

1 **Title: An engineered community approach for industrial cultivation of microalgae**

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9 **Running title: Community approaches for robust algal cultures**

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25 **Abstract**

26 In Nature, no species live in isolation. Traditionally, efforts to grow organisms for use in
27 biotechnology have focused on a single-species approach, particularly where a high-value
28 product is required in pure form. In such scenarios, preventing the establishment of
29 contaminants requires considerable effort that is justified economically. However, for algal
30 biotechnology, in particular where the focus is on fuel production, axenic culture is not
31 necessary, provided yields of the desired strain are not hampered by unwanted
32 contaminants. In the following article we review what is known about inter-specific
33 interactions of natural algal communities, the dynamics of which are likely to parallel
34 contamination in industrial systems. Furthermore, we discuss the opportunities to improve
35 both yields and the stability of cultures by growing algae in multi-species consortia.

36

37 **1. Background**

38 Microalgae (eukaryotic photosynthetic microbes) and cyanobacteria (oxygenic
39 photosynthetic bacteria), are a highly diverse collection of micro-organisms. They live in a
40 range of environments, including all aquatic ecosystems, both fresh-water and marine, and
41 species are also found in terrestrial habitats including on hard surfaces and snow. Many taxa
42 are capable of growing heterotrophically as well as phototrophically, and some obligate
43 heterotrophs also exist that, although ancestrally photosynthetic, have lost the ability to
44 photosynthesise. These include the dinoflagellate *Cryptocodinium cohnii*, which is of
45 commercial importance as a source of docosahexaenoic acid (DHA).¹ Algae are currently
46 cultivated on a relatively small scale for high value products such as the carotenoid

47 astaxanthin from *Haematococcus pluvialis* and the phycobiliprotein phycocyanin from the
48 cyanobacterium *Aphanizomenon flos-aquae*.² Certain strains are marketed as dietary
49 supplements, such as the cyanobacterium *Spirulina* sp. (*Arthrospira platensis*) and *Chlorella*
50 *vulgaris*.

51 Bulk growth of algae for products of lower value to displace commodities traditionally made
52 from fossil oil has received a lot of research attention.³ However, the scale-up required to
53 achieve this poses a wide range of problems, ranging from the energy costs of maintaining
54 large-scale photobioreactors, lower yields in large-scale cultures arising from factors such as
55 poor light penetration, mass transfer (where exogenous carbon dioxide is supplied, or
56 oxygen needs to be removed) and biological contamination, as well as the energy costs of
57 downstream processing and product.⁴⁻⁶ We concentrate here on consideration of how
58 understanding the ecology of the organisms under cultivation, that is their interaction with
59 others in the environment, can be harnessed to enhance productivity and thus increase
60 financial and environmental benefits achieved by cultivating algae.

61 **2. Applying community ecology to algal cultivation**

62 Most studies that target increasing yields in industrial cultures are aimed at an individual
63 species level, which assumes that cultivation will be in monoculture. However, because
64 contamination is inevitable without stringent sterile practice, which is neither cost-effective
65 nor likely to be achievable at industrial scale,⁷ understanding the growth dynamics of an
66 algal population growing in reactors is fundamentally an ecological problem.⁸ Moreover,
67 monocultures are by their nature unstable and prone to perturbation. Their genetic
68 uniformity encourages quick proliferation of pathogens and invaders, a common problem for
69 traditional single-crop agriculture (reviewed in Smith et al., 2014)⁹. Monocultures are

70 predicted to be unstable by classical theories of community ecology, which describe natural
71 systems as increasing in complexity over time (e.g. Elton, 1958)¹⁰. Given the chance, for a
72 given habitable environment, multiple species with diverse niche specificities will coexist
73 alongside each other, maximising the use of the available resources. “Invasions” by
74 organisms from neighbouring environments will continue until a “climax” stable state is
75 assembled, which is predicted to be resilient to change provided abiotic conditions remain
76 constant (May, 1977)¹¹.

77 Therefore, a new and emerging approach is to consider community approaches to
78 cultivation. The reasoning is that by starting with what would be an “end-point” consortium
79 in a natural system, it may be possible to avoid the development of unwanted alternatives.
80 In the following section we review the advantages of growing algae in consortia of species,
81 rather than as monocultures. The principles that we draw on are from aquatic community
82 ecology, and key concepts are summarised in Table 1.

83 **2.1 Maximising productivity**

84 One of the tenets of community ecology is that productivity is enhanced when diverse
85 organisms are grown together. This has been illustrated for a range of habitats, and
86 famously in a long-term experiment on grasslands. For a period of seven years, it was
87 demonstrated that 16-species grassland plots attained 2.7 times more biomass than the
88 respective monocultures.⁹ An aquatic experiment showed that diverse algal communities
89 (grown in biofilms) increased the uptake and storage of nitrate from streams, and
90 significantly increased in biomass content compared to monocultures.¹⁰ *Overyielding* is said
91 to occur when the biomass production of a consortium of species is greater than that of the
92 average monoculture of the species contained in the mixture.¹¹ *Transgressive* overyielding is

93 said to occur when the mixture outperforms even the most productive of the monocultures
94 of the constituent species.¹² There is evidence that, when functionally diverse algae ~~with~~
95 ~~complementary light requirements~~ are grown together, the resulting communities are more
96 productive than monocultures of individual species. Behl et al. analysed the rate of carbon
97 uptake and productivity for 85 assembled communities composed of species from four
98 functional groups: chlorophytes, diatoms, cyanobacteria and chrysophytes.¹³ The
99 researchers found that all algal communities consisting of species from two, three or four
100 different functional groups showed overyielding compared with their respective
101 monocultures, with transgressive overyielding in more than half of the assemblages studied.
102 ~~This is interesting as it suggests that positive interactions beyond resource use~~
103 ~~complementarity occurred between species. A possible way this could occur is through~~
104 ~~mutualistic interactions, reviewed in Section 2.3 below.~~

105 An important explanation for increased productivity in diverse cultures is through resource
106 use complementarity. When species that have different growth requirements are grown
107 together, competition between members of the community is reduced compared with that
108 experienced by individuals in dense monocultures. This allows more individuals to cohabit,
109 increasing the net biomass of the culture. One of the traits that distinguishes algal species is
110 the portfolio of pigments they use to absorb light. Although oxygenic photosynthetic
111 organisms use chlorophyll *a* as the major pigment in the photosystems, the accessory light
112 harvesting pigments differ (Figure 1). In cyanobacteria grown under iron replete conditions,
113 phycobilisomes on the surface of the thylakoid membranes contain the phycobilin pigments
114 phycocyanin and phycoerythrin. These pigments are also found in red algae, whereas green
115 algae (chlorophytes) contain chlorophyll *b*, as do all land plants. Chlorophyll *c* is the major

116 accessory pigment in the Chromalveolata. A possible explanation for overyielding of diverse
117 algae grown in cocultures as observed by Behl et al. therefore could be due to maximised
118 use of available light resource.

119 **2.2 Crop protection**

120 Contaminating organisms that invade algal cultures can reduce yields in different ways:
121 predators and pathogens are able to do so directly by killing the algae in culture, whilst
122 competing microalgae can take over as the dominant strain. The latter is a problem when a
123 specific algal strain is required, such as an oil producer or a strain with useful pigments. In
124 principle it could be possible to address all of these challenges by growing algae in culture
125 with carefully selected cohabiting species.

126 The effect of predators can be decreased through biomanipulation of the food web,
127 whereby an ecosystem is deliberately altered by adding or removing species. This is common
128 practice in the freshwater management industry, where the goal is to minimise algal
129 production.¹⁴ In the context of algal cultivation, which is the reverse scenario, if production
130 were to be hampered by invading zooplankton, the addition of zooplanktivores (such as
131 small fish) to the reactors might increase yields.^{8,15} However, this is unlikely to be possible
132 for closed photobioreactors, but may also not be practical in open ponds because most
133 reactors are very shallow, and would not be suitable for fish. An alternative solution is that
134 of crop protection through “interference”. By introducing multiple inedible algal species to
135 grow alongside the desired strain, the foraging efficiency of invading zooplankton may be
136 decreased due to the increased energetic costs of finding their desired prey.¹⁶ This technique
137 of pest control was recently investigated by Shurin et al. in a set of laboratory experiments,
138 the results of which are summarised in Figure 2.¹⁷ Communities containing 1, 2, 5 and 10

139 species of algae in various combinations were subjected to grazing by *Daphnia pulex*.
140 Although the total biomass of algal food resources increased with diversity, survival of
141 introduced *Daphnia* grazers declined markedly when 5 or 10 species of algae were grown
142 together.

143 However, there may be a cost to co-cultivation of a range of algal species when only a single
144 species is of commercial interest. It is possible to imagine a scenario where the growth of a
145 desired strain is decreased in a dense polyculture due to increased shading by co-cultured
146 strains. Where stability of a monoculture against invasions is the primary concern, this may
147 be enhanced by manipulating the abiotic environment to make the establishment of
148 competitors less likely. This is why extremophiles have been preferred in commercial
149 cultures, such as *Spirulina* sp., which is grown in highly alkaline conditions, or *Dunaliella*
150 *salina*, which is cultured in highly saline medium. A community solution to the problem of
151 competitors may be engineered through co-culturing with partners that produce allelopathic
152 chemicals. Chemical interactions are an important part of phytoplankton competition and
153 are particularly functionally important with dinoflagellates and cyanobacteria.¹⁸ These
154 organisms are able to produce chemicals that are toxic to most other algae in the
155 environment, allowing the former to bloom under the right conditions for growth, often
156 causing what are known as Harmful Algal Blooms, HABs.¹⁹ However, some species have
157 evolved to withstand the toxins produced during HABs and are able to cohabit with the toxin
158 producing strains. If either HAB-forming or HAB-tolerant species were identified as
159 interesting candidates for biofuel production, growth in consortia with toxin producing
160 strains could be a possible solution to competitive invasion. A similar approach is taken in
161 water treatment, where often barley straw is used to control populations of unwanted

162 algae. Toxins produced from the straw liquor are known to inhibit the growth of some algae
163 but not others.²⁰

164 Finally, bacterial contaminants often invade cultures of algae, as they are able to scavenge
165 algal exudates, which provide a source of carbon. If the bacteria compete with algae for
166 other nutrients, they often overtake the growth of the microalgae and can lead to the
167 establishment of anoxic conditions (REFS by Val). Bacterial fouling (surface growth) is very
168 severe in closed bioreactors, requiring these systems to be shut down and fully flushed
169 before operation can resume. This leads to yield losses and has an associated financial
170 burden. We have previously suggested that bacterial contamination may be decreased
171 through co-culturing algae with symbiotic (probiotic) bacteria that enhance algal growth.²¹
172 When bacteria are present in the culture medium, invading bacteria are less likely to
173 establish as the bacterial niche is already occupied. There is some empirical evidence from
174 fish aquaculture that supports this theory. For example, Sharifah and Eguchi report that
175 *Roseobacter* clade bacteria that are symbiotic with *Nannochloropsis oculata* (grown
176 commercially for fish food) successfully inhibited the growth of the fish pathogen *Vibrio*
177 *anguillarum*.²²

178 **2.3 Capitalising on mutualisms**

179 There is a range of ways in which it is possible to capitalise on mutualisms in industrial
180 biotechnology of microalgae. Mutualistic exchange of metabolites can replace external
181 inputs of scarce or expensive resources. For example, half of all algae are known to require
182 vitamin B₁₂ (cobalamin) for growth, while no eukaryotic microalgae are able to synthesise it.
183 Model laboratory consortia have been described in which vitamin B₁₂ dependent algae can
184 obtain cobalamin from vitamin B₁₂-synthesising bacteria, in exchange for a source of fixed

185 carbon,^{21,23} and indeed in the case of the *Dinoroseobacter shibae* partnership with its
186 dinoflagellate host, vitamin B₁ is also exchanged (Figure 3A). If this system were to be
187 employed industrially, the bacteria could replace exogenous addition of vitamins into the
188 medium, reducing material and energy inputs into the system. Other described mutualisms
189 include the provision of iron via siderophores from bacteria to algae in exchange for fixed
190 carbon.²⁴

191 It is possible to envisage a system where the mutualism between algae and bacteria
192 depends on provision of nitrogen by the bacteria, a macronutrient that is acknowledged as
193 one of the key drivers of microalgal productivity in natural systems.^{25,26} Modelling the
194 potential for algal biodiesel production in the USA indicated that the availability of nitrogen
195 and phosphorus fertilisers were the major limiting factors to large scale cultivation.²⁷ In a
196 recent study, *Azotobacter vinelandii*, a nitrogen-fixing bacterium, was genetically engineered
197 to excrete ammonium into the surrounding medium.²⁸ When the strain was co-cultured in
198 medium that did not contain exogenous carbon or nitrogen with oil producing microalgae
199 including *Chlorella sorokiniana*, *Pseudokirchneriella sp.* and *Scenedesmus obliquus*, the algae
200 were able to grow and accumulated lipid of up to 30% of their dry weight (Figure 3B). This
201 shows the potential for growing algae industrially in the absence of nitrogenous fertiliser
202 input by co-culturing with appropriate bacteria. As nitrogenous fertiliser is made through the
203 energy-intensive Haber-Bosch process that has been estimated to contribute up to 40% of all
204 energy inputs into microalgae biofuel systems,²⁹ provision of nitrogen *via* a symbiont could
205 significantly reduce the lifecycle energy and carbon footprint of the resulting fuel. It must be
206 noted that a sustainable alternative could be to grow algae on waste water that is rich in
207 nitrogen and phosphorus, thus recycling nutrients from domestic and agricultural effluent.³⁰

208 It is likely that the range of options for co-culturing algae with bacteria will increase as our
209 understanding of inter-specific interactions between these organisms improves. Evidence
210 suggests that microalgal interactions with bacteria are ubiquitous, although the physiological
211 basis for these is often not known. For example, Park et al. describe that 6 out of the 8
212 contaminants isolated from a *Chlorella elipsoidea* culture enhanced algal growth when co-
213 inoculated with the species in a controlled co-culture.³¹ Similarly, Do Nascimento et al.
214 described that the inoculation of *Rhizobium* strain 10II into cultures of oleaginous
215 microalgae *Ankistrodesmus* sp. strain SP2-15, resulted in up to 30% increased accumulation
216 of chlorophyll, biomass and lipids compared with axenic monocultures of the alga.³² The
217 bacteria influenced the metabolism of the microalgae, redirecting it towards lipid
218 accumulation.

219 **2.4 Improving the persistence of a desired strain**

220 A similar degree of regulation has been observed in the specific mutualism between the
221 vitamin B₁₂-dependent green alga *Lobomonas rostrata* and the soil bacterium
222 *Mesorhizobium loti*, where the ratio of algal to bacterial numbers equilibrated to around
223 1:30 in semi-continuous co-culture.²¹ ~~Regulation can be defined in accordance with Smith
224 and Douglas (1987) whereby a state of balance and stability between two organisms' growth
225 and population numbers is reached as a result of their symbiosis (living together).³³~~
226 Mathematical modelling of the dynamics of the two species in coculture revealed that the
227 population growth of one organism could be predicted entirely based on the expected
228 carrying capacity of the cocultured symbionts Grant et al. (2014) . Although the mechanism
229 remains unknown, the biological implication is that the symbionts are controlling the
230 amount of each other's growth when in coculture. Understanding regulatory mechanisms in

231 symbioses can benefit biotechnology by providing a mechanism for maintaining the long
232 term maintenance of a culture and its fidelity. If the growth of a desired algal is regulated by
233 a bacterium (or vice-versa) yields can be maintained despite a changing environment.

234 Environmental fluctuation, such as temperature and irradiance changes, is inevitable in all
235 large scale production systems, and could exert a selection pressure for a community of
236 algae to change from what is optimal for production (for example away from producing high
237 yields of lipids). Furthermore, if genetically modified organisms are considered, a changing
238 environment may exert pressure for the transformed strains to revert back to their original
239 form (the wild type) or drift randomly to an alternative genetic composition. For example, a
240 recent large scale effort to re-sequence strains of wild type *Synechocystis* sp. PCC6803
241 (originally from Berkeley as described by Stanier et al., 1971) maintained in various culture
242 collections around the world revealed that strains that had been presumed identical had in
243 fact accumulated mutations that are likely to have effects on glucose tolerance, metabolism,
244 motility, phage resistance and stress responses.^{34,35}

245 Culturing organisms that have been genetically engineered to be interdependent might
246 provide a selection pressure to prevent reversion, which would decrease the fitness of both
247 partners in the consortium. In fact, it was recently shown that engineered co-dependence is
248 stable even against the evolution of “cheaters” within the system,³⁶ although modelling
249 studies suggest that when the cost of cooperation is very high revertants will dominate.^{37,38}
250 Nonetheless it has been argued that co-dependence is so valuable to production it should be
251 genetically engineered.³⁹ Hosoda et al. engineered a syntrophic (cross-feeding) community
252 of *Escherichia coli*, where 2 strains co-habited: one auxotrophic for isoleucine and the other
253 for leucine.⁴⁰ Neither strain was able to survive on its own, but growth was possible in

254 synergistic co-culture. Kerner et al. engineered a similar system, where *E. coli* were either
255 tyrosine or tryptophan auxotrophs, but improved on the previous attempts by introducing
256 an element of control to the system.⁴¹ By tuning the metabolic exchange via gene expression
257 or chemical inducer they were able to regulate the growth rates and strain ratios. Finally,
258 more recently engineered inter-species associations have been demonstrated successfully.
259 An *E. coli* strain auxotrophic for glutamine was engineered to provide lipolic acid to
260 *Dictyostelium discoideum*, an amoeba, in exchange for the amino acid.⁴²

261 **3. Towards designing algal communities**

262 There is increasing awareness amongst the scientific community that microorganisms are
263 very social. Evidence is continuously emerging to demonstrate that microorganisms rely on
264 interactions with other species for a range of functions and communicate and cooperate to
265 perform activities such as dispersal, foraging, construction of biofilms, reproduction,
266 chemical warfare, and signalling.⁴³ Interactions range from necessary or advantageous to
267 growth, to competitive or even fatal. Ignoring the importance of interspecific interactions in
268 biotechnology dismisses the problems associated with contamination and misses the
269 opportunity to capitalise on the beneficial associations that can be harnessed to maximise
270 productivity.

271 We have identified four main advantages for using community approaches for the
272 cultivation of microalgae. It is possible to increase productivity of microalgal cultures (by
273 cultivating consortia of species that have complementary functional traits and therefore
274 overyield) or to decrease loss of productivity, by cultivating microalgae with species from
275 other life domains (such as non-photosynthetic bacteria and zooplanktivores), which can
276 increase resistance to predators and contaminants. We have highlighted the importance of

277 engineering co-dependence amongst introduced members to the consortium via mutualisms
278 with the benefit of reducing energy and material inputs. Finally, in agreement with Brenner
279 et al.³⁹ we believe that for a stable and robust culture, whenever a new organism is
280 introduced into a consortium, it should be contributing something useful to the culture
281 'economy' alongside receiving something in return for example through the division of
282 labour or specialisation. In that way interacting organisms rely on each other through trading
283 to establish a stable and long-lasting culture.

284 Of course the use of consortia of microbes in biotechnology is not novel; multi-species
285 systems are often employed to increase yields in microbial-based processes such as
286 anaerobic digestion, fermentation and bioremediation (reviewed in Sabra et al.)⁴⁴. In these
287 traditional systems microbial communities are allowed to develop naturally; the most
288 efficient assemblages are chosen for application and subsequently carefully maintained.
289 Although this approach is not common in algal biotechnology, recently Mooij et al.
290 demonstrated that by providing a selection pressure for algae to accumulate storage
291 compounds linked directly to fitness, communities rich in starch and/or lipid assembled
292 stochastically, and were able to outperform monocultures of known lipid producers.⁴⁵

293 These directed selection approaches will prove very useful to understanding the complex
294 and advantageous interactions of microorganisms. In parallel to these efforts, we proposed
295 a *Synthetic Ecology* approach to consortium assembly of cultures aimed to be more
296 productive and/or more resistant to contamination (15)(Kazamia et al., 2012a). Synthetic
297 ecology differs from the selection approaches by introducing an element of design and using
298 transferrable building blocks (namely specific species, engineered symbioses and growth
299 conditions) to assemble a desired community of microorganisms. ~~We believe that by~~

300 ~~focusing on species specific interactions and engineered metabolic exchanges we can~~
301 ~~advance the understanding of fundamental microbial physiology without compromising on~~
302 ~~creative solutions for biotechnology.~~ However, with all community approaches to
303 cultivation, their efficacy remains questionable until proven at scale. Stability of an
304 engineered consortium may face the same challenges as monocultures. A range of
305 unanswered questions remain: such as how much complexity within a consortium is
306 required before challenges faced by monocultures (instability, invisibility etc.) are
307 surpassed?

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