Title: An engineered community approach for industrial cultivation of microalgae

Authors: Elena Kazamia[1*], Anthony S. Riseley[2], Christopher J. Howe[2] and Alison G. Smith[1]

Affiliations:

1. Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, United Kingdom.
2. Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, United Kingdom.

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

*Corresponding author information:
Dr Elena Kazamia
Ek288@cam.ac.uk
+441223330219

Plant Metabolism Group
Department of Plant Sciences
University of Cambridge
Downing Street
Cambridge
CB2 3EA
United Kingdom

Abstract

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

1. Background

Microalgae (eukaryotic photosynthetic microbes) and cyanobacteria (oxygenic photosynthetic bacteria), are a highly diverse collection of micro-organisms. They live in a range of environments, including all aquatic ecosystems, both fresh-water and marine, and species are also found in terrestrial habitats including on hard surfaces and snow. Many taxa are capable of growing heterotrophically as well as phototrophically, and some obligate heterotrophs also exist that, although ancestrally photosynthetic, have lost the ability to photosynthesise. These include the dinoflagellate *Crypthecodinium cohnii*, which is of commercial importance as a source of docosahexaenoic acid (DHA).[1] Algae are currently cultivated on a relatively small scale for high value products such as the carotenoid astaxanthin from *Haematococcus pluvialis* and the phycobiliprotein phycocyanin from the cyanobacterium *Aphanizomenon flos-aquae*.[2] Certain strains are marketed as dietary supplements, such as the cyanobacterium *Spirulina* sp. (*Arthrospira platensis*) and *Chlorella vulgaris*.

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

2. Applying community ecology to algal cultivation

Most studies that target increasing yields in industrial cultures are aimed at an individual species level, which assumes that cultivation will be in monoculture. However, because contamination is inevitable without stringent sterile practice, which is neither cost-effective nor likely to be achievable at industrial scale,[7] understanding the growth dynamics of an algal population growing in reactors is fundamentally an ecological problem.[8] Moreover, monocultures are by their nature unstable and prone to perturbation. Their genetic uniformity encourages quick proliferation of pathogens and invaders, a common problem for traditional single-crop agriculture (reviewed in Smith et al., 2014)[9]. Monocultures are predicted to be unstable by classical theories of community ecology, which describe natural systems as increasing in complexity over time (e.g. Elton, 1958)[10]. Given the chance, for a given habitable environment, multiple species with diverse niche specificities will coexist alongside each other, maximising the use of the available resources. “Invasions” by organisms from neighbouring environments will continue until a “climax” stable state is assembled, which is predicted to be resilient to change provided abiotic conditions remain constant (May, 1977)[11].

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

2.1 Maximising productivity

One of the tenets of community ecology is that productivity is enhanced when diverse organisms are grown together. This has been illustrated for a range of habitats, and famously in a long-term experiment on grasslands. For a period of seven years, it was demonstrated that 16-species grassland plots attained 2.7 times more biomass than the respective monocultures.[9] An aquatic experiment showed that diverse algal communities (grown in biofilms) increased the uptake and storage of nitrate from streams, and significantly increased in biomass content compared to monocultures.[10] *Overyielding* is said to occur when the biomass production of a consortium of species is greater than that of the average monoculture of the species contained in the mixture.[11] *Transgressive* overyielding is said to occur when the mixture outperforms even the most productive of the monocultures of the constituent species.[12] There is evidence that, when functionally diverse algae ~~with complementary light requirements~~ are grown together, the resulting communities are more productive than monocultures of individual species. Behl et al. analysed the rate of carbon uptake and productivity for 85 assembled communities composed of species from four functional groups: chlorophytes, diatoms, cyanobacteria and chrysophytes.[13] The researchers found that all algal communities consisting of species from two, three or four different functional groups showed overyielding compared with their respective monocultures, with transgressive overyielding in more than half of the assemblages studied. ~~This is interesting as it suggests that positive interactions beyond resource use complementarity occurred between species. A possible way this could occur is through mutualistic interactions, reviewed in Section 2.3 below.~~

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

2.2 Crop protection

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

The effect of predators can be decreased through biomanipulation of the food web, whereby an ecosystem is deliberately altered by adding or removing species. This is common practice in the freshwater management industry, where the goal is to minimise algal production.[14] In the context of algal cultivation, which is the reverse scenario, if production were to be hampered by invading zooplankton, the addition of zooplanktivores (such as small fish) to the reactors might increase yields.[8,15] However, this is unlikely to be possible for closed photobioreactors, but may also not be practical in open ponds because most reactors are very shallow, and would not be suitable for fish. An alternative solution is that of crop protection through “interference”. By introducing multiple inedible algal species to grow alongside the desired strain, the foraging efficiency of invading zooplankton may be decreased due to the increased energetic costs of finding their desired prey.[16] This technique of pest control was recently investigated by Shurin et al. in a set of laboratory experiments, the results of which are summarised in Figure 2.[17] Communities containing 1, 2, 5 and 10 species of algae in various combinations were subjected to grazing by *Daphnia pulex*. Although the total biomass of algal food resources increased with diversity, survival of introduced *Daphnia* grazers declined markedly when 5 or 10 species of algae were grown together.

However, there may be a cost to co-cultivation of a range of algal species when only a single species is of commercial interest. It is possible to imagine a scenario where the growth of a desired strain is decreased in a dense polyculture due to increased shading by co-cultured strains. Where stability of a monoculture against invasions is the primary concern, this may be enhanced by manipulating the abiotic environment to make the establishment of competitors less likely. This is why extremophiles have been preferred in commercial cultures, such as *Spirulina* sp., which is grown in highly alkaline conditions, or *Dunaliella salina,* which is cultured in highly saline medium. A community solution to the problem of competitors may be engineered through co-culturing with partners that produce allelopathic chemicals. Chemical interactions are an important part of phytoplankton competition and are particularly functionally important with dinoflagellates and cyanobacteria.[18] These organisms are able to produce chemicals that are toxic to most other algae in the environment, allowing the former to bloom under the right conditions for growth, often causing what are known as Harmful Algal Blooms, HABs.[19] However, some species have evolved to withstand the toxins produced during HABs and are able to cohabit with the toxin producing strains. If either HAB-forming or HAB-tolerant species were identified as interesting candidates for biofuel production, growth in consortia with toxin producing strains could be a possible solution to competitive invasion. A similar approach is taken in water treatment, where often barley straw is used to control populations of unwanted algae. Toxins produced from the straw liquor are known to inhibit the growth of some algae but not others.[20]

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

2.3 Capitalising on mutualisms

There is a range of ways in which it is possible to capitalise on mutualisms in industrial biotechnology of microalgae. Mutualistic exchange of metabolites can replace external inputs of scarce or expensive resources. For example, half of all algae are known to require vitamin B_{12} (cobalamin) for growth, while no eukaryotic microalgae are able to synthesise it. Model laboratory consortia have been described in which vitamin B_{12} dependent algae can obtain cobalamin from vitamin B_{12}-synthesising bacteria, in exchange for a source of fixed carbon,[21,23] and indeed in the case of the *Dinoroseobacter shibae* partnership with its dinoflagellate host, vitamin B_1 is also exchanged (Figure 3A). If this system were to be employed industrially, the bacteria could replace exogenous addition of vitamins into the medium, reducing material and energy inputs into the system. Other described mutualisms include the provision of iron via siderophores from bacteria to algae in exchange for fixed carbon.[24]

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

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

2.4 *Improving the persistence of a desired strain*

A similar degree of regulation has been observed in the specific mutualism between the vitamin B_{12}-dependent green alga *Lobomonas rostrata* and the soil bacterium *Mesorhizobium loti*, where the ratio of algal to bacterial numbers equilibrated to around 1:30 in semi-continuous co-culture.[21] ~~Regulation can be defined in accordance with Smith and Douglas (1987) whereby a state of balance and stability between two organisms' growth and population numbers is reached as a result of their symbiosis (living together).[33]~~ Mathematical modelling of the dynamics of the two species in coculture revealed that the population growth of one organism could be predicted entirely based on the expected carrying capacity of the cocultured symbionts Grant et al. (2014) . Although the mechanism remains unknown, the biological implication is that the symbionts are controlling the amount of each other's growth when in coculture. Understanding regulatory mechanisms in symbioses can benefit biotechnology by providing a mechanism for maintaining the long term maintenance of a culture and its fidelity. If the growth of a desired algal is regulated by a bacterium (or vice-versa) yields can be maintained despite a changing environment.

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

Culturing organisms that have been genetically engineered to be interdependent might provide a selection pressure to prevent reversion, which would decrease the fitness of both partners in the consortium. In fact, it was recently shown that engineered co-dependence is stable even against the evolution of “cheaters” within the system,[36] although modelling studies suggest that when the cost of cooperation is very high revertants will dominate.[37,38] Nonetheless it has been argued that co-dependence is so valuable to production it should be genetically engineered.[39] Hosoda et al. engineered a syntrophic (cross-feeding) community of *Escherichia coli*, where 2 strains co-habited: one auxotrophic for isoleucine and the other for leucine.[40] Neither strain was able to survive on its own, but growth was possible in synergistic co-culture. Kerner et al. engineered a similar system, where *E. coli* were either tyrosine or tryptophan auxotrophs, but improved on the previous attempts by introducing an element of control to the system.[41] By tuning the metabolic exchange via gene expression or chemical inducer they were able to regulate the growth rates and strain ratios. Finally, more recently engineered inter-species associations have been demonstrated successfully. An *E. coli* strain auxotrophic for glutamine was engineered to provide lipoic acid to *Dictyostelium discoideum,* an amoeba, in exchange for the amino acid.[42]

3. Towards designing algal communities

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

We have identified four main advantages for using community approaches for the cultivation of microalgae. It is possible to increase productivity of microalgal cultures (by cultivating consortia of species that have complementary functional traits and therefore overyield) or to decrease loss of productivity, by cultivating microalgae with species from other life domains (such as non-photosynthetic bacteria and zooplanktivores), which can increase resistance to predators and contaminants. We have highlighted the importance of engineering co-dependence amongst introduced members to the consortium via mutualisms with the benefit of reducing energy and material inputs. Finally, in agreement with Brenner et al.[39] we believe that for a stable and robust culture, whenever a new organism is introduced into a consortium, it should be contributing something useful to the culture 'economy' alongside receiving something in return for example through the division of labour or specialisation. In that way interacting organisms rely on each other through trading to establish a stable and long-lasting culture.

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

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

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

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