chlorophyte.
437 *Archiv. Mikrobiol.* 32, 270–277. doi: 10.1007/BF00409348.

438 Almazroui, M., Nazrul Islam, M., Athar, H., Jones, P. D., and Rahman, M. A. (2012). Recent climate
439 change in the Arabian Peninsula: annual rainfall and temperature analysis of Saudi Arabia for
440 1978–2009. *Int. J. Climatol.* 32, 953–966. doi: 10.1002/joc.3446.

441 AlSarmi, S. H., and Washington, R. (2014). Changes in climate extremes in the Arabian Peninsula:
442 analysis of daily data: CHANGES EXTREMES OVER ARABIA. *Int. J. Climatol.* 34, 1329–1345. doi:
443 10.1002/joc.3772.

444 Cai, J., Lovatelli, A., Garrido Gamarro, E., Geehan, J., Lucente, D., Mair, G., et al. (2021). Seaweeds and
445 microalgae: an overview for unlocking their potential in global aquaculture development. FAO
446 doi: 10.4060/cb5670en.

447 Chen, Y., and Vaidyanathan, S. (2012). A simple, reproducible and sensitive spectrophotometric
448 method to estimate microalgal lipids. *Analytica Chimica Acta* 724, 67–72. doi:
449 10.1016/j.aca.2012.02.049.

450 Chen, Y., and Vaidyanathan, S. (2013). Simultaneous assay of pigments, carbohydrates, proteins and
451 lipids in microalgae. *Analytica Chimica Acta* 776, 31–40. doi: 10.1016/j.aca.2013.03.005.

452 Coward, T., Fuentes-Grünewald, C., Silkina, A., Oatley-Radcliffe, D. L., Llewellyn, G., and Lovitt, R. W.
453 (2016). Utilising light-emitting diodes of specific narrow wavelengths for the optimization and
454 co-production of multiple high-value compounds in *Porphyridium purpureum*. *Bioresource
455 Technology* 221, 607–615. doi: 10.1016/j.biortech.2016.09.093.

456 de Freitas, B. B., Overmans, S., Medina, J. S., Hong, P.-Y., and Lauersen, K. J. (2023). Biomass
457 generation and heterologous isoprenoid milking from engineered microalgae grown in
458 anaerobic membrane bioreactor effluent. *Water Research* 229, 119486. doi:
459 10.1016/j.watres.2022.119486.

460 Ekendahl, S., Bark, M., Engelbrektsson, J., Karlsson, C.-A., Niyitegeka, D., and Strömberg, N. (2018).
461 Energy-efficient outdoor cultivation of oleaginous microalgae at northern latitudes using
462 waste heat and flue gas from a pulp and paper mill. *Algal Research* 31, 138–146. doi:
463 10.1016/j.algal.2017.11.007.

464 Ferdouse, F., Holdt, S. L., Smith, R., Murúa, P., and Yang, Z. (2018). The global status of seaweed
465 production, trade and utilization. Available at: [http://www.fao.org/in-
466 action/globefish/publications/details-publication/en/c/1154074/](http://www.fao.org/in-action/globefish/publications/details-publication/en/c/1154074/).

467 Fick, S. E., and Hijmans, R. J. (2017). WorldClim 2: new 1-km spatial resolution climate surfaces for
468 global land areas. *Int. J. Climatol.* 37, 4302–4315. doi: 10.1002/joc.5086.

469 Fuentes-Grünewald, C., Ignacio Gayo-Peláez, J., Ndovela, V., Wood, E., Vijay Kapoor, R., and Anne
470 Llewellyn, C. (2021). Towards a circular economy: A novel microalgal two-step growth

471 approach to treat excess nutrients from digestate and to produce biomass for animal feed.
472 *Bioresource Technology* 320, 124349. doi: 10.1016/j.biortech.2020.124349.

473 Greene, C. H., Scott-Buechler, C. M., Hausner, A. L. P., Johnson, Z. I., Lei, X. G., and Huntley, M. E.
474 (2022). Transforming the future of marine aquaculture a circular economy approach.
475 *Oceanography* 35. doi: 10.5670/oceanog.2022.213.

476 Guillard, R., R. ., L. ed. (1975). "Culture of phytoplankton for feeding marine invertebrates," in *Culture*
477 *of phytoplankton for feeding marine invertebrates* (Boston, MA: Springer US). doi:
478 10.1007/978-1-4615-8714-9.

479 Henkanatte-Gedera, S. M., Selvaratnam, T., Caskan, N., Nirmalakhandan, N., Van Voorhies, W., and
480 Lammers, P. J. (2015). Algal-based, single-step treatment of urban wastewaters. *Bioresource*
481 *Technology* 189, 273–278. doi: 10.1016/j.biortech.2015.03.120.

482 Kapoor, R. V., Huete-Ortega, M., Day, J. G., Okurowska, K., Slocombe, S. P., Stanley, M. S., et al.
483 (2019). Effects of cryopreservation on viability and functional stability of an industrially
484 relevant alga. *Sci Rep* 9, 2093. doi: 10.1038/s41598-019-38588-6.

485 Lefers, R. M., Tester, M., and Lauersen, K. J. (2020). Emerging Technologies to Enable Sustainable
486 Controlled Environment Agriculture in the Extreme Environments of Middle East-North Africa
487 Coastal Regions. *Frontiers in Plant Science* 11, 1–7. doi: 10.3389/fpls.2020.00801.

488 Matsuzaki, M., Misumi, O., Shin-i, T., Maruyama, S., Takahara, M., Miyagishima, S., et al. (2004).
489 Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. *Nature*
490 428, 653–657. doi: 10.1038/nature02398.

491 Mayhead, E., Silkina, A., Llewellyn, C., and Fuentes-Grünewald, C. (2018). Comparing Nutrient
492 Removal from Membrane Filtered and Unfiltered Domestic Wastewater Using *Chlorella*
493 *vulgaris*. *Biology* 7, 12. doi: 10.3390/biology7010012.

494 Minoda, A., Sakagami, R., Yagisawa, F., Kuroiwa, T., and Tanaka, K. (2004). Improvement of culture
495 conditions and evidence for nuclear transformation by homologous recombination in a red
496 alga, *Cyanidioschyzon merolae* 10D. *Plant and Cell Physiology* 45, 667–671. doi:
497 10.1093/pcp/pch087.

498 Miyagishima, S. Y., and Tanaka, K. (2021). The Unicellular Red Alga *Cyanidioschyzon merolae* - The
499 Simplest Model of a Photosynthetic Eukaryote. *Plant and Cell Physiology* 62, 926–941. doi:
500 10.1093/pcp/pcab052.

501 Nirmalakhandan, N., Selvaratnam, T., Henkanatte-Gedera, S. M., Tchinda, D., Abeysiriwardana-
502 Arachchige, I. S. A., Delanka-Pedige, H. M. K., et al. (2019). Algal wastewater treatment:
503 Photoautotrophic vs. mixotrophic processes. *Algal Research* 41, 101569. doi:
504 10.1016/j.algal.2019.101569.

505 Rahman, D. Y., Sarian, F. D., van Wijk, A., Martinez-Garcia, M., and van der Maarel, M. J. E. C. (2017).
506 Thermostable phycocyanin from the red microalga *Cyanidioschyzon merolae*, a new natural
507 blue food colorant. *J Appl Phycol* 29, 1233–1239. doi: 10.1007/s10811-016-1007-0.

508 Ras, M., Steyer, J.-P., and Bernard, O. (2013). Temperature effect on microalgae: a crucial factor for
509 outdoor production. *Rev Environ Sci Biotechnol* 12, 153–164. doi: 10.1007/s11157-013-9310-
510 6.

511 Rumin, J., Nicolau, E., Gonçalves de Oliveira Junior, R., Fuentes-Grünewald, C., Flynn, K. J., and Picot,
512 L. (2020). A Bibliometric Analysis of Microalgae Research in the World, Europe, and the
513 European Atlantic Area. *Marine Drugs* 18, 79. doi: 10.3390/md18020079.

514 Selvaratnam, T., Henkanatte-Gedera, S. M., Muppaneni, T., Nirmalakhandan, N., Deng, S., and
515 Lammers, P. J. (2016). Maximizing recovery of energy and nutrients from urban wastewaters.
516 *Energy* 104, 16–23. doi: 10.1016/j.energy.2016.03.102.

517 Silkina, A., Ginnever, N. E., Fernandes, F., and Fuentes-Grünewald, C. (2019). Large-Scale Waste Bio-
518 Remediation Using Microalgae Cultivation as a Platform. *Energies* 12, 2772. doi:
519 10.3390/en12142772.

520 Singh, M., and Das, K. C. (2014). “Low cost nutrients for algae cultivation,” in *Algal biorefineries: Volume 1: Cultivation of cells and products*, eds. R. Bajpai, A. Prokop, and M. Zappi (Dordrecht:
521 Springer Netherlands), 69–82. doi: 10.1007/978-94-007-7494-0_3.

523 Toda, K., Takahashi, H., Itoh, R., and Kuroiwa, T. (1995). DNA Contents of Cell Nuclei in Two
524 Cyanidiophyceae: Cyanidioschyzon merolae and Cyanidium caldarium Forma A. *cytologia* 60,
525 183–188. doi: 10.1508/cytologia.60.183.

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528 **11 Supplementary Material**

529 Supplementary Data File 01. Data used to make Figure 1

530 Supplementary Data File 02. Data used to make Figure 2

531 Supplementary Data File 03. Data used to make Figure 3

532 Supplementary Data File 04. Data used to make Figure 4

533

534 **12 Data Availability Statement**

535 All data used in this manuscript can be found within the Supplemental Files provided.