Material Architectures for Improved Light and Carbon Supply in Microalgal Photobioreactors

by

Scott C. Pierobon

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Graduate Department of Mechanical & Industrial Engineering
University of Toronto

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This thesis explores the use of materials to improve light and carbon supply to microalgal cultures. These resources are the primary growth factors that limit productivity and, ultimately, the cost of biomass generation. Three unifiable approaches to supply are separately studied with performance evaluations based on culture densities achieved with the cyanobacteria *Synechococcus elongatus*, relative to controls. The potential performance of these supply approaches are compared with conventional approaches reported in the literature. The first study explores light supplied from thin, planar, glass waveguides that illuminate surface-grown biofilms using frustrated total internal reflection (FTIR). A biofilm with uniform density \(1.0 \pm 0.3 \times 10^9 \text{mL}^{-1}\) grew in a wide range of intensities that exceeded the high and low limits for suspension cultures. This extended growth regime is explained by semi-coherent, high-intensity light confinement in the waveguide, combined with intensity-smoothing by biofilm morphology. The performance suggests potential for greater growth areas and intensity ranges, with volumetric module densities on the order of \(10^8 \text{mL}^{-1}\). However, glass and other gas-impermeable materials used in reactors to improve light supply often negatively affect carbon supply and vice-versa. The second study explores materials that permit combined waveguide illumination and CO\(_2\) permeation. Use of cellulose acetate butyrate (CAB) waveguides doubled the density of suspension cultures relative to impermeable waveguides, but required elevated CO\(_2\). Fixed biofilm cultures, compatible with the established light and carbon supply approaches, can provide greater photosynthetic efficiency, but productivities suffer from respiration in the dark portions of the biofilm. The third study then uses cultures fixed in calcium-
alginate hydrogels to demonstrate photoactive light penetration in the low density bulk. Over time, microalgal growth within the hydrogel produces a thin, high-density surface layer due to high local light and CO₂ access. This hydrogel approach presents the opportunity for partial, skim-harvesting of this top layer. The effect on production of periodically harvesting the dense top layer was quantified. Over 18 days of cultivation, productivity was greatest in hydrogels formed with 150 mM Ca²⁺, and subject to 3-day harvest periods – especially in the first layer. Harvested biomass yields became more uniform with additional harvests. This growth format permits thick cultures that both experience photoactive light and produce at high density. Each of these three approaches has demonstrated improvements to light and/or CO₂ delivery to microalgal cultures, leveraging unique materials and approaches. Collectively, this thesis demonstrates proof-of-principle for next-generation approaches to achieving high-density microalgal photobioreactors.
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# List of Abbreviations

<table>
<thead>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>2PG</td>
<td>Phosphoglycolate</td>
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<tr>
<td>3PGA</td>
<td>3-phosphoglycerate</td>
</tr>
<tr>
<td>BPBR</td>
<td>Biofilm-based photobioreactor</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>CAB</td>
<td>Cellulose acetate butyrate</td>
</tr>
<tr>
<td>CCM</td>
<td>Carbon-concentrating mechanisms</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CO₃²⁻</td>
<td>Carbonate</td>
</tr>
<tr>
<td>Fd</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>FNR</td>
<td>Ferredoxin-NADP reductase</td>
</tr>
<tr>
<td>FTIR</td>
<td>Frustrated total internal reflection</td>
</tr>
<tr>
<td>H₂CO₃</td>
<td>Carbonic acid</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>Bicarbonate</td>
</tr>
<tr>
<td>HFM</td>
<td>Hollow fiber membrane</td>
</tr>
<tr>
<td>ISP</td>
<td>Illuminated surface biomass productivity</td>
</tr>
<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OD₇₅₀</td>
<td>Optical density at 750 nm</td>
</tr>
<tr>
<td>PBR</td>
<td>Photobioreactor</td>
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<tr>
<td>PE</td>
<td>Photosynthetic efficiency or light use efficiency</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>PPFD</td>
<td>Photosynthetic photon flux density</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinone</td>
</tr>
<tr>
<td>PQH2</td>
<td>Plastoquinone (reduced)</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>RuBisCO</td>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>STP</td>
<td>Standard temperature and pressure</td>
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<tr>
<td>TIR</td>
<td>Total internal reflection</td>
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<tr>
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<td>Total internal reflection fluorescence microscope</td>
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List of Appendices

Appendix 1: Evanescent photosynthesis: exciting cyanobacteria in a surface-confined light field

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1 Motivation

1.1 Climate change

The effects of climate change from global warming threaten global environmental and economic stabilities. The leading factor causing global warming is rising anthropogenic greenhouse gas emissions of mainly CO₂ from fossil fuel combustion. To mitigate these negative effects, a technological migration away from energy generation based on fossil fuel resources is necessary. The two major energy generation sources of global CO₂ emissions are concentrated at coal-fired electricity generation plants (see figures 1.1 and 1.3), and globally distributed among transportation modes - in descending order, as car use, road freight, aviation and shipping (see figures 1.1 and 1.2). A global migration away from coal-based electricity would benefit from already mature industries of alternative and renewable electricity generation (as indicated in figure 1.3) though this would likely take place on the order of decades. In contrast, although a migration away from fossil fuel-based transportation would benefit from continued developments in hydrogen fuel cell and battery technologies for road transport, these sources currently lack the energy density of liquid fuels required for aviation, shipping, and some freight modes. Furthermore, in the extreme case of emission-free global road transport, the remaining emissions from aviation, shipping and rail modes would still amount to 5.4% of yearly global greenhouse gas emissions from fossil fuel combustion (2010 data). A more realistic goal set by the United States Department of Transportation, for example, is to replace 30% of fossil fuels used for national transportation with sustainably generated, carbon-neutral liquid fuels by 2030.
Figure 1.1: Global energy flow in 2010. Reproduced from reference 23 with permission from Elsevier, copyright 2010.

Figure 1.2: Global greenhouse gas emissions by transportation mode. Source: IPCC Fifth Assessment Report, Working Group III, Climate Change 2014: Mitigation of Climate Change.
Figure 1.3: Lifecycle greenhouse gas emissions from electricity supplied by commercially available technologies. Source: IPCC Fifth Assessment Report, Working Group III, Climate Change 2014: Mitigation of Climate Change.
In order to meet both the immediate need to reduce the rate of global CO\(_2\) emissions and the likely continued need of liquid fuels for transport, renewable liquid fuel production technologies are required that are economically feasible, scalable, and sustainable\(^{12,24-26}\). A promising technology is sunlight-powered ‘bottom-up’ assembly of fuels using atmospheric CO\(_2\)(g). Direct bottom-up approaches using solid-state technologies have yet to produce complex (energy-dense) hydrocarbons and lack scalability, but achieve light-to-chemical conversion efficiencies in the tens of percent\(^{27-29}\). In contrast, photosynthetic organisms already perform bottom-up assembly of complex chemicals from CO\(_2\) but achieve light-to-chemical conversion efficiencies of 0.1 to a few percent with a theoretical maximum of ~ 12\%\(^{30-32}\). Co-opting these organisms for sustainable liquid biofuel generation presents additional ‘top-down’ challenges of meeting global demand with economically feasible production systems. For example, the global liquid fuel energy demand for only aviation, shipping and rail modes of transportation is 24 EJ/yr (2010 data), in contrast to 5 EJ/yr currently used globally for biomass-based energy, mainly as openly burned raw plant matter.\(^{33}\) To meet the energy demand of the above transportation modes with microalgal biomass will require production of approximately 10\(^{12}\) kg/yr, assuming average values for the energy- and combusted CO\(_2\)-content of liquid fuels used by these modes (~44 MJ/kg\(_{\text{fuel}}\) and ~1.37 kg\(_{\text{CO}_2}/\text{kg}_{\text{fuel}}\) respectively).

The use of terrestrial plant biomass for biofuel production is unsustainable due to competition with arable land for food supply, considering a steadily increasing global population and the emerging freshwater crisis\(^{34,35}\). Nevertheless, plant-based biofuel production is currently a mature industry\(^{36}\). For example, Brazil and the United States produce 87\% of global plant-based ethanol which is used to replace 41\% and 10\% of their national gasoline use for transportation, respectively (2011 data)\(^{36,37}\). Globally, plant-based ethanol replaces 7\% of gasoline-based transportation fuel by volume.\(^{37}\) In contrast, microalgae (single celled algae and cyanobacteria) do not require freshwater or arable land for cultivation, so these organisms are a sustainable source of biofuel\(^{38,39}\). Currently, microalgal biofuels are mainly generated by upgrading biomass lipids to biodiesel, though some operations use live microalgae to directly produce fuel (e.g. cells that excrete ethanol)\(^{40-43}\). The industry of microalgal biofuel production is still in its infancy, with a few businesses that are limited both in scale and economic success by the high costs of production\(^{44-48}\).

### 1.2 Economics

Microalgal biomass is produced in photobioreactors (PBRs), which enable control over key cultivation parameters of culture contaminants, temperature, pH, dissolved micronutrients, light and carbon (mainly as CO\(_2\))\(^{49}\). Reactors open to the environment (namely pond style) are less costly but
provide less control and so are less efficient and productive than closed photobioreactors (namely tube, column, or flat panel style)\textsuperscript{50}. Currently, microalgae in commercial and pilot plant scale reactors are cultivated as suspension cultures (free-floating cells)\textsuperscript{44}.

The economic success or failure of photobioreactor operation is determined by the overall cost of biomass, evaluated over the lifetime of the reactor, at a given scale of production. The generation of high-value biomass products in low-cost pond reactors has enabled several long-term, commercial-scale operations with demonstrated economic success; product examples include cosmetics, nutrition supplements, pigments, animal/aquaculture food\textsuperscript{51–53}. For relatively low-value biodiesel generation, however, there are only a limited number of well-reported studies in the research literature, of pilot-plant scale operations, each of which demonstrates different reactor configurations, operation parameters, or biomass processing pathways. Economic assessment of commercial-scale biomass production is thus difficult\textsuperscript{22,54}. In attempts to estimate the cost of commercial-scale biodiesel production, several techno-economic assessment studies have modeled lifetime production factors using normalized pilot plant data from the research literature. Although the methods used in these modeling studies inconsistently use system boundaries and result in large uncertainties, sometimes with non-comparable units, the studies together suggest a microalgal oil price point of 0.3 – 8 $US/L\textsuperscript{22,46,54–56}. This is an impressive figure, considering a two orders of magnitude greater price predicted two decades ago\textsuperscript{57}, and the current conventional diesel market price of \textasciitilde0.7 $US/L\textsuperscript{11}.

Despite the clear economic trajectory of microalgal biodiesel production towards a competitive price point with conventional diesel\textsuperscript{55}, current techno-economic assessment studies conclude that biodiesel production using modern photobioreactors is not profitable when reactors are operated in isolation at scale\textsuperscript{45,58–60}. Conversely, many studies indicate much improved chances for economic feasibility when reactor operations are integrated with other proven industries (e.g. wastewater treatment) or profitable operations (e.g. use of residual biomass to generate high-value products)\textsuperscript{48,61–67}. In both cases, the physical integration of photobioreactors with existing infrastructure helps mitigate the high up-front capital cost of photobioreactor facilities, which has been a major factor preventing initial investment\textsuperscript{48,68}.

For isolated photobioreactor operations, however, sensitivity analyses of techno-economic assessment studies consistently identify a key set of cost factors that largely prevent economic biodiesel production in pilot plant operations\textsuperscript{22,45,54,55,59,60,69,70}. The relative importance of these cost factors varies by study, and some of the largest do not directly relate to photobioreactor capital and
operational expenses, such as yearly operating factor (limited by sunlight), intrinsic culture performance (i.e. biomass productivity determined by cell-level light and carbon utilization), and facility construction expenses where considered separate from operation costs. The key photobioreactor capital and operational cost factors are presented below and, where possible, literature-reported ranges of their cost fractions are presented relative to the energy-equivalent lifetime of reactor operation, or to the energy used for cultivation only; they are: reactor maintenance and labor, reactor materials, biomass de-watering and lipid extraction, pumping energy, elevated CO₂ supply, and micronutrient supply.

- Reactor maintenance (16 – 21%) and labor (5 – 11%) are reported as two of the most significant cultivation energy costs.

- Capital costs for reactor materials are an embodied energy cost, reported as 6 – 95% of lifetime energy.

- De-watering and lipid extraction costs are commonly reported in the range 5 – 30% of the overall energy cost of production. After the de-watering and extraction stages, biomass upgrading costs to fuel are reported as 1 – 10% of total production energy.

- Electrical energy costs for pumping are significant. Most of this energy is used to overcome friction losses in order to achieve the flow rates required to circulate culture in closed and especially tube style reactors. The remainder of this energy is mainly directed to gas-pressurization for culture aeration by bubbling. These combined costs are reported as 22 – 92% of cultivation energy, or 1 – 4% of total production lifetime cost.

- Carbon-delivery costs are also significant. The source of CO₂ from flue gas or imported tanks is reported as 2 – 5% of lifetime operation cost and ~ 45% of cultivation energy cost.

- Micronutrient supply costs of nitrogen and phosphorous are reported as 6 – 40% of cultivation energy cost.

The large variability in the above costs reflects the continued need for parametrized data from pilot plant facilities with consistent, comparable designs, for the validation of lifecycle assessment models. In addition to the above cost factors, the two most important reactor design factors that influence biomass productivity, and so biomass cost, are culture-level efficiencies of light and carbon utilization.
Pilot-plant studies consistently demonstrate greater biomass productivities in reactors that better control the intensity range of light in cultures \(^{46,80,82}\). At this scale, control is enabled by the physical aspect ratios of the reactor. A ratio of culture area to ground reactor footprint on the order of ten reduces incident solar intensities to photosynthetically efficient intensities, whereas larger ratios of illuminated area to culture volume define a shorter light path length that improves photosynthetic efficiency by limiting the low intensity extreme in the culture \(^{46,80,83}\). Also, the efficiency of pilot plant \(\text{CO}_2(g)\) utilization by bubble delivery ranges from 4 to 90% \(^{58,60,69,76,79}\). The highest \(\text{CO}_2\) utilization efficiencies are observed in reactors that provide high-pressure, fine bubbles, with long residence times enabled by tall, vertical layouts \(^{79,81,84-86}\). Reactors that are thin and vertically oriented then achieve the highest light and \(\text{CO}_2\) utilization efficiencies \(^{46,80}\).

Specifically, vertical flat panel and tube style reactors achieve the greatest combination of light and \(\text{CO}_2\) utilization efficiencies, biomass productivities, and photosynthetic (light-to-chemical) efficiencies of \(\sim 2 - 3\%\) \(^{46,68,80,85,87,88}\). However, techno-economic studies that consider the capital and operational cost factors outlined above have inconsistently assessed these designs as being more \(^{46,89,90}\) or less \(^{77,88}\) cost-effective for biomass production than open pond and closed, horizontally oriented reactors. Recent insights into light- and carbon-related production factors and continued technology development are expected to resolve this inconsistency in the coming years \(^{46}\). For example, a recent analysis of vertical flat panel reactors identified optical path length and aeration rate as two of the most sensitive parameters for reducing the cost of biomass production, by 25 and 10\%, respectively, if each variable is halved (e.g. 2 to 1 cm optical path length) \(^{46,77,78}\).

Since microalgae are currently cultivated as suspension cultures in pilot plants, the volume of water managed by these photobioreactors is arguably the most far-reaching factor of the economics of biomass production \(^{68,91,92}\). Water management costs are intrinsic to each of the most important capital and operational cost factors outlined above, and culture volume is intrinsic to light- and \(\text{CO}_2\)-utilization efficiencies controlled by reactor aspect ratios, as just described. Specifically, reducing the water content of cultures lowers the costs of de-watering and lipid extraction, and reduces the size of reactors, which consequently lowers capital, maintenance, labor, and electrical energy costs. Simultaneously, reducing culture volume naturally informs modularly scalable, thin plate-style reactor designs \(^{93,94}\), with shorter optical paths and greater surface areas for greater light utilization efficiency and a greater potential for more efficient \(\text{CO}_2\) mass transfer to cells \(^{95}\). Factors affecting light- and \(\text{CO}_2\)-utilization efficiencies are presented in detail later (in sections 2.3, 2.4 and 2.5) along with a discussion of technologies for their supply, both currently used in conventional reactors and those developed in the work of this thesis. Cultures of large area and low water content are more
susceptible to thermal fluctuations that can reduce productivity \(^{96-98}\), which may necessitate additional insulation or thermal PID control in outdoor operations. However, the benefits of low water content are clearly demonstrated in studies of thin, flat panel outdoor pilot plant reactors, which can achieve the lowest total (operational and embodied) energy demands \(^{78}\), water use \(^{46,89}\) and frictional energy cost for culture circulation \(^{69}\).

To sustainably achieve economically scalable biodiesel production, the lifecycle cost of microalgal biomass generation must be considerably lowered. One techno-economic assessment study concluded that a 95% probability of economically successful biodiesel production using isolated pilot plant reactors requires 20-40% of current capital and 10% of current operation costs, combined with increased productivity that equivalently helps to offset these costs \(^{59}\). To efficiently reach these dramatic targets, a diversified research approach is needed. Novel photobioreactor technologies need to be explored alongside the continued development of conventional photobioreactor technologies based on suspension cultures, which have so-far placed the pilot plant cost of microalgal biodiesel within striking distance of the market price of conventional diesel \(^{11,22,46,54-57}\).

1.3 Thesis scope

Microalgae are tiny survival factories - they keep acquiring resources and generating products (themselves) until an essential resource is sufficiently depleted to slow growth \(^{99}\). To sustain this economically valuable production at high, non-limited rates, photobioreactors are used to manage the supply of resources to groups of microalgae. The most important resources by far are light and carbon (as \(\text{CO}_2\)), but, paradoxically, photobioreactors often supply these insufficiently, resulting in productivity- and density-limited cultivation. This limitation, combined with cultivation operation costs, has prevented economic production in pilot plant reactors \(^{48,50,100-102}\). One way to interpret the cause of insufficient supply rates is poor micromanagement of light and carbon supply paths to microscale groups of microalgae.

Conventional reactor designs manage source levels of light and carbon, but without much attention to their distribution in suspension cultures, coarsely managed by turbulent mixing. Newer reactor designs organize smaller, denser groups of microalgae and better control the supply paths of light and carbon, mainly by shortening them \(^{80,85,103}\). This development trajectory continues to improve reactor productivities, but pilot plant operation is still economically impeded by the high costs of managing large volumes of water containing small groups of microalgae associated with suspension cultures \(^{68}\). These costs are used to manage the dimensional mismatch between a volume of culture and its fundamentally area-based supply of light and gaseous \(\text{CO}_2\). To reduce water-associated costs
and manage resource supply to smaller, denser groups of microalgae, the field of photobioreactor design has recently started to develop a branch of reactors that cultivate microalgae as air-exposed surface biofilms, called porous-substrate photobioreactors (PSBRs) and previously “attached cultivation” and “twin-layer” (see figure 1.4). The high density of this culture format, compared to suspension cultures, poses a challenge for internal light and CO₂ distribution, but enables a minimal carbon supply path. Development of these reactors is currently in the proof-of-concept stage, in which biofilm cultivation approaches must first be proven by demonstrated productivities at laboratory and pilot plant scales before meaningful economic feasibility studies can be performed, for eventual performance comparison with existing photobioreactor technologies.

![Published articles per year](image)

Figure 1.4: Filtered Tomson Reuters™ Web of Science™ search results for peer-reviewed articles published between 1996 and 2016, containing the Boolean topic terms (a) “photobioreactor & microalgae & biofilm”, and (b) “photobioreactor & microalgae & (“twin layer” | “attached cultivation” | PSBR)”. Accessed December 2016.

In-keeping with this research trajectory (of micromanaging light and carbon in high-density cultures using short supply paths) and in response to the need for proof-of-concept studies, the thesis of this work is to demonstrate the utility of three light and carbon ‘supply architectures’ - defined here as the use-plus-operation of reactor materials - to support high-density microalgal cultures, while enabling modularly scalable reactor designs. Specifically, the presented studies utilize (i) thin, 160 µm glass sheets to supply light by frustrated total internal reflection; (ii) transparent nanoporous materials to simultaneously supply carbon and light by CO₂ permeation and scattered-light illumination; and (iii) periodically harvested air-exposed calcium-alginate hydrogels to achieve high-density production in a stratified density culture format that reduces respiration biomass
losses, compared to that of biofilms. In each study, the utility of the developed supply architectures is evaluated by the relative or absolute biomass productivity and growth morphology of the model microalgal cyanobacteria species *Synechococcus elongatus* PCC7942. Also, in the hydrogel study, architecture material costs over a short period of operation are reported and compared to that of PSBRs.

### 1.4 Thesis overview

Chapter 2 introduces standard photobioreactor performance metrics and operational considerations with respect to the optimal supply of light and carbon to microalgae, using a mathematical framework for discussion. The benefits and limitations of conventional and emerging culture supply technologies are discussed, including those developed in the work of this thesis. Considerations for organism-based evaluation of supply technologies are presented, and used to substantiate the choice of microalgal species used in all studies of this work. General light and carbon utilization factors of microalgae are discussed, with a focus on the specific utilization characteristics of this species.

Chapter 3 presents the light supply architecture that uses frustrated total internal reflection to illuminate microalgal biofilms. This chapter was published in the *Journal of Micromechanics and Microengineering*.

Chapter 4 presents the combined light- and carbon-supply architecture that uses the nanoporous, transparent material cellulose acetate butyrate to support growth of microalgal suspension cultures. This chapter was published in *Bioresource Technology*.

Chapter 5 presents the biomass productivity of periodically harvested microalgal hydrogel cultures. This chapter was submitted to *Bioresource Technology* in February 2017.

Chapter 6 summarizes the author’s contributions, identifies future work based on results of these contributions, and presents a future outlook for photobioreactor designs for industrial-scale microalgal production of low-value products.
2 Introduction

To begin, this chapter introduces the performance metrics used in the research literature to evaluate photobioreactor designs. The metrics then provide a basis for a discussion of general reactor operation modes and culture format factors that influence production, using a mathematical framework. Culture density is identified as a key production factor of both growth rate and cultivation time. The chapter is then split by the primary resources – light and carbon – into discussions of their culture-level supply. Optimal supply conditions are identified, and the benefits and limitations of current and emerging supply architectures are discussed. Detailed physical descriptions of the operating principles of supply architectures developed in this work are also included. Then, considerations for the choice of species and product used to evaluate supply architectures are discussed. The choice of species used in this work is substantiated. Specific effects of light and carbon supply on cell-level utilization by this species are presented, however, broad trends of these effects are valid for most microalgal strains.

2.1 Performance evaluation metrics

Literature studies of laboratory-scale photobioreactors are more numerous and cover a greater variety of designs and operation modes than those of pilot plant scale reactors. However, production and especially cost metrics evaluated at laboratory scales and limited operation periods are not generally predictive of pilot plant scale performances, at least due to challenges in assessment modeling (see Quinn and Davis (2015) for a review of current modeling methods). In these limited scale studies, the cost of generating biomass products is generally not quantified; instead, biomass productivity (the rate of biomass product generation) is considered the main performance metric. In addition to biomass productivity and cost, there are several standard literature metrics that enable fair performance comparisons of photobioreactors that may greatly differ in internal design, external layout, environment, operation scale, cultivation and harvesting schemes, and other operational parameters. In the focus of this work, on the performance effects of light and carbon supply and utilization in high-density reactor cultures, the following metrics are most useful:

BASE UNITS

- Ash-free dry weight of whole-cell biomass \( (g_{DW}) \), which corresponds to specific energy content \( (kJ/g_{DW}) \), depending on composition influenced by growth environment.
- **Area** (m²) of an illuminated reactor surface, or the ground occupied by a reactor (footprint).

- **Surface-to-volume ratio** (m⁻¹), which typically refers to illuminated surface area.

- **Optical path length** (m), which is the distance that light penetrates into a culture at which the intensity is some factor of the incident intensity. This factor is variably defined. Some examples include \(\frac{1}{2}, 1/e \approx 0.37\), or the light compensation point (see below).

### CULTIVATION

- **Growth rate** (d⁻¹), conventionally expressed as the *specific growth rate* (\(\mu\), d⁻¹) of the population on a per-cell bases in the exponential (also known as logarithmic) phase of microbial growth. This is not to be confused with the absolute growth rate, which is proportional to population size, \(N\), by the factor \(\mu^{99}\).

- **Lag time** (tₗₐ₉, h⁻¹) is the physiological acclimation period in which growth is slow or zero, occurring between the time of sudden cell exposure to a new environment, and the observed onset of exponential phase growth.

- **Cell density** (\(\rho\), mL⁻¹) is the number of cells per defined media volume.

- **Light compensation point** (\(\mu\)mol m⁻² s⁻¹) is the photosynthetic photon flux density (PFFD, \(\mu\)mol m⁻² s⁻¹) at which net photosynthesis is zero. The origin of the word 'density' in PFFD is due to the definition of flux in electromagnetism as a surface integral, instead of as a flow rate per area in the conventional mass-transfer sense. For the species used in this work, the light compensation point is approximately \(4 - 20 \mu\)mol m⁻² s⁻¹\textsuperscript{120,121}.

- **Light dilution factor** (no units) is the ratio of the indecent light surface area of a reactor to the reactor’s ground footprint area. For sunlit reactors, increasing the light dilution factor above unity is essential to improve overall photosynthetic productivity\textsuperscript{46,80}.

### HARVESTING

- **Cultivation period** (tₚₑ₉ᵣᵩ, d). For reactors that periodically harvest biomass, this is the duration of culture growth between harvest iterations, and is also known as the harvest period. Reactor downtime not related to periodic harvesting also enables this definition.
- **Dilution rate** \((D, s^{-1})\). For reactors of constant volume that continuously harvest biomass, this is the volumetric rate of biomass density removed, relative to the total volume of the culture.

**COST**

- **Cost of biomass** \((\$/kg)\) is implicitly an ash-free dry weight metric, though the subscript is often omitted in the literature \(^{41}\). Herein, currency is represented by the dollar sign.

**PRODUCTIVITY/EFFICIENCY**

- **Volumetric productivity** \((p, \text{g DW L}^{-1} \text{d}^{-1})\) of the liquid volume of the reactor.

- **Illuminated surface biomass productivity** \((\text{ISP, gDW m}^{-2} \text{d}^{-1})\) and **areal biomass productivity** \((\text{gDW m}^{-2} \text{d}^{-1})\). Care should be taken to distinguish between these when reactors are subject to constant light dilution factors (e.g. tilted panels with respect to external light capture), or do not occupy the full solar footprint (e.g. tube facilities).

- **Biomass yield on light** \((\frac{\text{gDW m}^{-2} \text{d}^{-1}}{\text{μmol m}^{-2} \text{d}^{-1}})\) is defined here in terms of absorbed **photosynthetically active radiation** (PAR, 400 - 700 nm) at a specific **photosynthetic photon flux density** (PFFD, μmol m\(^{-2}\) s\(^{-1}\)). However, other definitions exist in the literature \(^{122}\).

- **Biomass yield on CO\(_2\) or CO\(_2\) utilization efficiency** \(\left(\frac{\text{gDW m}^{-2} \text{d}^{-1} \left(\frac{\text{g carbon}}{\text{g DW}}\right) 44 \text{ g mol}^{-1}}{\text{gCO}_2 \text{m}^{-2} \text{d}^{-1}}\right)^{85,86}\), in which the rate of CO\(_2\) supply (denominator) may be alternatively expressed volumetrically, and biomass productivity (numerator) is expressed as carbon productivity using the molar mass ratio of CO\(_2\):C and the ratio of relevant carbon content to dry-weight biomass that embodies a final product. An accurate measure of relevant carbon content contained in cells requires knowledge of the molecular composition of the species at specific cultivation conditions, or, for products excreted by cells, knowledge of the biochemical pathways from consumed carbon to a final product. For biodiesel production based on cell biomass, **lipid content (%)** is currently the closest of such a measure, at least because it has been extensively quantified in many species at various cultivation conditions that influence the expression of fuel-upgradable lipids \(^{53,123}\).

- **Photosynthetic efficiency** or **light use efficiency** \((\text{PE, kJ biomass kJ light}^{-1})^{124}\), is equal to the biomass yield on light, expressed on an energy basis. Care must be taken to define both the light and biomass energy. The energy content of biomass can be of a specific molecule, or of a collection of molecules
in whole-cell biomass, which crucially depends on the species and cultivation conditions. The energy content of light can be classified by incident or absorbed radiation, in the full wavelength range or in the PAR bandwidth, from a solar or artificial lamp spectrum. These variable definitions of light energy used in PE calculations can lead to inaccurate performance comparisons.

- **Gross photosynthesis** (mass per time, variable units) is the rate of carboxylation.

- **Net photosynthesis** (mass per time, variable units) is equal to gross photosynthesis, minus the combined carbon loss rates as CO₂ from photorespiration and dark respiration.

### 2.2 Culture performance

For a fair evaluation of reactors, the above metrics must be applied in the context of additional knowledge of the underlying economic and physical factors that influence reactor performance. To gain this comprehensive understanding, a quasi-dimensional mapping of the physical relationships between the above metrics provides a useful framework for discussion; in the following equations, metrics are kept in their common units. The scope of this analysis is limited to the major productivity and cultivation factors, and to the general cost categories of capital and operation. We begin with the price point of biomass, as

\[
\frac{S}{\text{kg}} = \frac{S/\text{T}}{\text{kg}/\text{T}} = \frac{S}{\text{period}} + \frac{S}{\text{period}} \frac{\text{Capital}}{\text{period}} + \frac{\text{Operational}}{\text{period}} \frac{\text{period}}{A_{\text{culture}}},
\]

which is first split into cost and productivity components, with the former further split into capital and operational costs per operation period, \(t_{\text{period}}\), for a total reactor life time of \(T = i \cdot t_{\text{period}}\). In equation (1) and herein, reactor productivity is expressed on a per-area basis of the culture \(A_{\text{culture}}\), which is appropriate for high aspect ratio, thin cultures, such as those found in flat panel reactors and those demonstrated in the studies of this work. There are several possible relationships between operation period and productivity with regards to minimizing the cost of biomass. These relationships depend on the culture format and location of product in the culture.

In practice, reactor productivity is always less than peak cellular productivity. Peak productivity of a single cell occurs when light and carbon is used at optimal photosynthetic rates, which requires a local (micro-scale) environment of optimal light flux and carbon concentration. (Metabolic reasons for the existence of optimal photosynthetic rates are discussed in detail in sections 2.5.1 and 2.5.2). With aims to provide this local environment, reactor supply architectures manage the transfer of
resources - light flux, carbon concentration, and micronutrient concentration such as nitrogen and phosphorous - from external (macro) sources, *through the culture*, to the micro-scale of cell utilization. Crucially, cell-interception of resources (absorption of light or consumption of carbon) along these transfer paths leads to non-optimal light fluxes and carbon concentrations throughout the majority of the reactor, which slows cell production and only worsens with increased cell density. Cell growth proceeds in this self-limiting way to a quasi-steady state of zero net production, observed as a quasi-constant biomass content of the culture that depends on the specific species and limiting resources. Later, with sufficient resource limitation and/or accumulation of metabolic waste, the culture will begin to die off.

This characteristic of microalgal growth to reach resource-limited density puts culture productivity at odds with the period of reactor operation, where longer periods are desirable considering the law of economy of time. The optimal tradeoff maximizes the amount of generated product in an operation period, which minimizes the cost of biomass (equation (1)). This tradeoff is managed by cultivation and harvesting actions that remove biomass and/or refresh chemical nutrients. Cultivation and harvesting may occur independently (as in periodic batch operation) or simultaneously (as in continuous and semi-batch operations). Even in continuous operation then, the per-period operation cost term in equation (1) may always be defined, since periodic downtimes are needed in practice, at least for maintenance. The specific choice of reactor operation (cultivation and harvesting actions) that maximize absolute production in an operation period strongly depend on the culture format and location of products in the culture.

### 2.2.1 Culture format

Microalgae can grow as free-floating single cells or chains of cells, as in suspension cultures, or as colonies, leading to aggregate cultures such as biofilms and flocs. Since colony growth is also commonly observed in cultures immobilized by chemical matrices - such as the calcium-alginate hydrogels used in two studies of this work - colony growth is also considered aggregate cultures. Considerations for floc cultivation and harvesting are outside the scope of this work and not discussed; see reference for an overview. In biofilms and similarly immobilized cultures, cells are fixed near the surface of a substrate, physically separated from the bulk of liquid media, and so are not easily accessible for harvesting using fluid flow. In practice, these fixed cultures are simply harvested by mechanical scraping, periodically and to near-completion. Near-complete harvesting is most practical for thin cultures like biofilms, where the grown culture, typically 100 μm to a few mm thick, is reduced to a surface residue of cells after scraping. In contrast, suspension cultures...
are optimally continuously diluted to simultaneously harvest biomass and refresh dissolved nutrients so that micronutrient concentrations are constant.

### 2.2.1.1 Culture acclimation

Acclimation limits the productivity of both types of reactor operation modes – periodic harvesting of fixed cultures, and continuous dilution of suspension cultures. During small or slow non-limiting changes to the culture environment (i.e. a slow decrease in replete nutrient availability due to cell growth and consumption) productivity is generally constant and acclimation effects are low. However, large or sudden environmental changes lead to a lagging period of productivity, in which cells substantially divert energy from production to sufficiently acclimate their physiological state to better survive in the new environment \(^99,136,137\). This lag occurs in fixed cultures subjected to periodic harvesting; the beginning of each operation period is not optimally productive, since cells that are left over after a harvest must acclimate, for example, to the sudden increase in light and CO\(_2\) access. In suspension cultures subjected to continuous harvesting, culture circulation and mixing provides a quasi-steady-state environment of time-averaged light flux density and carbon concentration \(^138\).

Considering the mismatch between this average photosynthetic state and the fraction of time spent in higher/lower light intensities, suspension cultures subjected to continuous harvesting exhibit a constant, yet sub-optimal productivity. Furthermore, in worse, slower-mixing regimes, suspended cells may constantly strive to acclimate to new light environments, which may strongly limit productivity \(^93,120,139\).

### 2.2.2 Product location

In fixed and suspension culture formats, generated products may be contained (i) inside living cells or dead mature cells (e.g. lipids that are upgradable to biodiesel \(^41\)), or (ii) in the surrounding media due to excretion from living cells (e.g. genetically engineered strains that elute biofuel \(^52,140,141\)).

If products are contained inside cells (i), optimal production occurs in operation periods, \(t_{\text{period}}\), in which the biomass growth rate of the culture is at maximum (see figures 2.1(a) and (b)). For suspension cultures in which the volume of culture-plus-media is kept constant, this productivity is equivalent to the rate of change of reactor density containing some biomass fraction of the product, \(f_p\). In contrast, for fixed cultures in which density is approximately constant but volume increases with growth (i.e. biofilms), this productivity is instead based on cell number, \(N\). To show this dependence, total biomass produced in an operation period,
kg = A_{culture} \int_{0}^{t_{\text{period}}} [g_{DW} \text{m}^{-2} \text{d}^{-1}] dt,  \quad (2)

may be redefined as,

\begin{align*}
\frac{\dot{m}_{\text{product}}}{\text{m}^2} &= \int_{0}^{t_{\text{period}}} \left( \frac{\dot{m}_{\text{product}}}{\text{m}^2 \text{d}} \right) \, \text{d}t = \int_{0}^{t_{\text{period}}} \left( \frac{f_{\%} \cdot \rho_{\text{max}} \cdot \text{cell}}{d \cdot g_{DW}} \right) \, \text{d}t = \int_{0}^{t_{\text{period}}} \left( \frac{f_{\%}}{\text{m}^2} \cdot \mu \right) \, \text{d}t = \left\{ \int_{0}^{t_{\text{period}}} \left( \frac{f_{\%}}{\text{m}^2} \frac{\partial (\ln N(t))}{\partial t} \right) \, \text{d}t \right\}_{\text{volume}}.
\end{align*}

This maximum rate of production only occurs below a cell number (or density, \( \rho_{\text{max}} \)) threshold of nutrient-limitation and slower biomass growth. To ensure this point is never reached, whole-cell harvesting is required. For suspension cultures, harvesting is best achieved by continuous cell dilution with simultaneous refreshment of dissolved nutrients, as described previously, such that \( D_{\text{media}} = -D_{\text{culture}} = \mu_{\text{max}} \) (see figure 2.1(a)). For fixed cultures, however, semi-batch operation is required in which dissolved nutrients are optimally continuously refreshed, as above, but biomass is periodically harvested once the density threshold is reached at the end of the period of maximum growth rate. The optimal amount of culture to harvest at this point would be such that the next cultivation period begins at the maximum growth rate – \( \rho_{\text{min}} \) in figure 2.1(b).

Figure 2.1: Illustration of optimal production conditions in reactor operation periods, for cultures that contain products in cells (a, b) or excrete products from cells (c, d), when subjected to harvesting.
(red lines) that is continuous (a, c) or periodic (b, d). For cultures of constant density, the vertical axis may be replaced with number of cells.

If products are contained in the media, generated and excreted by cells (ii), optimal production occurs in operation periods that maximize the amount of accrued product, instead of the rate of production. For both suspension and fixed cultures, this result is achieved in some reactor state with an optimal arithmetic product of the number of cells and the distribution of the production rates of all cells, since

\[
\prod_{\text{product}} = \int_{0}^{\text{period}} \int_{1}^{\text{cell}} \left( \frac{\text{product}}{\text{cell}} \right) \cdot \text{cell} \cdot \text{d} \cdot \text{m}^2 = \left\{ \begin{array}{ll}
\int_{0}^{\text{period}} \int_{1}^{N} \left( \frac{\text{product}}{\text{cell}} \right) \cdot \text{N} \cdot \text{m}^2 \\
\int_{0}^{\text{period}} \int_{1}^{N} \left( \frac{\text{product}}{\text{cell}} \right) \cdot \rho \end{array} \right. .
\]

This optimal cell number may be greater than the density (or number) threshold of nutrient-limitation that, as argued above, defines the operation period of reactors whose products are contained inside cells. That is, achieving optimal production in a product-excreting culture may require a fraction of the cultivation period in slightly light- or CO\(_2\)-limited environments with \(\rho_{\text{optimal}} > \rho_{\text{max}}\). For these cultures, dissolved nutrients are optimally kept replete by continuously refreshing media, at \(D_{\text{media}}(\rho_{\text{optimal}})\). Also, harvesting is required to maintain this optimal cell number (or density), since not all metabolic energy can be diverted away from cell growth, even in cultures genetically engineered for excreting directly generated products\(^{142,143}\). For suspension cultures, as above in (i), cell harvesting is best achieved continuously, though at \(D_{\text{culture}}(\rho_{\text{optimal}})\), and simultaneously with nutrient refreshment and product collection (see figure 2.1(c)). For fixed cultures, as above in (i), cell harvesting must be periodic, but this is at odds with maintaining a constant, optimal density (equation (4)). The optimal harvesting scheme of fixed cultures for direct production is then not easily defined. At the beginning of each cultivation period, density (or cell number) will be lower than optimal, but photosynthetic rates in the cell distribution will be higher than at the end of each period - when, conversely, density exceeds the optimal amount (see figure 2.1(d)).

**2.2.3 Culture density**

The density terms in equations (3) and (4) indicate that the amount of product generated in an operation period, *regardless of culture format or product location*, is proportional to a density that either defines the optimal cultivation/harvest period, or cultivation state, respectively. Suspension
cultures can be maintained at ~ 10^6 mL\(^{-1}\) with good photosynthetic efficiency. However, the biofilm cultures typically exceed the maximum achievable density of suspension cultures by an order of magnitude or more (~10^8 mL\(^{-1}\)) and can maintain this density throughout cultivation. The outer layer of matrix-immobilized microalgal cultures are also capable of growing to greater densities than suspension cultures (see references and results in chapter 5). These fixed cultures then have potential to generate products more cost-effectively than the suspension cultures of conventional reactor designs. To achieve this potential, supply architectures must be much more effective than those of conventional reactors, since higher density cultures are increasingly limited by light and carbon availabilities. In developing these architectures, there is some room for increased capital and/or operation cost if their performance enables cost-offsetting productivities.

The quasi-dimensional analysis of production cost from this section is continued in the following two sections, in considering the relationships between light and carbon supply on reactor performance. Physical operating principles of the architectures developed in this work are presented, and compared to that of related architectures using examples from the literature.

### 2.3 Light supply

The reactor architectures developed in this work supply light and carbon on an equal area basis with the culture (\(A_{\text{culture}} = A_{\text{light}} = A_{\text{carbon}}\)). Considering this, the productivity term in equation (1) can be expressed as biomass yield on light, as

\[
\frac{\text{kg}}{\text{m}^2} \propto \frac{1}{A_{\text{light}} \cdot (\text{PPFD}) \cdot \left[\frac{\text{gDW}}{\text{m}^2 \cdot \text{d} \cdot \text{m}^2 \cdot \text{s}^{-1}}\right]},
\]

where the terms in braces are equivalent – the photosynthetic photon flux density (PPFD). The incident flux density on a single cell that optimizes its biomass yield depends on the state of its photosynthetic machinery. Light and chemical factors affecting this state are presented in section 2.5.1 for the model species used in this work.

On the level of the culture, however, the overall biomass yield on light is optimal at a specific distribution of photosynthetic states, corresponding to the distribution of light flux densities. This distribution is established by cell absorption in a convergent feedback loop. As discussed in section 2.2.1.1, acclimation to light in well-mixed suspension cultures is limited to a single, time-averaged photosynthetic state, resulting in a steady but sub-optimal biomass yield on light. In contrast, fixed cultures enable local light acclimation, resulting in a contiguous distribution of photosynthetic states.
with culture depth \(^{105}\). This effect potentially enables biomass yields closer to optimal \(^{106}\) and enhanced productivity due to the high density (as discussed in section 2.2.3). To exploit these benefits, the range of flux densities - from the incident illumination surface, through the full depth of the culture – must match the photosynthetic demand of each cell layer. This level of control over light is increasingly important in higher density fixed cultures \(^{103}\). For comparison, unmanaged light distribution in low-density suspension culture reactors results in large regions of photosynthetic inhibition and net-zero or -negative growth (in the dark) with the remaining few tens of percent of the culture producing, but sub-optimally \(^{103,126}\).

2.3.1 Architectures

In general, the efficient use of light for photosynthesis necessitates lower than solar intensities which can be up to \(\sim 2000 \mu\text{mol m}^{-2} \text{s}^{-1}\) \(^{150}\). Supply architectures that source sunlight must then dilute intensities before emission into the culture at the high intensity extreme. To fully control the low intensity extreme, architectures must limit the depth of the culture from the emission surface. As discussed in sections 1.2 and 1.3, conventional pilot plant photobioreactors dilute light with a large ratio of culture illumination area to reactor ground footprint, while the low intensity extreme is controlled by a large aspect ratio of illumination area to culture volume, resulting in a short light path. An alternative light supply architecture uses optical waveguides to dilute incident light and limit the low intensity extreme \(^{151-159}\). A waveguide’s dilution factor is simply the ratio of its emission-to-input surface areas \(^{160}\). Emission is achieved by breaking the condition of total internal reflection, which can happen in two ways, based on distinct phenomena that arise when light interacts with a surface. These phenomena are presently derived in the set of reference coordinates defined in figure 2.2, starting with a transverse plane wave at optical frequency, \(\omega\), traveling in the waveguide of index \(n_1\), at an incident angle \(\theta_1\) to the surface normal of the microalgal culture media of lesser index, \(n_2 < n_1\). Let both regions be semi-infinite, non-conducting, homogeneous, dielectrics, which is valid for culture media, and waveguides composed of glass or common plastics. When the transmitted wave is parallel to the interface, Snell’s law states that the incident (critical) angle, \(\theta_c\), is a maximum real number, as

\[
\frac{\sin \theta_1}{\sin \theta_2} = \frac{n_2}{n_1} = \sin \theta_c \sin 90^\circ.
\] (6)
Figure 2.2: Illustration of (a) refraction and (b) reflection that produces the near-surface evanescent field (red/green gradient region) at incident angles less, and greater than critical, respectively. The waveguide (red) and culture media (green) are represented by media with index $n_1$ and $n_2$, respectively.

Incident angles greater than critical, $\sin(\theta_1) > n_2/n_1$, cause total internal reflection (figure 2.2(b)), signified by an imaginary solution in Snell’s law for the transmitted angle. To clarify this, equation (6) can be rearranged with a Pythagorean trigonometric identity, as

$$\cos \theta_2 = \sqrt{1 - \left(\frac{n_1}{n_2}\right)^2 \sin^2 \theta_1} = \pm i \sqrt{\frac{n_1}{n_2} \sin^2 \theta_1} - 1 = \pm i \sqrt{\left(\frac{\sin \theta_1}{\sin \theta_c}\right)^2 - 1}. \quad (7)$$

Since only optical frequencies are considered, the magnetic permeabilities of the waveguide and culture may be set to the vacuum value of unity. This permits examination of the behavior of the transmitted light wave using only its electric field component. The electric field of a general transverse plane wave is,

$$\overrightarrow{E}_2 = \tilde{A} e^{i(k_2 \cdot \tilde{r} - \omega t)}, \quad (8)$$

with position vector $\tilde{r} = x \hat{x} + y \hat{y} + z \hat{z}$ and time-independent wave vector,

$$\overrightarrow{k}_2 = \frac{\omega n_2}{c} (\sin \theta_2 \hat{x} + \cos \theta_2 \hat{z}). \quad (9)$$

Substituting Snell’s law isolated for $\sin(\theta_2)$ and equation (7) into equations (8) and (9), and rearranging with a Pythagorean trigonometric identity gives,

$$\overrightarrow{E}_2 = \tilde{A} e^{i\left(\frac{x \omega n_1}{c} \sin \theta_1 - \omega t\right)} e^{\mp i \frac{\omega}{c} \sqrt{(n_1^2 - n_2^2) - n_1^2 \cos^2 \theta_1}}. \quad (10)$$
2.3.1.1 Total internal reflection

Equation (10) describes a transmitted wave that propagates in the same \( \hat{x} \) and opposite \( \hat{z} \) direction as the incident wave at the same angle (\( \theta_1 \)), indicating total internal reflection. This condition is simply broken with incident angles less than critical so that the transmitted wave is refracted according to Snell’s Law, as in figure 2.2(a). This phenomenon is used by modern waveguide-based light supply architectures, in a selective way, to achieve uniform culture emission\textsuperscript{151-159}. Two classes of these architectures are smooth, tapered, ’frustum’-shaped and surface-roughened waveguides. In each, the tapering angle, or distribution of roughened surface angles, respectively, is tuned to the length of the waveguide in order to achieve a distribution of relative incidence angles of guided rays along with length of the waveguide and uniform illumination across the waveguide emission surface\textsuperscript{151-158}. Specifically, the uniform emission profile in both architectures is due to low emission of high-intensity light near the input side of the waveguide, and vice-versa at the far side. One relative advantage of frustum-shaped waveguides is that they do not suffer from the back-reflections present in roughened-surface waveguides, due to an absence of extreme surface angles\textsuperscript{158}.

2.3.1.2 Evanescent field

Returning to equation (10), the non-propagating \( \hat{z} \)-component of the transmitted wave, not yet discussed, describes a near-surface field in the culture that exponentially decays with \( z \geq 0 \). This is the evanescent field – a near-surface phenomenon arising from the necessity of field continuity across the interface and the semi-infinite (unbounded) boundary condition of the culture. Since field intensity is proportional to amplitude squared, the evanescent field intensity also exponentially decays. For an incident beam of width much greater than its wavelength, this can be expressed as\textsuperscript{162}

\[
I = A^2 e^{-\left(\frac{z}{d}\right)},
\]

with penetration depth, \( d \), defined in terms of the exponential terms in equation (10) as,

\[
d = \frac{\lambda_0}{4\pi\sqrt{(n_1^2 - n_2^2)} - n_1^2\cos^2\theta_1}
\]

Equations (11) and (12) indicate that penetration depth is infinite at the critical angle (\( \theta_1 = \theta_c \)), corresponding to an infinite-period light wave traveling parallel to the interface. The intensity of the evanescent field at \( z = 0 \) can be up to several times that of the incident wave, depending on the incident angle and relative magnitudes of the \( \hat{z} \) and \( \hat{x} \) field components of the incident wave that
define light polarization, as shown in figure 2.3 (see reference 163 for derivations). Notably, this enhanced near-field intensity cannot propagate.

![Graph showing light supply](image)

Figure 2.3: Evanescent field intensity at a water-glass interface (z = 0) depends on light polarization components parallel (p-) and perpendicular (s-) to the plane of incidence. Reproduced from reference 164 with permission from Kluwer Academic Publishers, copyright 2002.

With constant illumination, energy flows into and out of the evanescent field, but no net energy flows into the culture, except for that which initially establishes the field 161. This may be verified by employing the Poynting vector, S, which is an abstraction of energy density flow per unit time. When averaged over a period much greater than $2\pi/\omega$, the $\hat{z}$-component of the Poynting vector at the interface in the first media (z = 0) is

$$\vec{S} \cdot \hat{z} = \frac{1}{2\omega} \text{Re}(\hat{z} \cdot \overline{k_2} |E_1|^2).$$

(13)

With $\overline{k_2}$ defined in equation (9), equation (13) becomes

$$\vec{S} \cdot \hat{z} = \frac{1}{2\omega} \text{Re} \left( \frac{\omega n_2}{c} \cos \theta_2 |E_1|^2 \right),$$

(14)

however equation (7) states that $\cos \theta_2$ is imaginary, so $\vec{S} \cdot \hat{z} = 0$ over time - there is no net direction of energy flow into the culture. In contrast, time-averaged values of $\vec{S} \cdot \hat{x}$ and $\vec{S} \cdot \hat{y}$ at z = 0 are finite, so energy propagates only within the reflection region in the plane of incidence 161-163,165,166.
2.3.1.3 Frustrated total internal reflection

The evanescent field can be used to break the condition of total internal reflection, to achieve emission from a waveguide at incident angles greater than critical. This requires finite boundary conditions in the culture, in contrast to the unbounded conditions that led to the $\hat{z}$ decaying and traveling wave components in equation (10). Physically, the boundary may be a particle of greater index than the culture media – consider a microalgal pigment – placed at some small gap distance $z > 0$ approximately within the penetration depth of the evanescent field. Within this gap, the $\hat{z}$-component of the field is described by a superposition of the original evanescent wave and an evanescent wave component that is reflected by the pigment. Outside this gap, away from the surface, the final transmitted field is purely propagating as conventional, radiative light. This may be verified again using the time-averaged Poynting vector, which is nonzero within the gap and normal to the waveguide interface, indicating energy flow into the culture. Detailed derivations and descriptions of this processes may be found in references 162 and 168. This process, where the presence of the particle ‘frustrates’ the TIR boundary condition to permit direct light emission, is called frustrated total internal reflection (FTIR); figure 2.4 shows a familiar example. It is noteworthy that this FTIR is not to be confused with the more popular acronym for Fourier transform infrared spectroscopy, which was not used in this work.

Figure 2.4: Example of frustrated total internal reflection. The air-exposed surface of water in a glass is viewed at an angle of total internal reflection, which obscures most of the hand on the far side of the image. Here, only the ridges of fingerprints (clearly seen in the red circle) penetrate local evanescent fields, which frustrates total internal reflections. At these locations, light exits the glass and reflects off the fingerprint ridges at angles viewable from the water surface. The troughs of fingerprints, however, are too far outside local evanescent fields, so light remains internally reflected there (in the red circle). Reproduced and modified from Wikimedia commons, credit: Olli Niemitalo.
The probability that a pigment within the penetration depth of the evanescent field will absorb evanescent energy is proportional to the intensity of the field, which is enhanced near $\theta_c$ and exponentially decays in $z^{165}$. However, FTIR emission of that energy does not simply follow the same exponential dependence. Thermodynamic laws deny enhanced emission - the rate of energy transmitted by the incident beam must be equal to the dissipation rate of the pigment, by fluorescence emission, heat, or other means, over all solid angles. Further, the emission direction may be highly anisotropic when the excited pigment dipole is in the near-field region, of 10 nm to approximately 100 nm, which can significantly perturb the evanescent field there $^{169,170}$. Figure 2.5 shows a specific near-field emission profile, but the main features are generally valid - most of the emitted intensity is directed back into the waveguide, especially near the critical angle, and back-reflections off the surface significantly contribute to the major lobe in region $n_1$ that results in far-field, radiative emission. On waveguides, as on most surfaces, there are often multiple layers of nanometer-scale particles that can occupy the evanescent field; for example, the components of a cell membrane. These intermediate layers limit evanescent penetration depth, but do not greatly change the nature of FTIR emission as just described $^{171}$.

![Figure 2.5: Radiation pattern of a dipole oriented at 45° to the surface normal (red line) located at one wavelength above the surface of glass ($n_2$) in air ($n_1$) (solid curves) and in the absence of the interface (dashed curves). Reproduced and modified from reference $^{170}$ with permission from OSA Publishing, copyright 2000.](image)

The high sensitivity of FTIR to the location and dipole orientation of emitting particles has been exploited for decades in total internal reflection fluorescence microscopes (TIRFMs), which are used to quantify energy transfer kinetics and track near-surface objects with high signal-to-noise ratio $^{165}$. Recently, in 2011, it was suggested that the location sensitivity of FTIR emission may enable effective light supply to high-density (biofilm) cultures $^{172}$, since higher density surface particles enable more FTIR events and high-density cultures are beneficial for production (as discussed in section 2.2.3).
Studies in 2012 – 2014 investigated this concept using a single spot or line of spatially coherent, monochromatic evanescent fields. They revealed similar photon flux density limits to microalgal growth and biomass yields on light, as compared to conventional radiative illumination. One study of this work, presented in chapter 3, used an area distribution of evanescent fields using coherent and incoherent sources. Various surface emission profiles were generated using the angle-tunability of FTIR emission, in contrast to the fixed emission profile of a waveguide that relies on sub-critical angles for illumination. Microalgal biofilm growth due to FTIR light was quantified and achievable light dilution limits of the FTIR light supply architecture were evaluated.

Recall from the beginning of this section (2.3) that control of the high and low extremes of light in reactors is needed to achieve high-density, productive cultures. The above paragraphs presented waveguide-based phenomena that can be used to dilute and distribute source light, in order to control the high extreme at waveguide surfaces. The low extreme may be controlled by inter-waveguide spacing, so that cultures are limited to the optimal optical depth defined by light attenuation.

In practice, absorbance, $A$, as commonly used (over-)simplified metric of attenuation that ignores scattering, reflection and the physiological state of the culture (e.g. wavelength-selective absorption by pigment content) and assumes a linear relationship of attenuation with culture depth. Absorbance may be defined by incident and transmitted photosynthetic photon flux densities, as

$$A = \log_{10} \left( \frac{P_{\text{FDD, incident}}}{P_{\text{FDD, transmitted}}} \right).$$  \hspace{1cm} (15)

Spectrometer sensitivity limits absorbance measurements to low density samples, but high density samples may be accurately measured at low density with a known dilution factor. A related quantity commonly used in the biology research literature is optical density, $OD$, which is absorbance measured over a 1 cm path length. In practice, this is assumed equal to the physical width of a standard 1 cm laboratory cuvette containing a low-density culture. Optical density can be used to estimate the growth and uniform biomass density of a culture, as

$$\frac{OD_t - OD_b}{t} \propto \frac{\partial \rho}{\partial \ell},$$  \hspace{1cm} (16)

since it linearly correlates to cell number and culture dry mass ($g_{DW}$).

In lab scale reactors, internal low and high light extremes have been managed by arrays of cylindrical light-scattering waveguides, but these still permit sub-optimal low light regions in suspension.
cultures\textsuperscript{153}. With waveguide-based illumination, the low light extreme is most effectively controlled by planar waveguides that fully bound the culture, and naturally inform a stacked-layer reactor design, as conceptually illustrated in figure 2.6. In this design, light emitted from a layer may pass through multiple adjacent layers before being absorbed. The photon flux density at any point in the stacked culture is then a geometric sum of decreasing light flux densities from more distant waveguides, due to culture attenuation\textsuperscript{178–180}. To achieve the same illumination in all layers, mirrors would be required on the interior surface of the outermost reactor walls. Cultivation of high-density cultures in the reactor, illustrated in figure 2.6, suggests the use of similarly thin waveguides on the order of 100 µm, which additionally keeps the capital cost of materials low. Adding layers in this design linearly increases areal density and productivity, as indicated by equations (3) and (4). The potential performance of a similar reactor design was recently demonstrated by Prof. Erickson’s group at Cornell University\textsuperscript{179,181}. In their design, stacks of light-scattering waveguides illuminated a dense suspension culture of ethylene-excreting \textit{Synechocystis} sp. PCC 6803 2x EFE and productivity was measured in different light regimes. However, waveguide surface intensity and spacing (2 mm) were not matched to suspension culture absorbance, resulting in 50 - 90% of the culture in unproductive intensities of <10 µmol m\textsuperscript{-2} s\textsuperscript{-1}. Nevertheless, peak productivity of the reactor was four times greater than a conventional flat-plate photobioreactor control with 3 cm light path\textsuperscript{178}.

Figure 2.6: Illustration of a stacked-layer reactor employing frustrated total internal reflection illumination from solid, planar waveguides and fixed culture. Light is tripped out of the waveguide and into the culture for photosynthetic use by the presence of biomass in near-surface evanescent fields, which frustrate the total internal reflection condition. Products may be contained in cells, or excreted by cells, as indicated. Reproduced and modified from reference\textsuperscript{147} with permission. © IOP Publishing 2014.

In figure 2.6, carbon is supplied in dissolved form with flow over high-density fixed cultures. However, carbon may be supplied by other means, as discussed in the next section.
2.4 Carbon supply

The productivity term of equation (1) can be expressed as the biomass yield on carbon, as,

\[
\frac{s}{kg} \propto \frac{1}{A_{\text{carbon}} \cdot k_L \cdot a \cdot \left( [\text{CO}_2] - [\text{CO}_2]_{\text{eq}} \right) \cdot \frac{\partial [\text{CO}_2]}{\partial t} \cdot \frac{\text{g}_{\text{DW} m^{-2} d^{-1}}}{12 \text{ g mm}^{-2}} \cdot \left( \frac{44 \text{ g mol}^{-1}}{12 \text{ g mol}^{-1}} \right) \cdot 44 \text{ g mol}^{-1} \cdot 12 \text{ g mol}^{-1} \cdot \left( \frac{\text{g CO}_2 m^{-3} d^{-1}}{\text{g CO}_2 m^{-3} d^{-1}} \right)}{10^{-5} - 10^{-6} \text{ m/s}},
\]

where the carbon supply interfacial area, \( A_{\text{carbon}} \) multiplied with the last term (not in braces) describes fixed carbon productivity, expressed as the carbon content of produced biomass using the molar ratio of CO\(_2\) to atomic C. The other terms in braces are equivalent volumetric rates; in the first of these, \( k_L a \) is the volumetric liquid phase mass transfer coefficient of CO\(_2\) (s\(^{-1}\)) where \( a \) is the specific gas-liquid interfacial area (m\(^2\)/m\(^3\)). The overall mass transfer coefficient, \( K_L \approx 10^{-5} - 10^{-6} \text{ m/s}, \) is dominated by this liquid phase component, as

\[
\frac{1}{K_L} = \left( \frac{1}{k_G \cdot H_{\text{CO}_2/\text{H}_2\text{O}}} + \frac{1}{k_L} \right) \approx \frac{1}{k_L},
\]

since gas-phase diffusivity of CO\(_2\) is four orders of magnitude greater than that of the liquid phase, and Henry’s Law constant for CO\(_2\) at room temperature is large \( (H_{\text{CO}_2/\text{H}_2\text{O}} \approx 170 \text{ MPa}) \). Of the remaining terms in braces, \([\text{CO}_2]\) represents the local concentration of dissolved CO\(_2\) species fixable by microalgae, \([\text{CO}_2]\)\(_{\text{eq}}\) represents the equilibrium concentration of fixable CO\(_2\) species in the aqueous phase (or the constant concentration at the gas-media interface), and \( \frac{\partial [\text{CO}_2]}{\partial t} \) represents the carbon fixation rate of the culture. Crucially, this rate is primarily a dual function of light (since photochemical energy is required to fix carbon) and the availability of carbon as dissolved CO\(_2\)\(_{\text{aq}}\) and HCO\(_3^-\). The local carbon concentration around a single cell that optimizes its biomass yield (equation (17)) then depends on its photosynthetic state, and the local proportion of available carbon species – these factors are discussed in section 2.5.2, for the model species used in this work.

On the level of the culture, however, the overall biomass yield on carbon is optimal at a specific combination of distributions of (i) photosynthetic states in response to light exposure, and (ii) carbon concentrations resulting from diffusion between sources and sinks - cells performing light-powered carbon fixation. As discussed in section 2.2.1.1, well-mixed cultures exist in a uniform photosynthetic state with a sub-optimal biomass yield on light. In turn, this limits the biomass yield on carbon, which can potentially be very close to optimal with respect to only the achievable near-uniform, optimal carbon concentration of a well-mixed culture. In contrast, fixed cultures feature depth-wise distributions of both carbon concentrations and light-acclimated photosynthetic states –
with a greater biomass yield on light compared to that of suspension cultures, as argued in section 2.3. Additionally, in fixed cultures, light and carbon sources should be supplied on the same side of the culture, so that the depth-wise trends of carbon availability and light-energy-driven fixation rates are matched. For example, deep in a biofilm culture where light and carbon fixation rates are low, carbon availability would also be limited due to consumption (carbon-interception) by cells in upper layers. This mitigates carbon consumption to exhaustion in lower layers and ensures a high carbon supply for demanding upper layers. Fixed cultures then offer potential for culture-level biomass yields closer to optimal, compared to that of suspension cultures, but only if supply architectures can keep up with the carbon demand from all layers.

In either case of nutrient - carbon or light - supply architectures are effective in terms of productivity and biomass yield when they enable control over the rate of supply and the transfer path in terms of the number of intercepting cells. The previous section discussed how waveguide architectures enable control of the supply rate of light (surface intensity) and light path (inter-waveguide spacing) by control over the high and low intensity extremes in cultures. The following section describes architectures that enable control of the supply rate and diffusion path of carbon in reactors.

2.4.1 Architectures

The relationships between terms of equations (17) and (18) physically describe the full path of CO$_2$ across multiple length scales, from supply in the gas phase, through liquid-phase diffusion and speciation, down to fixation by cells. Since the trajectory of this path is unavoidable, the terms of these equations are the targets of supply architectures with respect to improving the supply rate of carbon.

The base method of carbon supply is atmospheric CO$_2$(g) diffusion across a flat liquid interface to the culture, driven by a concentration gradient. Open reactors can access this mass transfer mechanism, but require additional CO$_2$ bubbling at elevated concentration, which is a ubiquitous method to improve mass transfer rates that targets the $[\text{CO}_2\text{eq}]$ term (CO$_2$-enriched gas mixtures). Laboratory and pilot plant scale photobioreactors often operate with CO$_2$(g) concentrations $> 1\%$ and typically in the 1 – 5% range to achieve peak production.

Another method to improve mass transfer rates that targets the $[\text{CO}_2\text{eq}]$ term, and is compatible with any architecture design, is the use of high alkalinity to shift the equilibrium concentration of CO$_2$ to increase $[\text{CO}_2\text{eq}]$. As discussed in section 2.5.2, the presence of OH$^-$ in solution enables the speciation of photosynthetically useful CO$_2$(aq) to also useful bicarbonate (HCO$_3^-$), and not useful
carbonate (CO$_3^{2-}$). With additional OH$^-$, the increased molar capacity of the carbonate buffer system draws CO$_2(g)$ into the liquid phase. Importantly, although speciation is dictated by pH (as shown in figure 2.14), the pH of the culture is not necessarily influenced by the availability of these inorganic carbon species $^{184}$. However, greater CO$_2(aq)$ concentrations are possible at higher pH, so this method is best suited to alkaliphilic phototrophs and other pH-tolerant species. This method is also suitable for the use of flue gas (10 – 15% CO$_2$), though dilution with air is required, as most species are not tolerant to > 2% CO$_2(g)$ $^{95}$. It has also been suggested that this method may integrate well with the high pH found in productive, high-density biofilms $^{104,183}$.

Related to both of the above approaches, bicarbonate salts can be added to increase alkalinity $^{183}$ (targeting the [CO$_{2eq}$] term of equation (17)) and the availability of HCO$_3^-$ for carbon fixation (targeting the [CO$_2$] term). However, this artificial increase of bicarbonate concentration in solution alters the micronutrient state of the culture, resulting in media- and species-specific effects on production $^{185,186}$. For example, in one study, the addition of bicarbonate did not affect growth, nutrient uptake, or lipid accumulation rates, but improved photosynthetic rates compared to controls $^{185}$.

In contrast to the above approaches, with a given CO$_2$ source the remaining liquid-phase mass transfer coefficient term of equation (17), $k_L$, can be targeted to improve the rate of CO$_2(g)$ $\rightarrow$ CO$_2(aq)$ transfer. Most architectures aim to improve $k_L$ by manipulating the components of its specific interfacial area, $A_{interface}$.

$$a = \frac{A_{interface}}{V_{culture}}$$

At constant culture volume ($V_{culture}$) the absolute interfacial area is most commonly increased by bubbling. However, as shown in the motivation section 1.2, the energy required for pumps that enable aeration (CO$_2$ supply and O$_2$ removal) by bubbling is a high cost of suspension culture reactor operation, which economically limits their performance. The mass transfer performance and limitations of bubbling-based aeration architectures, including their effects on light supply, have been thoroughly researched; see reference $^{187}$ for a concise review of modern approaches. Alternatively, at constant absolute interfacial area ($A_{interface}$) the culture volume may be reduced to increase the specific interfacial area and thereby the mass transfer coefficient. This is equivalent to reducing the mass transfer path length of CO$_2$, which is required of an effective carbon supply architecture, as argued in the introduction of this section (2.4). Using a large ratio of gas exchange area to culture volume is a common approach that has helped enable the highest densities and productivities achieved in suspension cultures $^{188}$, cultivated in thin, flat panel and other ‘short light path’ reactor designs, where the illumination regime provides the primary benefit,
as the name suggests\textsuperscript{80,181,189}. However, again, these reactors consume energy for aeration by bubbling.

In the past decade, bubble-free supply architectures that target both $k_L$ and $a$ components of the volumetric mass transfer coefficient have been co-opted from wastewater treatment technologies and implemented in photobioreactors\textsuperscript{84,190}. These are porous membrane contactors, in which the pores contain the gas-liquid interface, and mass transfer through them is by concentration-driven diffusion. Some of these systems have demonstrated superior $k_La$ values compared to bubbling\textsuperscript{49,95,191}. Other major advantages of membrane contactors are interfacial area control, energy-free carbon supply, and the supply of carbon already in dissolved CO$_2$(aq) form, which avoids CO$_2$($g$) losses of bubbling. A potential disadvantage is that the presence of the membrane between gas and liquid phases is an additional barrier to diffusion, quantified by its mass transfer coefficient, $k_M$ as

$$\frac{1}{k_L} = \left( \frac{1}{k_G^*H_{CO_2/H_2O}} + \frac{1}{k_M^*H_{CO_2/H_2O}} + \frac{1}{k_L} \right) \approx \frac{1}{k_L}. \quad (19)$$

However, as suggested by equation (18), $k_M$ may negligibly affect the overall mass transfer rate of CO$_2$. In extreme cases, contactor pores are entirely hydrophilic (water-filled) or hydrophobic (gas-filled). In either case, Carvalho and Malcata (2001) argued that $\frac{1}{k_M} \propto h$ would be roughly an order of magnitude smaller than $\frac{1}{k_L}$ or $\frac{1}{k_G}$, respectively, due to pore tortuosity and surface resistances, but only if the membrane thickness, $h$, is not too great\textsuperscript{49,191}. Also, the achievable supply rate of a membrane contactor may be significantly increased with larger pore size\textsuperscript{192} and a shear flow regime that significantly reduces the thickness of the boundary layer\textsuperscript{193}.

To-date, the most common type of membrane contactors employed in laboratory-scale photobioreactors are hollow fiber membranes (HFMs), which are widely used in water treatment\textsuperscript{194,195}. Example images are shown in figure 2.7. Gas supplied in the cores of HFMs can be at ambient pressure, with or without flow-through, or pressurized using end-capped fibers\textsuperscript{178,196}. Pressurizing HFMs increases carbon transfer but costs energy\textsuperscript{197}. However, pressures exceeding the fiber breakthrough pressure offer a means to HFM-mediated microbubble delivery to cultures\textsuperscript{193}, with greater surface area than typical ~mm-scale bubbles. In one such study, the $k_Ga$ of microbubble-forming HFMs was an order of magnitude greater than that of a standard microbubble diffuser, at the same flow and pressure (energy consumption) conditions\textsuperscript{191}. Without bubbling, HFMs have also been used as substrates for microalgal biofilm cultivation, with potential to exploit the biofilm density benefit to productivity, as discussed in section 2.2.3\textsuperscript{193,198}.  

\textsuperscript{80,181,189}
Since the performance of any carbon supply architecture depends on its $k_a$, this should ideally be included in comparative evaluations. Analytically, $k_L$ may be determined from the relationships between Sherwood, Reynolds, and Schmidt numbers, as

$$k_L = \alpha D^{-\frac{2}{3}} d^{(\beta-1)} u^\beta \nu^{\left(\frac{1}{3} - \beta\right)},$$

(20)

where $D$ is diffusivity, $d$ is characteristic length, $u$ is liquid velocity, and $\nu$ is kinematic viscosity. The coefficients $\alpha$ and $\beta$ must be experimentally determined for a specific reactor geometry and flow regime. Owing to experimental difficulty in determining these coefficients and the total interfacial area, $k_L a$ is often directly determined as a single term, by measuring the concentration change of a species in solution. For CO$_2$, this is explicitly

$$\frac{d[CO_2]}{dt} = k_L a \left([CO_2]_{eq} - [CO_2]\right),$$

(21)

with the same definitions of terms as in equation (17).

Recall from the introduction of this section (2.4) that control of the carbon supply rate and diffusion path are both needed to achieve productive cultures with high biomass yields on carbon. Approaches to enhance the supply rate were presented in the above paragraphs. Control of the carbon diffusion path is most simply enabled by architecture packing, analogous to the control over light paths enabled by inter-waveguide spacing that led to the stacked-waveguide reactor concept (figure 2.6). Also, recall that enhanced biofuel production in a similar stacked-waveguide reactor design was.
demonstrated by Prof. Erickson's group at Cornell University\textsuperscript{181}, as discussed in section 2.3.1.3. It is beneficial then, to use packable carbon supply architectures that are compatible with this stacked waveguide reactor design. To demonstrate such a reactor, the same group packed HFMs in-between the waveguides of their stacked reactor in a later study. They investigated HFM packing effects on biomass growth\textsuperscript{196,200} and chose 1 mm separation between parallel HFMs, with 24 HFMs per layer between waveguides resulting in a 90% ratio of carbon-to-light supply surface areas\textsuperscript{178}. However, with this enhanced capacity for carbon supply, the reactor only generated about half of the ethylene productivity of their waveguide-only reactor in similar light regimes\textsuperscript{178}. This reduced output was likely due to the light-absorbing nature of HFMs, and all membrane contactor materials – they are opaque. Based on the fiber diameters and packing, up to one-third of light emitted by waveguides in this reactor was absorbed by the HFMs. This loss compounded the existing light-limited performance due to the mismatch between low emission intensity and high culture density\textsuperscript{178}.

To improve the performance of the stacked-waveguide reactor, a transparent carbon supply architecture is needed. Nanoporous materials - in contrast to microporous membrane contactors - can be transparent and do not contain the gas-liquid interface, but completely separate them. Gas-liquid mass transfer in nanoporous materials is by atomic permeation - adsorption of gas species to one surface that diffuses through to the other side, driven by a concentration gradient. These nanopores are far more immune to clogging compared to the larger pores of membrane contactors and bubbling technologies\textsuperscript{195,197}. The gas transfer ability of nanoporous materials is quantified by permeability, $Dk$, as

$$Dk = D \cdot k = D \cdot \frac{S}{\Delta P} = D \cdot \left( \frac{\text{gas volume at STP, or mass in membrane}}{\text{volume or mass of membrane}} \right) \cdot \frac{1}{\Delta P} = h \cdot T,$$

where $D$ is the diffusivity (or diffusion coefficient), $k$ is the constant of Henry’s Law, $S$ is solubility, $\Delta P$ is the partial pressure drop across the membrane, and $h$ and $T$ are the membrane’s thickness and gas transmissibility, respectively. The ratio in brackets can be thought of as a molar volume ratio of the gas species in the material. Note that permeability is only defined for a given pressure difference, or pressure regime that is linear with mass flux across the membrane. There is no international standard for the combination of units that define permeability, however, the non-SI unit Barrer is exclusively used in the contact lens industry for $O_2$ permeability, and is commonly used in membrane technology industries: 1 Barrer = $10^{-11}$ (cm$^3$ at STP) cm$^{-1}$ cm$^{-2}$ s$^{-1}$ mmHg$^{-1}$.

The use of opaque nanoporous polymers for carbon supply in photobioreactors has been investigated\textsuperscript{201}, and a few photobioreactor patents contain the idea of using transparent, gas-permeable materials
that permit light capture and CO$_2$(g) fixation. However, to the best of the author’s knowledge, the use of transparent nanoporous membranes for carbon supply in photobioreactors is a novel contribution of this work. Section 4.5 presents a literature review of potential materials for this application, and the study of chapter 4 demonstrates enhanced culture growth using one material – cellulose acetate butyrate (CAB) – for CO$_2$ permeation alone, and then as an integrated gas-breathable waveguide that simultaneously supplies light and carbon. This dual-purpose material informs a stacked-layer reactor design illustrated in figure 2.8.

Figure 2.8: Illustration of a stacked-layer reactor employing planar nanoporous materials for combined light and CO$_2$ supply to cultures.

The above approaches to improve carbon mass transfer are also beneficial for removing oxygen waste generated by photosynthesis. Oxygen is toxic to microalgal cultures at high concentrations (~35 mg/L) which can easily accumulate in closed photobioreactors, since O$_2$ solubility in water is twenty times that of CO$_2$ in a range of physiological temperatures. Nanoporous material carbon supply architectures may enable O$_2$ removal if adsorption and diffusion specificity for O$_2$ is not greatly different than for CO$_2$ (see section 4.5). HFMs have shown promise for removing O$_2$ in closed tube reactors, which are especially susceptible to oxygen buildup. In fact, these tube lengths are limited by aeration (bubbling) nodes placed between them primarily to combat ‘oxygen holdup’ rather than restore pH-balance and replenish depleted CO$_2$. It has been suggested that dissolved oxygen concentrations in cultures should remain below 1 mM, or four times the equilibrium concentration in water. However, dissolved oxygen concentration in high density microalgal biofilms can be an order of magnitude greater than ambient equilibrium (e.g. 3.2 mM), which puts metabolic pressure on cells to undergo biomass-consuming photorespiration, as explained later in section 2.5.2.1. To exploit the density benefit of fixed cultures to productivity, photosynthesis must be maximized throughout the culture, and photorespiration avoided. The influence of external
light and carbon on cellular mechanisms of photosynthetic carbon fixation and respiration are presented in the next section, specifically for the model species used in studies of this work.

2.5 Species selection

The performance of supply architectures must be evaluated with respect to some metric of culture-level productivity of a microalgal species. Since light and carbon are the primary resources of all microalgae, these supply architectures are generally applicable, so their performance would ideally be evaluated with respect to the most generic productivities – the turnover rates of the primary production pathways. These are the photosynthetic electron transport chain and Calvin cycle, which are discussed in detail later in sections 2.5.1 and 2.5.2, respectively. There are several established techniques to measure chemical and energy flow through these pathways (see reference 209 for a review of approaches), however, these are based on indirect effects (e.g. fluorescence) and intermediate pathways that differ between species 104,210–212. The resulting species-specific and supply-regime departures from theory makes interpretation of these measurement results uncertain, and reduces the current utility of these pathways for evaluating supply architectures current 213,214. Instead, less direct but more robust productivity metrics of whole-cell biomass or excreted compounds are used to evaluate supply architectures in practice, as discussed in section 2.2.2.

Cellular biomass growth is an easily measured representation of overall metabolic productivity, while excreted directed products are the net outputs of specific pathway productivities and may be simply measured depending on their physical nature. For example, excreted products that are gaseous or volatile are easily detectable in sealed culture headspaces 52,178. Undirected production of excreted products (e.g. extracellular polysaccharides, as ‘slime’in Fig. 2.9) complicates quantification of both biomass and directed production metrics. Compared to eukaryotic microalgae, prokaryotic microalgae (cyanobacteria) are more generally applicable to evaluations by both biomass and direct productivities, due to the comparative ease of cyanobacterial genetic modification for directed production using a variety of techniques 215–217. Cyanobacteria have a lean metabolism and physical structure (see figure 2.9), with only two major ‘organelles’ - thylakoids and carboxysomes - that together enable more energy-efficient photosynthesis than eukaryotic microalgae, but with a smaller variety and storage capacity of cell compounds 62. With these characteristics, cyanobacteria may be considered ‘cell factories’, optimally cultivated continuously at high density for the generation of excreted products - including lipids for biodiesel 217. In contrast, eukaryotic microalgae may be
considered ‘warehouses’, optimally cultivated at high growth rates for the accumulation of cell biomass products - most notably lipids as biodiesel precursors \(^{215}\).

![Figure 2.9: Major components of a cyanobacteria cell. Reproduced from Wikimedia commons, credit: Kelvin Ma.](image)

For directed production, genetic manipulation of cyanobacteria need only apply to a few high-carbon flow pathways \(^{218}\). With limited chemical and energy resources, their flow through these pathways is necessarily balanced by lower metabolic turnover in other, not manipulated pathways. The resulting imbalance between fluxes of economically desired and ancillary pathways can be several orders of magnitude, for better or worse \(^{215}\). In the worst cases, cells may become inviable or vulnerable to out-competition, either by other cells, or by internal and still largely unknown cyanobacterial competing pathways (i.e. by back-reaction asymmetries and the accumulation of toxic products) \(^{219,220}\). However, in cyanobacteria, tens of percent of fixed carbon can typically be directed to a product of interest \(^{221-223}\) and up to 80% has been achieved in one species \(^{224}\).

In recent years, genetic manipulation of cyanobacteria has increased the number of directed products and their productivities, and has led to several breakthroughs in production limitations, as covered in many recent reviews \(^{52,141,221,225}\). For example, the recent introduction of sugar transporters in *Synechococcus elongatus* enabled heterotrophic growth, which can sustain cells overnight in solar photobioreactors, enabling up to double the productivity \(^{226}\). The same strain has been engineered to
overproduce in the cell and excrete a variety of carbohydrates, short chain alcohols, and free fatty acids. In other species, the recent achievement of engineered limonene production is an important milestone toward generating bio-jet fuel. With continued advances in directed production (no doubt soon to be enabled by the powerful CRISPR/Cas system recently identified in many cyanobacteria, which would expand carbon pathway investigations and manipulations) the evaluation of reactor supply architectures based single-compound productivities of engineered strains will become less informative to reactor design than those based on the more generic, traditional biomass productivity metric of wild-type strains.

Given that the biomass productivity of a wild-type cyanobacteria is a useful metric for evaluating light and carbon supply architectures, the following discusses two requirements of a selected species. As discussed in section 2.2.2, the productivity of a microalgal culture begins to decrease from its maximum when a key resource becomes unavailable – most likely primary resources of light or carbon. It is essential then to evaluate light and carbon supply architectures with respect to culture productivity in these limiting regimes. This ensures good architecture performance in more productive regimes. For this, a species with sensitive growth response to low light and carbon is required. Also, recall that the architectures developed here (FTIR waveguide illumination and gas-breathable waveguides) are compatible with high-density, fixed cultures, which are potentially more productive than suspension cultures (as discussed in section 2.2.3). Since the immobilization matrices can alter microalgal metabolism, such as calcium-alginate used in the hydrogel study of this work (chapter 5), a biofilm-forming species is most generally useful.

The cyanobacterium *Synechococcus elongatus* fits each requirement: *S. elongatus* exhibits clear biomass growth trends in a wide range of light intensities (~10 – 500 µmol m⁻² s⁻¹) and CO₂(g) concentrations (<5%), including CO₂-rare media (see figures 2.1 and 2.19). Also, among cyanobacteria, *S. elongatus* is unique in being an obligate photoautotroph, meaning it generates biomass using dissolved carbon dioxide species as its sole source of inorganic carbon – an additionally relevant tool to mitigate climate change. In the studies of this work, the sensitive biomass growth response of *S. elongatus* to light, and carbon supplied by the developed architectures only, enabled a clear evaluation of architecture performances. *S. elongatus* is also easily grown in the laboratory, requiring minimal micronutrients. Additionally, the microalgal genera *Synechococcus* is one of the four most intensively explored by genetic manipulation. In fact, the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) that enables carbon fixation has only been studied using tobacco, spinach and *Synechococcus*. This genera is also one of the largest among
Species selection

cyanobacteria. In particular, *S. elongatus* accounts for ~17% of primary production in marine environment, compared to 25% attributed to all marine cyanobacteria.

Figure 2.10: Net O₂ evolution response to light (A) and to inorganic carbon in high light (450 μmol m⁻² s⁻¹) (B) of *Synechococcus* sp. grown at high and low carbon concentration (2-4% CO₂ and air bubbled, respectively). Reproduced and modified from reference with permission from the American Society of Plant Biologists, copyright 1982.

The specific strain used in the studies of this work is *Synechococcus elongatus* PCC7942 (herein *S. elongatus* unless otherwise noted); the wild-type was used for suspension and hydrogel cultures, whereas the biofilm-forming knockout mutant T2SEΩ was used for biofilm cultures. Its metabolism characteristics regarding the utilization light and carbon supplied by reactor architectures are discussed in the next two sections.

### 2.5.1 Light utilization

In this work, architectures were developed toward improving reactor-level productivity by controlling the high and low extremes of light supplied to fixed cultures. Within such a light distribution, individual cells may experience slow and quick changes to light, due to culture growth and periodic harvesting, respectively, over a wide range of light intensities. Since the productivity of a single cell depends on the state of its photosynthetic machinery, it is important to understand how these states are influenced by light in order to ensure an optimal biomass yield on light and, related by equation (5), ultimate biomass cost.

Light attenuation by a single cell is due to absorption by its various components, including pigments that strongly absorb a narrow range of wavelengths to momentarily gain an excited energy state. When an excited pigment is brought into electronic coupling with another molecule, before its...
excitation lifetime ends, the energy becomes delocalized over both molecules, with changes to the electron orbitals and atomic structure of both. This interaction between structural and electronic changes is the basis of the transfer of absorbed light energy in cells, as excitons (delocalized charge separations). Additionally, quantum coherence between excitons with moderate environmental coupling is required to maintain long excitation lifetimes (ms compared to fs-nS for in-vitro pigments) that enable long-range exciton transfer \textsuperscript{242}; for example, across the tens of nanometers between light-harvesting and water-splitting complexes in cyanobacteria \textsuperscript{243,244}. These structures are described in detail below. In general, light absorption and long-range exciton transfer are both enabled by pigments coupled to each other via proteinaceous support structures with precise atomic orientations. Figure 2.11 shows example in-vivo absorption spectra of \textit{S. elongatus} with wider peaks than isolated pigments due to overlapping absorption bands of interconnected pigments and proteins in the cell.

Figure 2.11: Absorption spectra of \textit{S. elongatus} suspension cultures before (initial) and after 65 hours exposure to indicated CO\textsubscript{2} concentrations in air (−/+ signs indicate controls). The presence of phycocyanin and chlorophyll-a absorption peaks, at 620 and 680 nm, respectively, indicate viable cultures. Low-wavelength noise is due to detector sensitivity. Reproduced and modified from reference \textsuperscript{245} with permission from Elsevier, copyright 2016.

A simplified photosynthetic light harvesting and electron transfer pathway in \textit{S. elongatus} and other cyanobacteria is shown figure 2.12 and is now described. Light-harvesting and exciton transfer first occurs in antennae structures called phycobilisomes. These contain highly ordered, stacked pigments
of phycocyanin (excitation 550 – 650 nm) and phycoerythrin (excitation 480 – 570 nm), which are open-chain structures lacking the Mg\(^{2+}\) centers of chlorophylls. Exciton transfer from the antennae is facilitated through a series of chlorophyll-a molecules and binding proteins in the D1 and D2 subunits of the photosystem II (PSII) transmembrane complex. The chlorophylls in these subunits also aid in light capture mainly by red-wavelength absorption. Excitons are funneled to a pair of chlorophyll molecules that bridge D1 and D2 but behave quantum mechanically as a single molecule, called P680 for the 680 nm-equivalent exciton energy. An electron is donated by the chlorophyll-a of D1, through intermediate carriers, to reduce the stable molecule plastoquinone ($\text{PQ} \rightarrow \text{PQH}_2$) residing in a pool in the transmembrane space. The reaction center chlorophyll is quickly reduced by the water-splitting enzyme function of the Mn\(^{4+}\) cluster of D1, which prevents electron recombination between the intermediate carriers and the reaction center \(^{246}\). Reduced plastoquinone is then oxidized by the cytochrome b\(_{6f}\) complex, which concomitantly moves protons into the thylakoid lumen and reduces the luminal carriers cytochrome c6 and plastocyanin. Meanwhile, a neighboring transmembrane protein complex, photosystem I (PSI) also harvests light using chlorophylls, since it lacks a phycobilisome, and funnels the excitons to the P700 chlorophyll-pair reaction center. The electron donated by a PSI chlorophyll-a is transferred through intermediates to ferredoxin (Fd). By this point, the luminal carriers cytochrome c6 and plastocyanin can reduce this PSI reaction center chlorophyll-a. The remainder of the process is the electron transfer from ferredoxin to ferredoxin-NADP reductase (FNR) which recycles NADP\(^+\) to NADPH. Meanwhile, protons accumulated in the lumen by water splitting and plastoquinone oxidation are used by ATP reductase to recycle ADP to ATP \(^{247,248}\). These energy carriers, ATP and NADPH, are the primary metabolic connection between photosynthesis and carbon fixation (discussed in the next subsection).
2.5.1.1 Photoinhibition

The above paragraph describes the operation of the photosynthetic electron transport chain at moderate light intensity, with balanced excitation and reduction rates of reaction centers. In this regime, assuming no other limiting factors, the turnover rate of reaction centers linearly increases with light intensity, but so does photoinhibition – strictly, the light-induced inactivation of PSII function. Specific targets of direct photodamage are the Mn⁴ oxygen-evolving complex of PSII (electron donor) and an electron-accepting series of bound plastoquinones in D1 and D2 subunits. Concomitantly with increasing light intensity, an imbalance between excessive exciton generation and lesser availability of plastoquinone reductant causes PSII reaction centers to wait for longer periods in their excited state before turnover. When the wait is longer than the few-nanosecond lifetime of excited P680, it may rephrase to the lower energy state of triplet chlorophyll, which has a several microsecond lifetime. In the presence of oxygen, triplet chlorophyll relaxes by energy transfer to O₂ to form singlet oxygen (¹O₂). Although the lifetime of ¹O₂ is on the order of 100 ns with a mean diffusion distance of only 10 nm, it is highly damaging to the D1 subunit in close proximity, and/or to PSII repair by inhibiting mRNA translation of de novo synthesis of D1. In cyanobacteria, PSII repair is also inhibited by stressors of high salt, low-CO₂, and temperature at both extremes. Other oxidative species (e.g. H₂O₂, OH⁻, O₂⁻) are also generated near PSII and...
elsewhere in related photoinhibitory process, but the mechanisms just described are of prime or equal importance. The complexity of these interacting chemical- and light-induced damage effects on PSII function and repair has made it difficult to determine a generalized mechanism that defines photoinhibition in a range of conditions; Vass (2012) presents for a review of current models.

Aside from reaction center effects, high-intensity light also generates excess excitons throughout non-reaction center chlorophylls of PSI and PSII, and the pigments of phycobilisomes, which can relax by fluorescence emission. The deliberate use of super-saturating intensities to achieve fluorescence emission is a widely used approach to simply identify cells in fluorescence microscopy, as used in the studies of this work. Alternatively, in pulse-amplitude-modulated fluorometry, this approach is used to quantify fluorescent quantum yields that in turn is used to determine relative electron transport rates, which can enable estimations of the effects of light on non-photosynthetic metabolic processes.

The continuity of photoinhibition with light intensity necessitates constant engagement of mechanisms that protect against direct light damage and reduce the presence of reactive oxygen species. Partly to reduce direct photodamage, S. elongatus and other cyanobacteria can express two forms of the D1 subunit that are resilient to different light intensity ranges. There are also several mechanisms to protect against reactive oxygen species. Prevention of their formation starts at excited phycobilisome pigments and non-reaction center chlorophylls, which may be non-photochemically quenched by orange carotenoid protein and high-light induced proteins before the excitons can be transferred to reaction centers. Further down the transfer chain, cyanobacteria can also shift the donor-acceptor redox potential difference of excited P680 to reduce the rate of \( ^1O_2 \) formation. Finally, generated \( H_2O_2 \) and \( O_2^- \) may be scavenged by enzymes in the water-water cycle at PSI using electrons donated by PSII.

### 2.5.1.2 Photoacclimation

Photoinhibition protection mechanisms are suitably fast-acting for the seconds-to-minutes fluctuations in natural environments that span full sunlight and darkness, either at illuminated surfaces (e.g. flickering shadows) or by mixing of cells through depth-wise light gradients. In artificially lit reactors, however, incident light intensities are constant, or may be optimally pulsed with respect to the rate of photosynthetic turnover. Mixed, suspension reactor cultures of moderate density experience large intensity fluctuations in periods on the order of ~ 100 ms to seconds. Chemical- and photo-acclimation, in contrast, involve more extensive and integrated metabolic re-arrangements in response to slowly changing nutrient availabilities, typically over
hours to days in both natural and reactor settings. For example, in slowly increasing high light, microalgae trim their light-harvesting structures and/or reduce pigment expression (see figure 2.13), which increases light penetration and overall productivity, reduces fluorescence losses with consequent increase to photosynthetic efficiency, and additionally aids in photoinhibition protection. Genetic engineering methods that reduce the size of light-harvesting structures have also enhanced photosynthetic efficiencies and growth density \textsuperscript{258}. In well-mixed suspension cultures, these natural and genetic approaches to photoacclimation are global. However, in fixed cultures, natural light acclimation is local and stratified such that lower pigment content is found in cell layers closer to the light-exposed surface. This enables better light penetration and explains the promising achievements, in recent studies of biofilm-based photobioreactors, of several times greater productivities and photosynthetic efficiencies compared to suspension cultures in similar lighting conditions \textsuperscript{104,105}.

![Figure 2.13: Phycocyanin (A) and chlorophyll-a (B) content from whole-cell spectra over a range of growth irradiance for \textit{Synechococcus elongatus} grown at 35°C. Cells grown with low (< 0.1 mM, open symbols) and high (~ 4 mM, closed symbols) inorganic carbon concentrations. Reproduced from reference \textsuperscript{259} with permission from the John Wiley & Sons, copyright 2006. *](image)

### 2.5.2 Carbon utilization

Once CO\textsubscript{2(g)} is in solution, it will remain dissolved as CO\textsubscript{2(aq)} without the availability of OH\textsuperscript{-}. In this condition, the presence of water will enable CO\textsubscript{2(aq)} to slowly gain H\textsuperscript{+} and equilibrate as carbonic acid (H\textsubscript{2}CO\textsubscript{3}) in trace amounts, so concentrations and rate constants of CO\textsubscript{2(g)} to H\textsubscript{2}CO\textsubscript{3} often implicitly include that of CO\textsubscript{2(aq)} and vice-versa. With increasing availability of OH\textsuperscript{-} from water splitting, however, H\textsubscript{2}CO\textsubscript{3} dissociates in relatively quick succession into bicarbonate (HCO\textsubscript{3}\textsuperscript{-}) and then carbonate (CO\textsubscript{3}\textsuperscript{2-}), which can both dominate the fraction of total dissolved inorganic carbon, Ci – the sum of all four species. Figure 2.14 shows the pH-dependence of Ci equilibrium fractions in pure
water. This distribution is not greatly influenced by additional micronutrients in BG-11 media used to culture freshwater \textit{S. elongatus} \textsuperscript{260}. Figure 2.14 then suggests that most Ci is HCO\textsubscript{3}\textsuperscript{-} in the pH range in which \textit{S. elongatus} and most cyanobacteria thrive. However, the \(~0.1 ~ 0.2~ \text{mM}\) concentration of HCO\textsubscript{3}\textsuperscript{-} resulting from BG-11 equilibrium with atmospheric CO\textsubscript{2(g)} (400 ppm) is considered rare (\(\text{<} 0.1~ \text{mM}\) \textsuperscript{259} \textsuperscript{260}). Additionally, \textit{S. elongatus} and other microalgae exclusively use CO\textsubscript{2(aq)} to fix carbon in the Calvin cycle, illustrated in figure 2.15 \textsuperscript{261}. This strong contrast between rare environmental CO\textsubscript{2(aq)} and its necessity for carbon fixation is resolved by several carbon-concentrating mechanisms (CCMs) in cyanobacteria (and other microalgae), which actively uptake HCO\textsubscript{3}\textsuperscript{-} and concentrate and convert it to CO\textsubscript{2(aq)} for carbon fixation.

![Figure 2.14: Speciation of CO\textsubscript{2} in water. The shaded region indicates the pH range of healthy \textit{S. elongatus} cultures.](image)

Figure 2.14 : Speciation of CO\textsubscript{2} in water. The shaded region indicates the pH range of healthy \textit{S. elongatus} cultures.
Figure 2.15: Carbon dioxide fixation in the Calvin cycle. Reproduced from Wikimedia commons, credit: Mike Jones.

In *S. elongatus*, uptake of environmental CO$_2$(aq) may be diffusion-driven across the outer and cell membranes (see figure 2.9), but before further diffusion into the cell, thylakoid enzymes use NADPH to facilitate conversion of CO$_2$(aq) to HCO$_3^-$, further facilitating diffusion of CO$_2$ into the cell$^{237,262}$. In contrast, uptake of environmental HCO$_3^-$ requires active transport, due to resistance to the negative charge. Specifically, an actively established Na$^+$ gradient is used with cell transmembrane symporters to bring HCO$_3^-$ into the cell. Once inside the cell, most $\text{Ci}$ remains as HCO$_3^-$ due to slow interconversion kinetics with CO$_2$(aq)$^{263}$. This mitigates diffusive losses, since the effective permeability of the outer, cell and thylakoid membranes for HCO$_3^-$ is three orders of magnitude less than for CO$_2$ $^{264,265}$. Once inside the cell, HCO$_3^-$ and CO$_2$(aq) diffuse into microcompartments called carboxysomes, containing RuBisCO and carbonic anhydrise (CA) (see figure 2.16). Here, the interconversion rate between HCO$_3^-$ and CO$_2$(aq) near RuBisCO is greatly improved, catalyzed by CA
Diffusive losses of CO$_2$(aq) from the carboxysome is restricted by currently unclear mechanisms – see reference 264 for a discussion.

RuBisCO is the enzyme that catalyzes CO$_2$(aq) into a central biomass precursor. RuBisCO is among the most globally abundant proteins, but often is the rate-limiting enzyme in photosynthesis due to low CO$_2$ affinity and selectively over O$_2$ 238,265,266. Compared to plants, cyanobacterial forms of RuBisCO have lower CO$_2$ affinity and selectively, but higher CO$_2$-saturated catalytic capacity; ~12 CO$_2$/s in *S. elongatus* compared to ~3 CO$_2$/s in Tobacco 267,268. To exploit this capacity, cyanobacterial CCMs work to provide CO$_2$(aq) at saturating concentrations local to RuBisCO.

Figure 2.16: Electron micrograph of *Synechococcus elongatus* PCC 7942 showing beta-carboxysomes (dark icosahedral shapes) and schematic of a carboxysome showing internal RuBisCo (green) and carbonic anhydrase (red). Scale bar 500 µm. Reproduced and modified from Wikimedia commons, credits: Raul Gonzalez and Cheryl Kerfeld (left); User Toyeates (right).

The following discussion of *S. elongatus* CCMs are schematically illustrated in figure 2.17. In this species, the proteins involved in HCO$_3^-$ and CO$_2$(aq) uptake mechanisms are expressed with variable amounts and affinities in response to environmental Ci and light 269. In Ci-replete media, constitutive, low-affinity transporters are expressed. At low Ci, additional and higher-affinity transporters are expressed, which require substantial energy to operate 263,268,269 and thereby constrain the acclimation response of cells. For example, figure 2.18 shows that the acclimation (lag) period of high- and low-Ci grown *S. elongatus* to a sudden increase in light is on the order of hours or minutes, respectively 211. However, these high-affinity transporters are very effective; in Ci-rare media, CO$_2$ concentrations near RuBisCO are 1000 times higher than external concentration 259. Considering the
poor O₂/CO₂ specificity of RuBisCO, this concentration also helps suppress photorespiration \(^{265, 269}\). In contrast, in Ci-replete media, the CO₂ concentration near RuBisCO is 20 - 60 times greater than the environment \(^ {259}\). Together, *S. elongatus* CCMs enable a near-constant carbon fixation rate in a wide range of environmental Ci - including near-exhausted media (figure 2.19) - and light intensities required for CCM expression and metabolic energy \(^ {259, 263, 268-271}\).

Figure 2.17: Basic components of the CCM in freshwater B-cyanobacteria strains including *Synechococcus* sp. PCC7942. Adapted from Price, *et al.* (2002), with permission from CSIRO Publishing, copyright 2002 \(^ {272}\).
Species selection

Figure 2.18: Growth rates of high-Ci (~ 4 mM, black symbols, solid line) and low-Ci (< 0.1 mM, white symbols, dashed line) cultures of *S. elongatus* measured at various times relative to the shift from low light (50 µmol m⁻² s⁻¹; time < 0) to high light (500 µmol m⁻² s⁻¹; time > 0). Reproduced from reference 211 with permission from the American Society of Plant Biologists, copyright 2004.

Figure 2.19: Net CO₂ uptake rates at steady-state photosynthetic conditions against CO₂ concentration in cells of WT Synechococcus sp. PCC7942. Experiments were performed at pH 7.8. Reproduced modified from reference 272 with permission from CSIRO publishing, copyright 2002.

2.5.2.1 Photorespiration

Photorespiration is an essential process in cyanobacteria and plants that minimizes energetic and organic carbon losses, due to unavoidable RuBisCO oxidation of ribulose-1,5-bisphosphate, which produces toxic phosphoglycolate (2PG) in an oxygen environment. In cyanobacteria, 2PG is generated at 1 – 10% of the rate of RuBisCO carboxylation, with lower rates observed even in high (5%) CO₂(g) access, and higher rates especially seen during (aiding) acclimation from high- to low-CO₂ environments. Cyanobacteria have three metabolic pathways to detoxify, or recycle 2PG into useful 3-phosphoglycerate (3PGA) - the central metabolic product of the Calvin cycle (see figure 2.15). Figure 2.2 shows the simplified 2PG-recycling process, with CO₂ and ammonia produced. This negative biomass productivity is not limited to photorespiration; CO₂ release is necessary to form many biomass precursors. In the cyanobacterium *Synechocystis*, for instance, photorespiration losses are much smaller than carbon and energetic losses by malic enzyme oxidative pentose phosphate pathway activity. Since 2PG recycling requires photosynthetic energy in the form of ATP and NADPH, photorespiration has a weak dependence on light and is generally a steady background process.
Figure 2.20: Schematic overview of photosynthesis and photorespiration. Reproduced and modified from reference 277 with permission from the American Society of Plant Biologists, copyright 2010.

2.5.2.2 Dark respiration

In contrast to the function of photorespiration to mitigate biomass loss by consuming metabolic energy, the function of dark respiration is to provide metabolic energy (ATP) in the absence of photosynthesis by consuming biomass. In cyanobacteria, the dark respiratory electron ‘chain’ uses some of the same transmembrane structures as the photosynthetic electron transport chain, with variable reaction sequences 248. For both processes, the plastoquinone pool acts as a central redox buffer, as illustrated by the photorespiratory action of SDH and NDH-1 dehydrogenases in figure 2.210 247. Since there is no or negative biomass production during dark respiration, it is essential to maintain photobioreactor cultures at light-penetrating densities for efficient biomass generation. However, biofilm-based reactors suffer from significant dark respiration when thicknesses exceed the light compensation point, at $\geq 250 \, \mu m$ 104–106,278, leading to demonstrated reduced biomass productivity 92,102,106,108, negative growth rates 105,278 and supersaturating oxygen concentration that competes with photosynthesis in well-illuminated, adjacent cell layers 104,278. In contrast, the hydrogel cultures used in a study of this work (section 5) permit photoactive illumination throughout by stratification of culture densities; high-density growth in the top layer shades a lower density bulk.
In summary, this chapter argued that in all modes of photobioreactor operation, high density cultivation is beneficial for productivity and production cost only if photosynthetic rates are maintained by light and carbon supply architectures that can match the demand rates of the culture. Several approaches to improve light and carbon supply are presented from the literature and compared to the potential of the two supply architectures developed in this work, presented in chapters 3 and 4. The current chapter also indicated that the ability of fixed cultures to acclimate to local light and carbon environments may enable higher photosynthetic efficiencies than suspension cultures and potentially greater gross photosynthesis. However, in high density biofilms, respiration losses to net productivity may limit production. These losses may be reduced by harvesting biofilms before growth to light-limiting thickness, or by using alternative fixed-culture formats that enable high-density growth but permit photoactive light penetration in the bulk. A combination of these harvesting and cultivation approaches is presented in the study of chapter 5.
3  Evanescent cultivation of photosynthetic bacteria on thin waveguides

This chapter was originally published in Journal of Micromechanics and Microengineering, volume 24, pages 045017(8) (2014), and has been adapted with permission. © IOP Publishing. All rights reserved. The applicant was the primary author for this work and played the primary role in experiment design, execution, data collection, data analysis, and paper writing. The efforts of all other authors are gratefully recognized, they are: Matthew D. Ooms and David Sinton.


Waveguides with thicknesses similar to biofilms (10 – 100 µm) provide an opportunity to improve the bioenergy density of biofilm photobioreactors, avoiding the fundamental light- and mass-transport productivity limitations of planktonic photobioreactors. This report investigates the biofilm growth of a mutant of *Synechococcus elongatus* (PCC 7942) in evanescent light fields that can be scaled over large planar areas. In this study, areas of 7.2 cm² are illuminated via frustrated total internal reflections on planar waveguides. The resulting photosynthetic biofilm growth showed resilience to surface intensities exceeding photosynthetic limits and a more uniform cell density distribution \(1.0 ± 0.3 \times 10^9 \text{ mL}^{-1}\) than predicted from surface light distribution profiles. These results indicate potential for larger area biofilms using the uniform lighting conditions identified. The combination of evanescent illumination with biofilms indicates a modular reactor cell density on the order of \(10^8 \text{ mL}^{-1}\), representing a two orders of magnitude improvement over current facility architectures, with significant potential for further improvement through denser biofilms.

3.1  Introduction

Concerns over climate effects of rising atmospheric CO₂ concentration from fossil fuel combustion are motivating the development of carbon-neutral, sustainable fuel generation technologies to supplement global energy demand while mitigating near-term CO₂ generation. Microalgal-based biofuels, generated from biomass products of photosynthesis-driven CO₂ sequestration, are a carbon-neutral and more sustainable alternative to terrestrial crop-based biofuels.

Current widespread microalgal cultivation technologies involve planktonic cultures (i.e. microalgae in solution) in controlled environments of photobioreactors (PBRs). In these reactors, volumetric productivity suffers from fundamental light- and fluid-regime restrictions on densities, growth rates and the scalability of reactor dimensions. Specifically, light absorption by high density cultures
creates an internal intensity gradient, resulting in a photosynthetically inactive fraction of the liquid reactor volume that limits density and channel cross-sections \(^{126}\). In addition, slow mass transport in macroscale PBR channels causes regions of nutrient limitation and toxic product holdup (e.g. O\(_2\)) that inhibit photosynthesis, slow growth and limit reactor channel lengths \(^{285,286}\). Both the light- and fluid-regime limitations to productivity are tied to scale (increasing with reactor size) and thus present barriers to large-scale deployment of these technologies. Over 40 years of PBR design and development to mitigate these limitations have improved volumetric productivities, but with marginally increasing efficacy and cost-effectiveness upon reactor scale-up \(^{156,177,286-289}\). Volumetrically cost-effective biofuel production on the industrial scale requires a PBR design that uniformly illuminates culture (lacking a photosynthetically inactive fraction) and does not rely on macroscale mass transfer - these requirements are satisfied by biofilm-based photobioreactors (BPBRs).

In contrast to planktonic cultures, biofilms have microscale thickness that is independent of reactor channel cross-sections and so naturally favors microscale spacing between biofilms for high reactor density and microfluidic perfusion through this space for rapid mass transfer \(^{172}\). Furthermore, the limited thickness of biofilms enables near-uniform culture illumination that precludes an inactive fraction and ensures efficient light usage, even at film densities greatly exceeding that of planktonic cultures. Despite this potential to overcome the fundamental productivity limitations of planktonic PBRs, biofuel productivity studies in BPBRs have only recently begun (2010) and are limited to biohydrogen production in non-scalable or single-biofilm reactors that are not suited to integration at high density \(^{154,290-293}\). As conceptually illustrated in figure 2.6, multilayered planar waveguides provide a route to scalable, high energy density BPBRs using photosynthetic biofilms optically excited by frustrated total internal reflection (FTIR). In this illumination mechanism, the evanescent light field of inherently nanoscale depth that extends from the surface of a waveguide, where a photon undergoes total internal reflection (TIR), is perturbed by coinciding near-surface biomass of refractive index greater than the surrounding media. This frustrates the TIR condition and allows the photon to escape the waveguide. As demonstrated here, evanescent fields that enable FTIR illumination can be distributed controllably and with dramatic scale over planar waveguide surfaces, with implications for inherently large-scale energy challenges. In comparison, the distribution of scattered light illumination from roughened (lossy) waveguides is fixed in fabrication and so is less flexible than FTIR illumination to evaluate and tune intensity-dependent growth. It is noteworthy that substrate roughness, one of several factors influencing initial microalgal and bacterial
attachment, does not necessarily influence developed bacterial or axenic microalgal biofilm density, though near-substrate biofilm morphology may be affected.

There have been several recent advances involving the evanescent growth of microalgae. Jung, et al. showed that the efficiency of FTIR photon usage for cyanobacteria biomass growth is comparable to that of direct, radiative illumination. In recent work towards the proposed BPBR architecture, Ooms, et al. demonstrated evanescent intensity-dependent growth of the model cyanobacteria *Synechococcus elongatus* (PCC 7942) in a single 0.07 cm² FTIR laser spot on a glass prism waveguide. A subsequent study demonstrated successful growth of the same strain employing the evanescent fields of surface plasmon resonance in the same illumination setup. This species has demonstrated compliance to genetic engineering approaches for direct biofuel synthesis and flourishes on an economically minimal nutrient supply of trace elements and dissolved CO₂. While exciting, these recent demonstrations were restricted to small areas and/or low-density conditions. In the present work, planar waveguides enable evanescent growth area scale-up through multiple FTIRs and quantification of high-density microalgal biofilm growth as a function of local evanescent field intensity. A biofilm-forming mutant of *S. elongatus* cultivated over 7.2 cm² of thin glass waveguides, 0.16 mm thick, enabling a modular architecture for scale-up (stacking) at high density.

### 3.2 Methods

#### 3.2.1 Microorganism and cultivation

Cultures of a biofilm-forming mutant strain of *Synechococcus elongatus* (PCC 7942), herein referred to as T2SEΩ, donated by Prof. Rakefet Schwarz (Bar-Ilan University, Israel), were maintained at 30°C under continuous illumination of 15 µmol m⁻² s⁻¹ of photosynthetic active radiation (PAR, 400 nm – 700 nm) in modified BG-11 media, containing solutions of: NaNO₃ 1.5 g, MgSO₄·7H₂O 65 mg, CaCl₂·2H₂O 36 mg, K₂HPO₄ 306 mg, Na₂EDTA·2H₂O 1 mg, Iron (III) ammonium citrate 6 mg, Citric acid 6 mg L⁻¹ sterilized by autoclave and Trace Metal Mix A5 1 mL L⁻¹ sterilized by filtration, buffered to pH 8.0 with NaOH.

#### 3.2.2 PMMA waveguide apparatus, operation and growth analysis

Figure 2(a) shows a cross-section illustration of the 20 mL capacity polymer waveguide reactors made from laser-engraved poly(methyl methacrylate) (PMMA) with waveguide dimensions (6 x 70 x 70) mm³. Reactors were seeded with 0.2 OD₇₅₀ nm wild-type *S. elongatus* culture, sealed with a gas-permeable, vapor-resistant silicone membrane (Rodgers Corp., BISCO HT-6240), placed in 32°C atmosphere and isolated from continuous sources of external light. Light from a 656 nm peak light
emitting diode (LED) (OSRAM GmbH, LH W5AM) was butt-coupled through an aperture into PMMA waveguides. Growth continued for 3–5 days until the growth area at the 2nd FITR reflection did not increase. SLR camera (Nikon D60) images of reactors were used to determine relative growth, quantified by green channel pixel values (0 to 1) in which red and blue channel values respectively exceeded 0.925 and 1.5 (as defined in mat2gray() in MathWorks MATLAB).

3.2.3 Glass waveguide apparatus, operation and growth analysis

The 10 mL capacity glass coverslip waveguide reactors were made by laser-engraved stacked PMMA sheets bonded with chloroform with a silicone layer for gas exchange. Glass coverslip waveguides (0.160 x 24 x 60) mm³ (Ted Pella, Inc., 260360) were immersed in 0.1 OD_{750 nm} T2SEΩ culture and suspended above the reactor base by a laser-engraved step in a supporting PMMA layer. Cells settled by gravity onto the coverslip and biofilm formation was self-triggered between 48 and 72 hours of continuous illumination under 25 µmol m⁻² s⁻¹ PAR (Skye Instruments, SKP 200 and SKP 215). Without external illumination, a 660 nm peak diode laser (5 – 250 mW) beam was coupled into a short waveguide edge using a plano-concave/-convex lens pair (Thorlabs Inc., LK1336RM-A and LJ1703RM-A) to produce a thin 0.16 mm x 12 mm beam geometry at θ_{in} = 45° with respect to the normal of the glass input face. Following 6 days of continuous exposure to evanescent illumination, the reactors were moderately agitated by hand to remove weakly adhering cells from biofilms. Coverslips were then removed and quickly transferred to a bath of fresh media in a holder designed for optical sectioning in laser-scanning confocal microscopy (Carl Zeiss, Inc., LSM-510). Chlorophyll-a in biofilms was excited (λ_{EX} = 543 nm, λ_{EM} > 650 nm) and image stacks were taken in a 7x7 grid located at 5, 10, 20, 30, 40, 50 and 55 mm along the waveguiding axis and every 2 mm in the center 12 mm portion perpendicular to the waveguiding axis. Cell density was determined as described in 3.5.2. Following confocal analysis, biofilms were removed from media, dried in ambient conditions and placed on a fluorescent lamp to image visual pigmentation. Relative growth was quantified as for PMMA reactors.

3.2.4 Determination of waveguide surface intensities

Evanescent field maps were measured independently from the growth experiments in PMMA and coverslip reactors using fluorescent solution and particles, respectively.

In PMMA waveguide reactors, 10 mL of 20 mM fluorescein sodium salt solution (Sigma-Aldrich, Inc.) covered the waveguide surface. A cone of light rays supplied from a 505 nm LED (OSRAM GmbH, LV
W5AM) was butt-coupled into the waveguide in the identical setup as used for growth. FTIR regions were visualized by the locally excited solution, imaged for relative intensity.

In coverslip waveguide reactors, a 404 nm laser was butt-coupled to the waveguide as described for the growth experiments with input angles ranging from $\theta_{in} = 20^\circ$ to $45^\circ$ in $5^\circ$ increments. Polystyrene fluorescent microspheres (Cospheric LLC, FMY) were seeded into the reactor at 0.54 $\mu$g mL$^{-1}$ and settled onto the surface with a surface particle density similar to that of cell-seeded growth reactors (to the extent possible without significant background signal generated by self-excited fluorescence). One of the two output beams (owing to the odd or even total number of internal reflections) was focused through a matched achromatic doublet lens pair (Thorlabs, Inc., MAP103030-A) and passed to a power detector (Thorlabs, Inc., S120C) and screen pair. Specifically, the screen provided the relative beam intensity map and the detector provided the intensity calibration. The difference in waveguide input and output power, minus measured coupling and waveguide-edge losses, was solely attributed to upper surface losses due to the presence of the microspheres. Surface power losses were normalized to the summed fluorescence signal of corresponding images and distributed among pixels, weighted by their fluorescence signal, to yield surface intensity maps (W/m$^2$).

3.3 Results and discussion

3.3.1 PMMA waveguide growth results

Figure 3.1 shows relative evanescent field intensity and growth results on planar, 6 mm thick PMMA waveguides using an LED source. The setup shown in figure 3.1(a) provides a simple experimental system whereby growth in response to FTIR light can be observed at multiple, discrete reflections (see also growth time lapse Movie 3.1). Periodic relative evanescent field intensity peaks are clearly delineated in figure 3.1(b) using a shallow fluorescein solution. Green pigmentation thresholding was applied to images of growth results, such as the one shown in figure 3.1(c). Figure 3.1(d) shows significant positive correlation between relative growth due to evanescent light and normalized fluorescence intensity ($p < 5 \times 10^{-10}$). Growth in the first 18 mm is partly affected by scattered light due to coupling losses and so was excluded from figure 3.1(d). These results indicate the efficacy of growth using multiple FTIRs in planar waveguides. It is noteworthy that the incoherent cone of LED light rays resulted in evanescent fields with penetration-depths ($1/e$) on the order of 150 nm, calculated using classical electrodynamics theory (assuming 90° grazing incidence and $\lambda = 660$ nm). This is small compared to field depths resulting from spatially coherent light guided near the critical angle ($> 2 \mu$m). The observed growth in small penetration depth fields points to future applicability of evanescent illumination using incoherent sunlight. While these results clearly demonstrate planar
growth in multiple FTIR spots, the waveguide is impractically thick as compared to the biofilm (6 mm vs. 0(10) µm). In order to achieve practical BPBR module densities, much thinner planar waveguides are required.

Figure 3.1: Planar PMMA waveguide with LED input setup and FTIR growth results. (a) Reactor schematic and light path, (b) FTIR regions visualized through fluorescence with apparent spreading of uncollimated, waveguided light, (c) corresponding microalgae growth (time lapse growth in Movie
3.1), and (d) correlation between measured normalized field intensity and relative growth results from five identical experiments, each averaged along the center waveguiding axis. Growth in and just downstream of the 1st FTIR region is partly attributed to scattered light due to coupling losses.

### 3.3.2 Glass waveguide growth results

The reactor based on a thin glass waveguide (24 mm x 60 mm x 0.16 mm) is shown schematically in figure 3.2(a). Initially distinct and tightly-spaced surface reflections in figure 3.2(b), termed striations, give way to more uniform surface illumination due to beam spreading. Two beams exit the waveguide owing to an odd or even total number of internal reflections. Striations are visible near the input side of the fluorescence-based evanescent surface intensity maps at each coupling angle, shown in figure 3.2(c). For direct comparison, plots of normalized axial evanescent field intensity maps are shown together as intensity profiles in figure 3.2(d). A small intensity increase over the last 20 mm of the waveguide, observed at coupling angles ≤ 35°, is attributed to light back reflecting off the far end of the waveguide, which is potentially useful for growth. Within the first 35 mm of waveguides, two angle-dependent intensity trends can be identified, with important implications for the utility of this illumination approach. At coupling angles ≤ 35° surface intensity is near-uniform along the length of the waveguide and decreases with angle. This trend suggests that uniform surface illumination on longer waveguides is possible, by conserving waveguided light using small coupling angles. For example, at 30°, 25° and 20° coupling angles the length of waveguides with surface intensities within the growth limits of wild-type *S. elongatus* 296, are estimated to be (110 ± 20) mm, (340 ± 220) mm and (700 ± 440) mm respectively, requiring (70 – 160) mW of monochromatic red light (633 – 660 nm) (see supplementary information section 3.5). In this work, the other dimensional limitation to demonstrated biofilm growth area is the 12 mm wide laser beam profile. Use of coupling angles ≤ 20° and wider beam profiles potentially enable the scalable, large area, uniform illumination required for high-density BPBR modules. In contrast, at coupling angles ≥ 35° a clear tradeoff between uniformity and peak intensity enables non-uniform profiles for the quantification of biofilm growth as a function of surface intensity.
Results and discussion

(a) Diagram of the optical setup showing glass output and input faces, a coverslip, and a housing.

(b) Diagram showing the detected beam.

(c) Images showing the detected beam at different angles: 45°, 40°, 35°, 30°, 25°, and 20°.

(d) Graph showing the normalized evanescent field near-surface intensity (m²) as a function of the center waveguiding axis (x, mm) for different angles: 45°, 40°, 35°, 30°, 25°, and 20°.
Results and discussion

Figure 3.2: Thin glass waveguide with laser input setup and evanescent field characterization. (a) Reactor schematic showing exploded assembly view and (b) internal light path with coupling angle (θ). (c) Images of fluorescence intensity of surface-settled fluorescent microspheres on a single waveguide, indicating relative evanescent surface intensities as a function of labelled laser angle. (d) Corresponding plot of cross-sectional averaged near-surface light intensities, normalized to input power, in center 12 mm along the waveguiding axis dimension (x) at coupling angles (descending with decreasing normalized intensity at x = 0).

Figure 3.3 shows the results of T2SEΩ growth experiments in the thin glass waveguide reactor at 45° coupling angle with the corresponding surface evanescent field intensity profile plotted for reference. At the input end of the waveguiding axis (x = 0), average density increases with length, effectively leveling out in the region x > 20 mm. This trend is generally supported by previous experiments showing growth at monochromatic red intensities between 66 W/m² and 12 W/m², corresponding here to the portion of the waveguide between x = 20 to 57 mm (cut-off value intersections indicated by dotted black lines in figure 3.3(a)) 296. While the general growth trend is to be expected from the measured light intensities (figure 3.2(d)) and previous intensity limits to growth 296, the presence of substantial biofilm density at x < 20 mm is noteworthy. In effect, the transition from photoinhibition to growth is blurred in these reactors and this is attributed to a few potential factors. First, as shown in the intensity profiles, the surface light intensity is highly non-uniform (with light striations exhibiting a periodicity of ~ 500 µm) in the context of a ~ 20 µm thick biofilm. The surface intensity between striations will be well below the average local value. The amplitude of the light variations is also likely underestimated by the limited resolution of the intensity measurement method applied here. In short, cells harvesting locally low light levels between striations may proliferate in an otherwise photoinhibitory regime. Second, light scattering by cells within the biofilm smooth the influence of surface intensities on growth. Lastly, developed biofilms displayed significant heterogeneity, as quantified by the error bars in figure 3.3(a). Heterogeneous biofilms locally influence downstream light intensities and subsequent growth. Importantly, these results indicate the efficacy of FTIR cultivation of biofilms in thin glass, planar waveguide BPBRs, resulting in remarkably uniform biofilm densities (1.0 ± 0.3) x 10⁹ mL⁻¹ overall and (1.2 ± 0.8) x 10⁹ mL⁻¹ within photosynthetic limits, in response to nonuniform surface intensities. Downstream of the upper intensity limit on the waveguide (x > 20 mm), a similar variation in biofilm density and thickness between waveguide regions that guide light semi-coherently (where striation peaks are visible) and incoherently (including in PMMA reactors) suggest that surface intensity is a more important factor
for biofilm growth than evanescent field penetration depth. This supports the observation in PMMA reactors that incoherent light can be used for evanescent illumination.

Figure 3.3: Biofilm growth results in thin glass photobioreactors at \( \theta_m = 45^\circ \). (a) Plot of average cross-sectional biofilm cell densities obtained from chlorophyll-excited confocal stack images (circles, with error bars showing maximum and minimum density, \( n = 3 \)). The microsphere fluorescence-derived surface light field intensity is shown at left for reference (solid line). Photosynthetic intensity thresholds for the wild-type of this species are shown at \( x \) locations indicated by black dashed arrows. (b) Confocal depth-wise image sections of a representative biofilm with (c) relative growth (green pigmentation) image of the biofilm over the entire waveguide surface, showing low growth in the striation region (left side). Cross-sectional averages include the center 12 mm region perpendicular to the waveguiding axis, indicated by the dashed lines in (c).
In the context of photobioreactor systems, these growth results provide some indication of the potential of BPBRs. Specifically, modules of the proposed architecture each include a waveguide for illumination, a biofilm, and a microchannel for mass transport. Employing the thin waveguides here, the biofilm thicknesses grown, and moderate 50 µm microchannel depths results in module cell densities on the order of $10^8 \text{mL}^{-1}$, maintained at system-level upon modular scale-up. Direct comparison with existing technologies, such as tubular PBRs, is complicated due to strain-specific performance, cell size differences, light utilization variability within the culture and the very different geometries and harvesting modes of these facilities. An optimization study that provided detailed tube-reactor geometry and light utilization levels\textsuperscript{126}, combined with typical planktonic cell densities ($10^8 \text{mL}^{-1}$), points to a facility-level density on the order of $10^6 \text{mL}^{-1}$. In comparison, the proposed approach provides at least two orders of magnitude improvement in facility-level density. The far-red light used in this work can substitute for sunlight since energy from blue photons is ultimately down-converted to the energy required to drive the photosynthetic reaction centers of the cell through fluorescence and/or heating\textsuperscript{297}. This approach also offers the potential to avoid light and mass-transfer limitations of traditional reactors to achieve efficient biomass production, or, more suited to this architecture, volumetrically cost-effective direct biofuel production from engineered microalgae. Module density enhancement is possible, primarily by increasing biofilm cell content, for example, by longer growth times or thicker biofilms. Further enhancement by limiting the thickness of the waveguide and perfusion layers is limited by mechanical strength and shear forces, respectively.

### 3.4 Conclusions

In this work, biofilms of a mutant strain of *Synechococcus elongatus* (PCC 7942) were illuminated by multiple FTIR beams, using evanescent light fields distributed over 7.2 cm$^2$ of sub-millimeter thick planar waveguides. Despite local heterogeneities, biofilms developed in photosynthetically useful and inhibiting intensities were remarkably uniform, with overall densities (1.0 ± 0.3) x $10^9 \text{mL}^{-1}$ and thicknesses (18 ± 9) µm. No intensity limit to growth was observed, indicating potential for longer waveguides and greater growth areas. Growth results in semi-spatially coherent and incoherent FTIR beams together suggest that surface growth is relatively less dependent on evanescent field penetration depth than on intensity, and that this dependence is somewhat alleviated by the developed biofilm morphology. The biofilm densities achieved suggest BPBR module densities on the
order of $10^8 \text{ mL}^{-1}$ – at least two orders of magnitude greater facility-level density than in current PBRs.

### 3.5 Supplementary information

#### 3.5.1 Estimation of waveguide lengths

The length of waveguides supporting only surface intensities (12 – 66) W/m² were estimated by extrapolation of fits of the intensity maps of figure 3.2 to the form $a \exp(b \times x) + c \exp(d \times x)$. Figure 3.2(d) data was first smoothed using a 5 point moving average filter to reduce intensity oscillations (striations) at waveguiding axis lengths $x < 30$ mm. The range of smoothed data used in these fits was $1 \text{ mm} < x < 15 \text{ mm}$ and $25 \text{ mm} < x < 35 \text{ mm}$, in order to avoid regions of back-reflection ($x > 35 \text{ mm}$) and a region of artificially high fluorescent microsphere concentration ($15 \text{ mm} < x < 25 \text{ mm}$) due to disturbances arising from the measurement method. The error ranges of extrapolated waveguide lengths supporting *S. elongatus* photosynthetic intensities of $(110 \pm 20) \text{ mm}$, $(340 \pm 220) \text{ mm}$ and $(700 \pm 440) \text{ mm}$, respectively for $30^\circ$, $25^\circ$ and $20^\circ$ coupling, reflect 95% confidence bounds for the fitting coefficients a to d.

#### 3.5.1.1 Addendum to estimation of waveguide lengths

The above fitting analysis may overestimate the length of 160 μm glass waveguide that can support photosynthetic surface intensities for *S. elongatus*, due to the compounding uncertainties of manually excluding the data ranges above and extrapolating intensity decay profiles to lengths over ten times the 60 mm length over which fluorescence profiles were measured. Conversely, however, the above fitting analysis may underestimate the predicted photoactive waveguide lengths, due to the use of 12 W/m² as the lower intensity threshold of *S. elongatus*. This intensity is equivalent to 66 μmol m⁻² s⁻¹ at the peak 660 nm wavelength of light used in the growth experiments, yet *S. elongatus* growth is strongly positive at 25 μmol m⁻² s⁻¹ (4.7 W/m² of 660 nm light)¹²⁰. Another set of fits were then performed to better account for these competing effects on estimates of 160 μm glass waveguide lengths that can support photosynthetic surface intensities for *S. elongatus*. The decay profiles of $25^\circ$ and $20^\circ$ coupling angles were used, since these profiles were the most uniform of those measured, which is desirable for surface growth. The revised fits used data that maximized the decay of intensity with length ($x$) in these profiles, and 25 μmol m⁻² s⁻¹ was used as the low-intensity bound. Fits were of the same form ($a \exp(b \times x) + c \exp(d \times x)$) as above, and applied to the same smoothed and 5-point moving averaged data sets as above. The range of data used was restricted to the steep intensity fall-off regions $0.55 \text{ mm} < x < 14 \text{ mm}$ and the 1 mm range about the lowest point in the range
25 mm < x < 40 mm, which avoided the artificially high intensity regions described above. The revised maximum and minimum estimates of waveguide lengths that reflect 95% confidence bounds for the fitting coefficients (a to d) are 618 mm and 1170 mm for 25° coupling, and 200 mm and 650 mm for 20° coupling, respectively. The revised minimum length estimates are 2.81 and 0.83 times the original fit minimum lengths for 25° and 20° coupling, respectively. The revised fitting method improves upon assumptions of the original method, and provides a more conservative estimate of the length of waveguide that would support photosynthetic intensities of this species: at least 20 cm.

3.5.2 Individual biofilm growth results in glass waveguide reactors

Figure 3.4 shows growth results for individual biofilms with densities plotted for each (right axis) and the measured evanescent field intensity shown for reference (left axis). In all cases, biofilm cell density was calculated by applying a consistent and arbitrary intensity threshold to chlorophyll-a fluorescence in all confocal slices and dividing the number of filled voxels in slices by the mean number of voxels of 10 well-defined cells found in confocal stacks (480 ± 90 voxels/cell). At the first instance of evanescent illumination, after biofilm formation under direct light, biofilm density was uniform, as indicated by the average density of direct light control reactors across all waveguide grid locations (0.4 ± 0.1 x 10⁹ mL⁻¹).
Figure 3.4: Example of inhomogeneity between individual biofilms grown in separate reactors (identical growth settings for three biofilms; red, green, blue), shown by relative green pigmentation of visual images (dashed lines, arbitrary units) and mean biofilm density from confocal stacks averaged over the center 12 mm portion perpendicular to the waveguiding axis dimension (x) (points with standard deviation error bars). The extents of cross-sectional density for each biofilm is shown as light patches. Mean evanescent field surface intensity profile is included for reference (solid line).

Movie 3.1: Time lapse video from bright field imaging of the entire polymer waveguide surface. Images were taken every 30 min with < 1 s exposure time, during evanescent excitation. Link: stacks.iop.org/JMM/24/045017/mmedia
4 Breathable waveguides for combined light and CO$_2$ delivery to microalgae

This chapter was originally published in Bioresource Technology, volume 209, pages 391-396 (2016), and has been adapted with permission. © Elsevier. All rights reserved. The applicant was the primary author for this work and played the primary role in experiment design, execution, data collection, data analysis, and paper writing. The efforts of all other authors are gratefully recognized, they are: Jason Riordon, Brian Nguyen and David Sinton.

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Suboptimal light and chemical distribution (CO$_2$, O$_2$) in photobioreactors hinder phototrophic microalgal productivity and prevent economically scalable production of biofuels and bioproducts. Current strategies that improve illumination in reactors negatively impact chemical distribution, and vice versa. In this work, an integrated illumination and aeration approach is demonstrated using a gas-permeable planar waveguide that enables combined light and chemical distribution. An optically transparent cellulose acetate butyrate (CAB) slab is used to supply both light and CO$_2$ at various source concentrations to cyanobacteria. The breathable waveguide architecture is capable of cultivating microalgae with over double the growth as achieved with impermeable waveguides.

4.1 Introduction

Microalgal-based photobioreactors (PBRs) are a promising technology for renewable biofuel and high-value bioproduct generation. Given the strong dependence of microalgal photosynthesis on light intensity and chemical concentration (CO$_2$, O$_2$), optimal production necessitates tight control of these conditions throughout. While open pond PBRs benefit from design simplicity, they have not been cost-effective due to light and chemical supply limitations that cause gradients throughout the reactor that limit photosynthetic efficiency and culture density. In contrast, closed PBRs allow greater photosynthetic productivities and densities, due to improved light distribution and CO$_2$ and O$_2$ transport. However, strategies that benefit light distribution often negatively impact chemical control, and vice versa.

Delivering the optimal amount of light to cells is critical to reactor design. Illumination control in closed PBRs has traditionally been accomplished by turbulent perfusion. Turbulent mixing and bulk flow moves cells through a light gradient for time-averaged intensity exposure. While effective, this fluid circulation is energy-intensive, and is impractical at large scales. For example,
in tube pilot plant reactors, liquid pumping requires 53–60% of operational power \(^{51,78}\). These approaches bring cells to light. An alternative illumination strategy is to distribute light to cells via waveguides in reactors. Waveguiding distributes light through large volumes of culture, and shifts operational cost (pumping) to capital cost (material). Recently, reactors using planar waveguides have demonstrated improved light distribution, photosynthetic productivity, cell density, and modular scalability \(^{147,178}\). However, waveguides developed to date occupy significant reactor volumes (5–33%), limiting volumetric productivity \(^{178,299,300}\). Furthermore, all waveguide reactor strategies reported to-date are impermeable, choking cultures of vital \(\text{CO}_2\).

Control of the chemical environment is also critical to photobioreactor operation. One traditional approach is to bubble \(\text{CO}_2\) at a single node and circulate the culture through the reactor loop. This approach suffers from chemical hold-up in the reactor, that is, \(\text{CO}_2\) depletion and \(\text{O}_2\) accumulation. The alternative approach is to bubble \(\text{CO}_2\) throughout reactor panels. Bubbling throughout the illuminated culture in flat panel reactors effectively removes chemical gradients and enables the greatest productivities, densities and photosynthetic efficiencies. However, bubbles on the top surface of the reactor panels scatter light strongly. Thus, improved chemical transport is achieved at the expense of light distribution and usage \(^{94}\). Bubbling is also expensive. In tube pilot plant reactors, active aeration and \(\text{CO}_2\) costs are respectively 7–16% and 15–44% of nutrients-plus-utilities costs \(^{51}\). Further, aeration and \(\text{CO}_2\) costs are each 5–10% of capital-plus-operation costs \(^{55,77}\). Alternative aeration architectures based on nanoporous membrane contactors achieve single-phase gas-to-liquid mass transfer, and cost-effective \(\text{CO}_2\) delivery and utilization \(^{197,301,302}\). Recently, hollow fiber membrane contactors have demonstrated enhanced mass transfer of \(\text{CO}_2\) to cells in photobioreactors \(^{81,178,200}\). With high density cultures, however, a high density of hollow fibers is required to avoid \(\text{CO}_2\) limitations \(^{178,200}\). While membrane contactors clearly show promise for photobioreactors, and have been deployed at scale in waste water treatment \(^{190,301}\), all current efforts involved opaque membrane materials that block light.

In this work, an integrated illumination and aeration architecture is demonstrated, using a single transparent, hydrophobic, nanoporous material - a breathable waveguide - to simultaneously distribute light and \(\text{CO}_2\) throughout a microalgal culture. Use of a single surface for combined delivery leverages the similar length scale of light and \(\text{CO}_2\) dispersion (mm to cm) in high density cultures \(^{94,303}\). The phototrophic biomass growth of batch cultures of \(S.\ elongatus\) were evaluated in carbon-limited and -replete conditions, using cellulose acetate butyrate first as a contactor for \(\text{CO}_2\) permeation alone, and then as a breathable waveguide. Positive growth response to \(\text{CO}_2\) permeation
was observed and double the growth was achieved with waveguide illumination and breathability as compared to waveguide illumination alone.

4.2 Materials and methods

4.2.1 Microorganism and cultivation

The microalgae used for all experiments was a T2SE-null mutant of the freshwater cyanobacteria *Synechococcus elongatus* PCC 7942 with kanamycin resistance, donated by Professor Rakefet Schwarz (Bar-Ilan University, Israel). The mutation permits biofilm growth, but this capacity was not used. This species was selected for its high-affinity carbon uptake in CO$_2$-rare media, which enabled clear evaluation of CO$_2$-limited growth \(^{236,237}\). Additionally, *S. elongatus* is a model organism in photosynthetic studies, and is one of four microalgal species that have received great attention as direct producers of high-value biofuels and bioproducts, due to their amenability to genetic modification. Ethanol, isobutyraldehyde, and free fatty acid production in *S. elongatus* have been demonstrated \(^{221,295,304-306}\).

The culture was grown in modified BG-11 media (Sigma-Aldrich, C3061), supplemented with 50 µg/mL kanamycin and 80 mM HEPES buffer, pH-adjusted to 8.0 with NaOH and kept at a temperature of 27 ± 1 °C. The culture was maintained in a shaken incubator flask with ambient 0.05 ± 0.01% CO$_2$ headspace. Culture depth and 0.1 optical density at 750 nm (OD$_{750nm}$) were maintained by daily dilution with fresh media. The incubator and experiment reactors were illuminated with red light (632 – 638 nm), which enables strong growth response in lieu of the solar spectrum, and is standard practice when using monochromatic sources \(^{120,179,189}\). In the incubator, an array of 632 nm LEDs (OSRAM Opto Semiconductors) and light-diffusers provided uniform area illumination.

4.2.2 Breathable waveguide material

Cellulose acetate butyrate (CAB) was chosen as a breathable waveguide material for this study, due to its optical transparency, biocompatibility, relatively high gas permeability (32 and 6.2 Barrer to CO$_2$ and O$_2$, respectively), and relatively low cost and water impermeability. A comparative table of permeabilities and costs of a selection of other candidate breathable waveguide materials is presented in Supplementary Material.
4.2.3 Breathable reactor design and operation

Five breathable reactors were fabricated, as shown in Fig. 4.1(a). For each reactor, constant output from a 634 ± 14 nm LED (Avago Technologies) and collimator lens (Thorlabs) provided uniform-area intensity top-down through 13 mL of culture in a black-anodized 25 mm ID aluminum cylinder. A glass window above the culture sealed 3 mL of initially ambient atmosphere (0.05% CO₂), such that 0.7 ± 0.1 mM of total inorganic carbon (herein Ci - the sum of CO₂(aq), H₂CO₃, HCO₃⁻ and CO₃²⁻ species) was initially available to cells. The bottom of the culture contacted a highly gas-permeable, white, hydrophobic membrane (Pall, Versapor 200 R), which reflected diffuse light into the culture. A test material could be fastened directly below. The Versapor membrane served to maintain identical illumination conditions, independent of membrane test material. Both membranes sealed the culture against a polyethylene terephthalate (PET) sheet, with a CO₂ permeability of 0.27 Barrer (Goodfellow, ES303020). Flowing CO₂ diffused into the culture from below. Ambient pressure, air-balanced CO₂ mixtures were flowed at 1.4 L/min with 75 ± 2% relative humidity. Water vapor added to the gas stream mitigated water loss due to permeation (< 5%).

Five cases were tested in triplicate: (i) CAB contactor with 0.05 ± 0.01% CO₂, (ii) CAB contactor with 0.50 ± 0.06% CO₂, (iii) CAB contactor with 5.00 ± 0.02% CO₂, (iv) impermeable glass disk (negative control) and (v) no additional material with 5.00 ± 0.02% CO₂ (positive control). All cultures were initially at 0.10 ± 0.02 OD750nm. After 65 hours of growth, individual culture densities were measured with 0.001 OD750nm uncertainty (custom software written in Ocean Optics OceanView). To mitigate the impact of transitory growth rates on steady-state growth, cultures were not exposed to a change in photon flux when experiments were initiated, as discussed in Supplementary Material. To avoid the possibility of micronutrient-limited growth, experiments were halted after 65 hours so that densities did not exceed ~ 0.7 OD750nm.

4.2.4 Breathable waveguide reactor design and operation

The breathable waveguide reactor is schematically illustrated in Fig. 4.1(b). A 638 nm laser diode was focused by a plano-concave/-convex lens pair (Thorlabs) into a 0.3 mm × 24 mm line beam profile. The beam was coupled at 0° angle into a 1 mm-thick CAB sheet, which emitted continuous surface intensities spanning 20 – 40 µmol m⁻² s⁻¹. Waveguide losses were quantified, as discussed in Supplementary Material. The CAB sheet supported 20 mL of media (no headspace) and a 24 mm × 93 mm × 2 mm slab of S. elongatus hydrogel culture. A PMMA block gently pressed the hydrogel against the waveguide. The hydrogel was prepared by mixing 10 mL each of 0.2 OD750nm culture and 4% wt/vol alginic acid sodium salt (Sigma-Aldrich) in modified BG-11 media. The alginate was
crosslinked by setting the mixture on top of a gel containing 4% wt/vol agarose (Sigma-Aldrich) and 100 mM of CaCl₂ in deionized water. Use of a hydrogel culture ensured an initially uniform distribution of cells at 0.1 OD₇₅₀ nm in the reactor. Hydrogels are commonly used to immobilize cells for biomedical applications. CO₂ was flowed below the reactor at the same conditions as for breathable reactors, in addition to 0% CO₂ flow (99.998% N₂). Duplicate cultures were grown at 0.05 and 0.5% CO₂ source concentrations, and single cultures were grown at 0 and 5% CO₂ source concentrations. After 65 hours, the hydrogel was removed and a center region of uniform density was cut out and de-gelled by addition of 10% wt/vol sodium citrate in order to evaluate optical density due to waveguide CO₂ permeation.

Figure 4.1: Photobioreactor schematics. (a) Breathable reactor, with photograph shown at right. A shaker table was used to prevent cells from settling. (b) Breathable waveguide reactor, with photograph at right showing surface emission from 1 mm × 10 cm × 10 cm CAB waveguide in air (optical input is 70 ± 5 mW). All reactors were maintained at a temperature of 27 ± 1 °C; ambient
Results and discussion

4.3 Results and discussion

4.3.1 Breathable reactors with bulk LED illumination

Permeation experiments were conducted to assess the growth of *S. elongatus* due to CO₂ permeation through 0.25 mm thick transparent CAB contactors. After 65 hours of incubation, various levels of CO₂ permeation affected final culture density, as shown in Fig. 4.2(a). The growth results are plotted with negative and positive control reactors which used glass or no material in place of CAB, respectively. Growth increased with increasing CO₂ concentration applied below the permeable membranes. The high density achieved in the 5% CO₂-permeated CAB reactors matched that of the positive control case (5% CO₂ with no CAB contactor), indicating that the presence of the CAB contactor did not detectably reduce cell growth. The small densities achieved in the ambient (0.05%) CO₂-exposed CAB reactors did not detectably outperform the negative control reactors (0% CO₂ permeated). The more moderate (0.5%) CO₂ concentration led to strong, carbon-efficient growth, at 76% of the final density of the 5% CO₂ cases, with only a tenth of the CO₂ supplied. Measurements of pH taken before and after experiments confirm similar relative abundance of HCO₃⁻ in all breathable reactors, as discussed in the Supplementary Material. Further, the presence of strong pigment absorption peaks in all final culture spectra confirm the health of cultures (as shown in Fig. 2.11). These results demonstrate that CAB contactors can effectively deliver useful concentrations of CO₂ to cultures with moderately elevated CO₂ levels supplied, while occupying minimal reactor volume.
Results and discussion

Figure 4.2: Effect of CO₂ concentration on microalgal growth using breathable reactors. (a) Culture density growth over 65 hours with CAB contactor, and control membranes of high (+) and zero (-) gas permeability, exposed to CO₂(g) concentrations as indicated. Inset schematic of reactor illumination and CO₂ supplied from membranes. Optical densities and error bars represent the mean and the standard deviation of triplicate reactor experiments. (b) Calculated depth-wise light intensity distribution in reactors based on measurements. Shaded regions represent the maximum range of measurement uncertainties. The dashed line indicates the light intensity threshold below which the growth rate of *S. elongatus* is non-ideal.

While membrane contactors provided CO₂ for culture growth (Fig. 4.2(a)), the cultures nonetheless suffered from self-limiting shading, particularly at higher densities. The light distribution within each
of these cultures at the end of the experiment are shown in Fig. 4.2(b), in comparison to the starting distribution. Initially all cultures had over 100 μmol m\(^{-2}\) s\(^{-1}\) of light, an intensity associated with light-replete, steady-state growth rates \(^{120,211}\). However, as cultures grew and density increased, growth in all reactors became light-limited. While continuous culture circulation during cultivation mitigated the effect of light gradients, fluid flow merely reduces the impact of top-down illumination gradients without solving the problem. The light mapping in Fig. 4.2(b) illustrates the coupled nature of light and CO\(_2\) delivery, and makes the case for distributing both light and CO\(_2\) more effectively within cultures using breathable waveguides.

### 4.3.2 Breathable waveguide reactors with waveguide illumination

To test the effectiveness of the breathable waveguide architecture, experiments were performed where light was supplied from the surface of a CAB waveguide. Hydrogels containing microalgal culture were positioned directly above the waveguide, and surrounded by a PMMA housing. Since this housing did not fully block atmospheric gas permeation, only the central regions of hydrogel cultures were collected and analyzed, in which growth is primarily caused by CO\(_2\) permeation through the waveguide below. In order to fully accommodate laser light input into the edge of the waveguide, the CAB slab used was four times thicker (1 mm) than those in section 3.1, with four times less CO\(_2\) transmissibility. The relative growth trends obtained with this setup can inform the feasibility of combined waveguide permeation and illumination.

The final optical densities of hydrogel cultures incubated over 65 hours (Fig. 4.3) used various CO\(_2\) source concentrations with the breathable waveguide architecture: simultaneous waveguide illumination and CO\(_2\) permeation. The overall positive trend of increasing growth with increasing source concentration is consistent with results from breathable reactor experiments (Fig. 4.2(a)), albeit within a different reactor configuration. More than double the final density achieved with 5% CO\(_2\) source concentration, as compared to negative controls (0% CO\(_2\)), indicates carbon-replete hydrogel cultures. Weaker growth at reduced CO\(_2\) source concentrations indicates relative carbon-limitation, where 0.05% and 0.5% CO\(_2\)-permeated cultures marginally outperformed controls. In the analyzed region of all hydrogel cultures, the presence of strong pigment absorption peaks indicated viable culture health, similar to that observed of final cultures in breathable reactor experiments (Fig. 2.11). Overall, the positive growth results from breathable waveguide and permeation experiments together demonstrate the utility of a breathable waveguide architecture to distribute both light and CO\(_2\) to microalgae.
Results and discussion

4.3.3 Future prospects

The combined delivery of light and CO₂ to microalgae from the single breathable waveguide studied in this work is a significant step towards high-density production in stacked reactors. The performance of stacked slab waveguide architectures reported to-date have been limited by poor CO₂ dispersion, due to impermeable waveguides or low-permeability sources that occupy additional culture volume. In contrast, use of breathable waveguides stands to significantly improve chemical distribution within stacked reactors without additional density limitations. Results from this work suggest that elevated CO₂ source concentrations are necessary to ensure adequate permeation through CAB waveguides. However, future designs could rely on thinner materials for improved CO₂ transmissibility, or alternative materials for improved permeability, such as those presented in Table 4.1. Together, these design improvements have the potential to sustain cultures with light and passive transport of ambient CO₂. Such an ideal breathable waveguide reactor would avoid the operational costs of culture circulation, active aeration, and elevated CO₂ that currently limit the economic scalability of biofuel and bioproduct production in traditional photobioreactors.
4.4 Conclusions

In summary, the present work demonstrates use of a breathable waveguide to simultaneously supply light and CO$_2$ to microalgae. Microalgal growth response to CO$_2$ permeation in CAB reactors was positive, with higher growth occurring at higher CO$_2$ concentrations. Use of CAB required elevated CO$_2$ concentration (5%) to enable carbon-replete growth. Simultaneous breathable waveguide illumination and CO$_2$ permeation in reactors enabled double the growth as achieved with waveguide illumination alone. In contrast to other, separate, often conflicting, light and chemical control strategies, the breathable waveguide architecture enables optimal use of culture area for combined delivery of light and CO$_2$.

4.5 Supplementary Information

Table 4.1: Permeation properties of a selection of transparent, biocompatible materials.

<table>
<thead>
<tr>
<th>Material</th>
<th>CO$_2$</th>
<th>O$_2$</th>
<th>Water</th>
<th>Cost $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permeability (Barrer)</td>
<td></td>
<td>(× 10$^6$ $\text{USD/m}^3$)</td>
<td></td>
</tr>
<tr>
<td>Cellulose acetate (CA)</td>
<td>2.4 – 23 2,13,17</td>
<td>0.80 – 1.6 2,13,17</td>
<td>5300 – 6700 13</td>
<td>1.0 13</td>
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<tr>
<td>Cellulose acetate butyrate (CAB)</td>
<td>32 – 49 3, a</td>
<td>5.0 – 10 3,9 a</td>
<td>n/a b</td>
<td>0.5 13</td>
</tr>
<tr>
<td>Cellulose triacetate (CTA)</td>
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<td>1.0 – 1.7 20,21</td>
<td>10000 21</td>
<td>0.2 11</td>
</tr>
<tr>
<td>Dupont™ Teflon® AF 1600</td>
<td>520 1</td>
<td>270 – 340 8,27</td>
<td>1100 8</td>
<td>40.5 26</td>
</tr>
<tr>
<td>Dupont™ Teflon® AF 2400</td>
<td>2800 – 3900 8,22</td>
<td>960 – 1600 8,22,27</td>
<td>4000 8</td>
<td>43.1 26</td>
</tr>
<tr>
<td>High-density polyethylene (HDPE)</td>
<td>0.40 – 1.7 13,18,25</td>
<td>0.40 – 0.53 13,25</td>
<td>13 13</td>
<td>0.1 13</td>
</tr>
<tr>
<td>Low-density polyethylene (LDPE)</td>
<td>6.0 – 15 6,13,18,25</td>
<td>1.6 – 5.1 6,13,16,18,24,25</td>
<td>91 – 110 13,24</td>
<td>0.1 13</td>
</tr>
</tbody>
</table>
Polycarbonate (PC)  0.93 – 26 $^{23}$  0.27 – 7.0 $^{23}$  1300 $^{13}$  0.3 $^{13}$

Polydimethylsiloxane (PDMS)  3100 – 4500 $^{2,4,10,14,15,19}$  600 – 800 $^{2,15,19}$  40000 $^{2}$  0.4 $^{13}$

Poly(methyl methacrylate) (PMMA)  0.77 – 3.1 $^{5,20}$  0.13 – 0.23 $^{5,13,20}$  670 $^{13}$  0.1 $^{13}$

Water  1900 $^{c}$  47 $^{c}$  –  –

1 Barrer = 10$^{-11}$ (cm$^3$ STP) cm$^{-1}$ cm$^{-2}$ s$^{-1}$ mmHg$^{-1}$

$^a$ CAB permeability measured for this study was 32 Barrer to CO$_2$ and 6.2 Barrer to O$_2$ (ISO 15015-2).

$^b$ Not available in Barrer unit equivalents. Water vapor transmission rate of 0.25 mm CAB membranes used in this study are 65.5 g m$^{-2}$ day$^{-1}$.

$^c$ Gas permeability in water at 25°C estimated with Henry's law solubility and diffusivity (Gevantman, 2015; Sander, 2014; Cussler, 1997).

$^d$ Lowest costs of commercially available materials found by the authors. Material formats of Dupont™ Teflon$®$ and CTA are granular and for other materials are planar sheets of thickness ≥ 100 μm (Goodfellow).

–, not applicable

Permeabilities are measured between 20°C and 35°C and valid for film thicknesses ≥ 100 μm. Large permeability ranges reflect tunability by material composition (Lonsdale 1985). Water permeabilities suggest the permeabilities of the air-water interface accessible to open photobioreactors.

**Light distribution within breathable reactors**

To ensure identical photon flux between incubation and initial experiment cultures, the vertical light gradients in the incubator and breathable reactors were calculated using measurements of the specular and diffuse reflectance, and absorbance of the empty reactors, combined with absorbance of the culture at the illumination wavelength (634 nm). For empty reactors, absorption due to the
window and light path was measured at the position of an absent Versapor membrane using a light meter sensor (Li-Cor, LI-190SA). The reflectance of the Versapor membrane was also measured (Ocean Optics, FOIS-1 Integrating Sphere). LED emissions were tuned to achieve total equal continuous illumination in the incubator (2830 µmol m⁻² s⁻¹ cumulative intensity per mm) and initially in reactors (2810 ± 50 µmol m⁻² s⁻¹), to within 2%.

Light distribution within the breathable waveguide reactor

Optical input power to the 1 mm × 10 cm × 10 cm CAB waveguide was 70 ± 5 mW, and with the hydrogel present, output power from the beam path was 40 ± 10 mW. Scattered light losses from the other edges of the slab summed to < 1 mW. Power from a collimated diode laser beam (638 nm) was swept from 10 to 150 mW to measure the waveguide attenuation in air as 53000 ± 2000 dB/km. This loss is mainly due to internal scattering that ejects light, primarily from the closest (top and bottom) surfaces, which enabled effective photosynthetic use in this application. Surface intensities for growth were estimated by placing the light meter sensor close to the waveguide wetted with a thin layer of water. The sensor was also rotated to capture the angular nature of surface emissions resulting from internal scattering. At the input power above, the water-wet waveguide surface intensities at different positions spanned 20 – 40 µmol m⁻² s⁻¹.

Maintaining constant pH within breathable reactors

Over the course of the three day growth period, the pH of the 0.05, 0.5 and 5% CO₂-permeated reactors went from 8.0 ± 0.1 to 8.04 ± 0.03, 8.0 ± 0.1 to 8.04 ± 0.03 and 8.04 ± 0.03 and 7.55 ± 0.01 (mean ± s.d.), respectively. The slight drop in pH observed for the 5% CO₂-permeated reactor indicates an increased Ci. However, this change is minor, largely due to the 80 mM HEPES buffer used in all culture media. The similar relative abundance of HCO₃⁻ in all breathable reactors enabled direct comparison of growth differences resulting from mass balances between CO₂ flux and cell consumption in reactors containing different absolute Ci concentrations.
Figure 4.4: Photographs of hydrogel cultures grown in breathable waveguide reactors with waveguide permeation at CO$_2$ source concentrations indicated. Optical densities were measured of uniform density regions of hydrogels within the dashed lines visually selected. Red arrows indicate waveguide input and transmission direction.

References for Supplementary Table 4.1


5 Periodic harvesting of microalgae from calcium alginate hydrogels for sustained high-density production

This chapter is a paper submitted to Biotechnology and Bioengineering in February 2017. The applicant was the primary author for this work and played the primary role in experiment design, execution, data collection, data analysis, and paper writing. The efforts of all other authors are gratefully recognized, they are: Jason Riordon, Brian Nguyen, Matthew Ooms and David Sinton.

High-density biomass production is currently only realized in biofilm-based photobioreactors. Harvest yields of whole biofilms are self-limited by daughter-upon-parent cell growth that hinders light and leads to respiratory biomass losses. In this work we demonstrate a sustainable multi-harvest approach for prolonged generation of high-density biomass. Calcium-alginate hydrogel cultures loaded with Synechococcus elongatus PCC7942 achieved production densities comparable to that of biofilms (10^9 mL^-1) and optimal total productivity in harvest periods of two or three days that allowed high-density surface growth without self-limiting cell buildup or surface death. Hydrogel rigidity from cross-linking calcium concentration had a strong influence on surface growth and harvest yields, especially in the first harvests. Subsequent harvests achieved more uniform biomass yields and distributions, unaffected by bulk respiration or light penetration. Collectively, these results demonstrate the feasibility of sustained, high-density biomass production by periodic harvesting within microalgal hydrogel cultures.

5.1 Introduction

Photobioreactors provide a route to produce valuable bioproducts as well as global dependence on fossil fuels. Conventional suspension culture cultivation in pilot-plant photobioreactors, however, have not economically produced biomass for biofuels, due to high water management costs and poor light and CO_2 access. Biofilm-based photobioreactors enable high-density cultivation with ~100× lower water content than conventional photobioreactors, however, the daughter-upon-parent stacking of cells starves lower layers of light and CO_2, which causes a gradual decline in productivity over time. Lower layers consume biomass by respiration, driven by high-density oxygen production, while growth occurs only in the top ~250 - 500 µm of the biofilm – a scenario that necessitates batch harvesting at this thickness for optimal production.
Hydrogel-immobilized microalgal cultures have gained traction in wastewater treatment applications for the separation of culture and media, and offer the same fixed-culture benefits as biofilms for biomass production: low water requirements, high-density growth, photoacclimation for improved light access, and air-exposed cultivation for improved CO₂ access. Hydrogel cultures have also shown promise for biomass reclamation, protection, and generation including biofuel production. With microalgal cultures, hydrogel immobilization can profoundly change cell morphology, metabolites, physiological state and growth response. Such changes can be beneficial, with specific productivity benefits depending on the species, loading density, and composition of the hydrogel matrix and cross-linking agents. Microalgae in hydrogels have exhibited comparable or higher concentrations of chlorophyll and carotenoids, a greater variety of lipids, lower senescence, and comparable growth rates, lag times and light utilization efficiencies relative to that of suspension cultures. Microalgal growth in hydrogels, however, mainly proceeds as in biofilms - in a self-limiting near-surface colony layer of comparable thickness and density.

This paper demonstrates the feasibility of sustained, high-density biomass production in rigid microalgal hydrogel cultures by periodic harvesting of the developed high-density surface layer. This approach avoids self-limiting growth, enabling long-term sustained production, as illustrated in Fig. 5.1. Specifically, we use rigid, planar calcium-alginate hydrogels loaded with the cyanobacterium *Synechococcus elongatus* exposed to light and ambient CO₂ from above and in diffusive contact with media from below. We first demonstrate the recovery of whole-hydrogel cultures to partial culture harvesting, and evaluate growth kinetics using the established three-phase linear model. Next, we evaluate how harvest periodicity influences overall productivity, using 2-, 3-, 6- and 18-day periods, as compared to a conventional cell suspension. We evaluate the effect of calcium cross-linker concentration on harvested biomass yields. Confocal microscopy is used to probe the distribution and viability of near-surface cells within hydrogels during cultivation. Finally, the effects of respiration and light-limitation in sub-surface layers on harvested biomass yields are evaluated relative to surface growth. These results demonstrate the feasibility this approach for continuous biomass generation, with benefits of high-density production for low water management costs, partial harvesting that preserves the culture for regrowth, and low-density regions for deep light penetration and low-density oxygen production.
5.2  Methods

5.2.1  Microalgal strain and suspension cultivation

The wild-type freshwater cyanobacteria *Synechococcus elongatus* PCC 7942 (herein *S. elongatus*) was incubated in shaker flasks at 27 ± 1 °C under a continuous photosynthetic photon flux density (intensity) of 100 - 200 µmol m⁻² s⁻¹ (400 - 700 nm) and with ambient 0.05 ± 0.01% CO₂ in the headspace. This species has been used in the direct production of biofuels, in addition to biomass production demonstrated here. Cells were cultured in BG-11 media (Phytotechnology Laboratories, B1511) at 2× concentration supplemented with 40 mM of HEPES buffer and adjusted to pH 8.1 with NaOH.
5.2.2 Preparation and growth conditions of hydrogel cultures

Procedures for preparing, sampling, and harvesting hydrogel cultures are schematically illustrated in Fig. 5.2. All hydrogel cultures were prepared by cross-linking a solution of 2% w/v alginate (Sigma, A0682) in 1.0 OD750 cell culture with calcium that diffused from agarose gels in contact with the solution, to equilibrium concentrations of 150, 300 or 400 mM Ca²⁺. This alginate composition enabled rigid hydrogels due to the high guluronic acid content of ~50% consecutive residues. Hydrogel cultures were of 60 mm diameter with thicknesses of 1.0, 4.0 or 4.6 mm. Further formulation and preparation details are described in the supplementary material. These hydrogel formulations are similar to the optimal formulation reported of submerged 2% calcium-alginate hydrogel cultures, of *C. vulgaris* for growth and retention (0.3:1 and 180 mM, respectively) and *S. elongatus* for photosynthetic activity (90 - 180 mM).

Figure 5.2: Illustration of methods for hydrogel culture preparation, density sampling, and harvesting. Included are photographs of a biopsy sample and a partially harvested hydrogel.
5.2.3 Hydrogel culture growth conditions

After preparation, hydrogel cultures were gently washed with fresh media and placed on agarose gel supports (2% w/v balance media), which raised cultures above the liquid level of a surrounding 2×BG-11 media bath in re-sealable tissue culture flasks (150 cm², TPP product No. 90552). The function of the supports is to mediate the diffusion of dissolved chemicals between media baths and cultures, which is equivalent to the function of the porous membrane in porous substrate photobioreactors (PSBRs). Since 2×BG-11 contains 0.2 mM Ca²⁺, calcium diffused out of the hydrogels which contained much higher calcium concentrations. The diffusion of dissolved nutrients from media into hydrogel cultures was driven by cell consumption, since evaporative volume loss of media baths was < 0.5 mL/day. Media was refreshed at days 1 and 5 in the post-harvest growth response experiment, and every 6 days in the harvest period experiment. During experiments, the atmosphere in all flasks was 0.05 ± 0.01% CO₂, ~ 85% relative humidity, and a temperature of 27 ± 1 °C. Flasks were uniformly and continuously illuminated from above by fluorescent lamps (Sunblaster T5 HO 6400k) above a light-diffuser. Incident intensity on all hydrogel cultures during experiments was continuous at 180 ± 10 µmol m⁻² s⁻¹ of photosynthetically active radiation (400 – 700 nm). This intensity is in-between growth saturation and inhibition intensities (80 - 500 µmol m⁻² s⁻¹) which ensured good photosynthetic efficiencies for this species. To minimize the role of light gradients across the samples (flask shape led to a bias), each flask contained two pairs of identical hydrogels, placed diagonally with respect to the direction of bias (except for two flasks used for confocal imaging in the post-harvest growth response experiment). Hydrogel cultures of different initial calcium concentration were placed in different flasks in the post-harvest growth response experiment, and in the same flasks in the harvest period experiment. Further details of hydrogel culture layouts in flasks for density sampling and confocal imaging measurements are described in the supplementary material, and below.

5.2.4 Suspension culture controls

Operating in parallel in both experiments, suspension cultures of the same species were cultivated for the same duration and in the same incident light, CO₂, temperature, and humidity conditions as experienced by hydrogel cultures, from the same initial loading densities. Suspensions were maintained 4.0 mm deep on shaker tables. Small evaporative losses of media (< 1% vol/vol day⁻¹) were balanced by addition of fresh media every 3 and 6 days in experiments of the post-harvest growth response and harvest period, respectively. This suspension culture thickness is practically
optimal for productivity, being even thinner than the most productive, thinnest flat-panel reactors.

5.2.5 Density sampling and measurement

Immediately before measuring the optical density of a hydrogel, the top surface was washed with media from the surrounding bath using a transfer pipette, with runoff containing any cells spilling back into media baths. Biopsies of the full depth of hydrogels were sampled before and after each harvest (see below). The number and location of extracted biopsies was chosen to mitigate several potential sources of density sampling bias, described in detail in the supplementary material (Fig. 5.7). Extracted biopsies were liquefied over 10 min by immersion in a sodium citrate tribasic solution of 5% wt/vol (balanced media) in a 1:1 mass:volume ratio (Fig. 5.2). Liquefied samples were filtered through 20 µm cell strainers to remove dust and other large contaminants. Liquefied samples were further diluted with fresh media in additional whole-volume ratios, as needed, to keep measured optical densities in the linear spectrometer response range of < 0.7 OD\textsubscript{750} (OceanOptics, USB2000+). Optical densities were measured with a maximum uncertainty of 0.032 OD\textsubscript{750}. Hydrogel absorbance was calculated based on thickness, optical density, and the number of whole-volume dilutions. Optically measured volumetric culture densities were converted to cell number or dry weight densities. Calibration curves and generation procedures are provided in supplementary material (Fig. 5.8). The areal density of a harvested layer is the volumetric density of a hydrogel multiplied by its thickness before harvesting, subtracted from the same product after harvesting.

5.2.6 Cell harvesting

At each harvest, hydrogels were removed from flasks and washed with media from the surrounding bath as above. Each hydrogel was then placed upside-down on a mount that pressed down from the top lightly, to maintain gel shape (Fig. 5.2). An extra-wide microtome blade (80 mm, VWR 95057-834) was pushed through the mounted gel by hand, with rapid transverse reciprocating motion in order to remove 650 ± 100 µm from the light-exposed surface of the hydrogel. Images of harvested hydrogel cultures and harvest cut roughness are presented in supplementary material (Fig. 5.9 and 5.10). The remaining hydrogel was washed again with bath media before being placed into its flask. This harvest thickness was the minimum reproducible amount, due to deformation of the 2% alginate gels during slicing. The 15% uncertainty in harvest thickness was estimated based on the cumulative effect of inconsistencies in hydrogel deformation during harvesting. Discovered in practice, this effect
prevented harvesting of the final three 2-day periods on days 14, 16, and 18. Instead, the 2-day harvest periodicity is reported up to day 14, or 2 days after the 6th harvest iteration in the results.

5.2.7 Calculation of light intensities in hydrogel cultures

Photosynthetic photon flux densities transmitted through each harvested layer and the adjacent 650 µm layer below were calculated based on optical density measurements using the equation,

\[ T = I_o \sum_{\lambda=400 \text{ nm}}^{700 \text{ nm}} f_s(\lambda) f_l(\lambda) 10^{-\left(\frac{OD_1(\lambda)d_1 - OD_2(\lambda)d_2}{1 \text{ cm}}\right)} \tag{23} \]

where \( T \) is the transmission intensity, \( I_o \) is the total incident intensity on the layer, \( f_s(\lambda) \) is the normalized response of the spectrometer sensor (Li-Cor LI-190R), \( f_l(\lambda) \) is the normalized, average lamp spectra determined at hydrogel positions in culture flasks with typical amounts of condensation on the interior of the lids, and \( OD(\lambda) \) is the absorbance at 1 cm path length of dissolved \( S. \ elongatus \) hydrogel culture, including hydrogel structure, and \( d \) is the thickness of the culture. The subscripts 1 and 2 indicate a time before and after harvests, respectively. Calculations of light transmitted by the layer below harvested layers are estimated by assuming uniform measured optical densities in hydrogels post-harvest, and using equation (23) without the subscript-2 terms where \( d_i \) is 650 µm.

5.2.8 Effects of hydrogel composition on light absorption and contamination

To isolate the effects of light absorption by the hydrogel structure on the optical density measurements of dissolved hydrogel cultures, an experiment identical to the harvest period experiment was run in series with blank hydrogels, in which cell-free media replaced \( S. \ elongatus \) culture. Identical sampling, washing, harvesting, and measurement procedures were applied. Blank hydrogel optical densities did not appreciably change over time, and thus values were averaged and subtracted from the optical density data from the harvest period experiment (see procedure described in supplementary material). Among all blank hydrogels, average optical densities of 0.22 ± 0.02 mean ± s.d. \( OD_{750} \) were typically <5% of \( S. \ elongatus \) optical densities, and absorbed < 15 µmol m\(^{-2}\) s\(^{-1}\) of incident light. No contaminating cell growth was optically detected, since blank hydrogel densities did not appreciably increase over time, and no regions of contaminant growth were visually observed in cell-loaded hydrogels.
5.2.9 Hydrogel staining, confocal imaging, and analysis

In the harvest period experiment, confocal fluorescence images of hydrogel depth profiles were taken before and after each harvest, and more often in 6- and 18-day harvest periods. Images were taken from different 8 mm diameter biopsies of hydrogel cultures that stained with SYTOX Green fluorescent dead-cell stain. Details of the cultivation layout, staining protocol, imaging and image processing are found in the supplementary material. Processed fluorescence images were averaged along the hydrogel width to obtain linear traces, which were then smoothed before being normalized over time for each hydrogel type, defined by harvest period and calcium concentration. These traces are presented in the results section alongside processed images in which fluorescence amplitudes were normalized locally among hydrogel types.

5.3 Results & Discussion

Experiments were designed to demonstrate and assess the multi-harvest hydrogel cultivation approach by (i) exploring post-harvest growth response, (ii) determining the role of harvest periodicity on overall productivity, (iii) observing cell distribution and viability within hydrogel harvests, and (iv) evaluating the effect of light in sub-surface layers.

5.3.1 Determination of post-harvest growth response

To evaluate the growth recovery of whole *S. elongatus* hydrogel cultures following partial culture harvests, 4.0 mm hydrogel cultures were formed with either 150 or 300 mM Ca\(^{2+}\) and subjected to 0, 1 or 2 harvests, where the top 650 ± 100 µm of the hydrogel was removed, and the remaining areal density of the hydrogel measured over time, as shown in Fig. 5.3. Since the number of data points (12 - 14) was limited by hydrogel area and the unbiased sampling scheme (see supplementary information), points were clustered following the 1st or 2nd harvest to best evaluate how whole cultures recover post-harvesting. Densities herein are expressed on a per-area basis of 650 ± 100 µm harvests, or whole hydrogel thicknesses that are reduced by each harvest. In the once-harvested cultures, growth recovery was rapid, with densities matching (or at times exceeding) that of non-harvested cultures. To model specific exponential growth rates and lag times of whole hydrogel cultures and a suspension (control) culture, the simple and robust three-phase linear model of microbial growth was applied to the beginning of each period following a harvest (6 – 8 data points) \(^{322,323}\), since no hydrogel-immobilized growth kinetics model exists. Model results are reported in Table 5.1.
Figure 5.3: Recovery of S. elongatus growth in 4.0 mm air-exposed hydrogels, formed with 150 and 300 mM Ca$^{2+}$. Top 650 ± 100 µm layers are harvested once (orange lines), or twice (purple lines) in a 3-day harvest period. Hydrogel cultures that are not harvested (grey and black lines) are shown for comparison.

Thinner hydrogel cultures achieved greater specific growth rates (0.28 ± 0.05 d$^{-1}$ for 1.0 mm hydrogels and at most 0.18 ± 0.03 d$^{-1}$ among 4.0 mm hydrogels), since harvested layers of all hydrogel cultures contained 20% - 65% of all cells and transmitted > 40 µmol m$^{-2}$ s$^{-1}$ to the bulk at the time of harvest (see methods section 2.7). The overall positive growth of whole cultures (Fig. 5.3), and robust post-harvest surface growth at light-limited intensities$^{120}$ indicates the feasibility of periodic partial culture harvesting within rigid hydrogels for biomass production.

5.3.2 Determination of optimal harvest period

5.3.2.1 Harvested biomass productivities

To evaluate the role of harvest periodicity on the biomass productivity of high-density surface layers, 4.6 mm hydrogel cultures formed with 150 or 400 mM Ca$^{2+}$ were subjected to 2-, 3-, 6- and 18-day harvest periods where the top 650 ± 100 µm of the hydrogel was removed, and the remaining areal density of the hydrogel measured over time, as shown in Fig. 5.4(a). Harvest periods of 2- and 3-days
removed more biomass than regrew between harvests. However, the volumetric density of all cultures increased during cultivation, with an 18-day range of culture densities of $0.3 - 9.0 \times 10^9$ mL$^{-1}$ for whole-hydrogels and $0.08 - 3.1 \times 10^9$ mL$^{-1}$ for harvests, which is on the same order of magnitude as previously reported submerged $S.\ elongatus$ biofilms $^{147}$.

The total biomass productivity of all harvested layers, shown in Fig. 5.4(b), is displayed as the sum of the biomass yields of each successively harvested layer, normalized to the total period of cultivation (either 12 or 18 days; see methods section 2.6). This normalization permits visualization of the relative differences in biomass yields of individual harvests as the relative heights of stacked-bars in Fig. 5.4(b), for each type of hydrogel defined by harvested period and initial calcium concentration. The heights of stacked-bars in Fig. 5.4(b) are directly proportional to the drops in areal density caused by harvests in Fig. 5.4(a). All hydrogel cultures exhibited higher biomass productivity than the 4.0 mm suspension culture (dashed line, Fig. 5.4(b)) and the 2- and 3-day harvest periods resulted in the greatest total biomass productivity at each calcium concentration. Among hydrogels formed with 150 mM Ca$^{2+}$, the 1st harvested layer achieved the greatest individual layer productivity in all cases, with the maximum of $1.0 \text{ gDW m}^{-2} \text{ d}^{-1}$ achieved in hydrogels subjected to a 3-day harvest period. This productivity is high for $S.\ elongatus$, comparable to that of suspension cultures supplemented with elevated carbon concentrations and in similar illumination conditions $^{325-327}$. 
Figure 5.4: Growth of *S. elongatus* in air-exposed, initially 4.6 mm hydrogels formed with 150 and 400 mM Ca$^{2+}$ in which the top 650 ± 100 µm layer is harvested in 2-, 3-, 6-, or 18-day periods. (a) Areal cell density of hydrogels, where dashed-line drops indicate harvests. Error bars represent
compounded uncertainties, in descending relative magnitude, of harvest layer thickness, optical
density measurement, and conversion factors between optical and cell or dry-mass densities. Inset
schematic shows the harvest iteration labeling scheme. (b) Total areal biomass productivity in 18
or 12 (*) days of total cultivation of harvested hydrogel layers and that of a 4.0 mm deep
suspension culture (horizontal dashed line). The relative biomass yields of individual harvest
iterations within each harvest period and calcium concentration are shown with cumulatively
propagated error bars.

5.3.3 Harvested biomass yields

The 1st harvested biomass yields of hydrogels formed with 150 mM Ca$^{2+}$ were much greater than
those formed with 400 mM at each harvest period (Fig. 5.4(b)). This result is in agreement with
literature studies, where higher photosynthetic activity at lower calcium concentration is observed
due to greater nutrient diffusion enabled by a lesser degree of calcium-alginate cross-linking, causing
a more flexible immobilization matrix$^{320,324}$. In subsequent harvests, biomass yields were remarkably
uniform, with variances among 2- and 3-day periods of 0.02 - 0.08 g$_{DW}$ m$^{-2}$, which is explained by
delayed effects of calcium dilution on hydrogel flexibility and growth.

It is established that loosely-bonded Ca$^{2+}$ in alginate hydrogel cultures exchange with chelating
agents and monovalent cations in media (e.g. Na$^{+}$ and phosphates)$^{321}$, which improves flexibility,
nutrient diffusion and growth$^{314,316,320,328}$. Loosely-bonded calcium ions diffused out of all hydrogels
during cultivation, since cultures were in diffusive contact with media baths and agarose supports
that totaled > 3× the total volume of hydrogels and which initially contained much lower
concentrations of calcium (0.2 mM Ca$^{2+}$) and higher concentrations of monovalent cations (134 mM
Na$^{+}$, and 230 mM potassium phosphate) than hydrogels. Although calcium dilution in the extreme
can result in hydrogel destabilization and cell leakage when exposed to liquid flow, increasingly with
combined alginate content < 1% w/v and higher cell-loading densities$^{314,316,324}$, all hydrogels
maintained mechanical integrity throughout experiments.

In hydrogels formed with 400 mM Ca$^{2+}$, biomass yields in Fig. 5.4(b) before day 6 were significantly
lower than yields after day 6. Specifically, yields before and after day 6 for 2-day periods (3rd harvest)
were 0.7 ± 0.1 and 1.1 ± 0.2 g$_{DW}$ m$^{-2}$ (N = 6, t(4) = 3.5, p ≤ 0.025, CI$_{0.95}$ -0.81, -0.09), and for 3-day
periods (2nd harvest) were 0.72 ± 0.05 and 1.4 ± 0.2 g$_{DW}$ m$^{-2}$ (N = 6, t(4) = 5.1, p ≤ 0.007, CI$_{0.95}$
-1.0191, -0.3009), respectively. This six-day delay period to significant growth is attributed to calcium
dilution that weakened the rigid hydrogel structure and, under a threshold concentration,
significantly enhanced growth rates$^{320}$. This result is in agreement with observations by Simpson et
al. (2004) in a comprehensive study on the effects of calcium dilution on cell growth in calcium-alginate hydrogels of high and low guluronic residue content. The authors showed that a ~ 5 day delay period to improved growth of murine insulinoma cells in structurally similar hydrogels (2% w/v alginate of 56% consecutive guluronic residues, cross-linked with 10 and 100 mM Ca$^{2+}$) is due to calcium dilution.

In hydrogels formed with 150 mM Ca$^{2+}$, calcium dilution did not improve growth. In contrast, biomass yields decreased to uniformity after the 1st harvest, with variances of 0.01, 0.07 and 0.08 g DW m$^{-2}$ for 2-, 3- and 6-day periods, respectively. Lower 2nd harvest biomass yields were also observed in the post-harvest growth response experiment, in both types of hydrogels formed with < 400 mM Ca$^{2+}$. Reasons for the 1st-to-2nd harvest production drop in these hydrogels are unclear. Evidence of minor cell leakage from hydrogels formed with 150 mM Ca$^{2+}$ does not account for the decrease in yields observed in these cultures (see supplementary material). One possible explanation for the production drops is growth lag in 2nd and later harvests due to acclimation to ambient CO$_2$ upon surface exposure; only the 1st harvested layers of all hydrogel cultures were continuously exposed to ambient CO$_2$, yet these layers experienced a sudden increase in surface light intensity upon the start of experiments similar to that experienced by later harvested layers due to surface exposure. Overall, these results show that calcium plays a key role in limiting surface-layer microalgal growth in alginate hydrogels of high guluronic residue content.

5.3.4 Estimate of light use efficiency of biomass generation

The light use efficiency of biomass generation is an important parameter to assess photobioreactor performance. The light use efficiencies of harvested layer yields were calculated based on optical density measurements and calculated light intensities in harvested layers. The calculations assumed a linear rate of density increase of harvested layers between density measurements. The range of calculated efficiencies was 0.04 – 0.48 g$_{DW}$ mol$^{-1}$ of photosynthetically active radiation. Efficiencies were greater in hydrogels formed with the lower (150 mM) calcium concentration, with the greatest efficiencies achieved in the 1st harvest of the 18-day periods. These light use efficiencies are comparable to those of other microalgal species, including biofilms of *Halochlorella rubescens* in a PSBR exposed to ambient air.

5.3.5 Fluorescence profiles

To provide further insight into the growth process of *S. elongatus* in hydrogels, specifically near the surface of harvested layers, confocal microscopy was used to collect representative depth profiles of
hydrogel cultures before and after harvesting. Fig. 5.5 shows processed depth profile images of pigment autofluorescence - mainly chlorophyll-a and phycocyanin - and associated width-averaged autofluorescence traces in the top 300 µm of cultures immediately prior to harvests. Images and traces were normalized among hydrogels formed with the same calcium concentration and harvest period.

Images in Fig. 5.5 that show a near-surface autofluorescence layer indicate a high-density colony layer. Hydrogel-immobilized cultures typically exhibit a high-density near-surface colony layer at similar depths to those observed in Fig. 5.5, which can contain > 90% of cells due to growth in replete light and nutrient access, minimally limited by matrix diffusion \(^{132,135}\). Overall, the developed near-surface colony layers were 50 - 160 µm in thickness and the maximum diameter of colonies within - at 18 days of cultivation without harvesting - were approximately 18 and 50 µm in hydrogels formed with 150 and 400 mM Ca\(^{2+}\), respectively, based on pigment and SYTOX Green fluorescence (Fig. 5.11). Although pigment autofluorescence is not quantitatively predictive of biomass concentration due to variations in pigment expression (e.g. photoacclimation) \(^{331,332}\), the location and size of high-density cell colonies in the near-surface layer is clear.

Figure 5.5: Representative images of pigment fluorescence profiles in the top 300 µm of hydrogel cultures formed with 150 mM or 400 Ca\(^{2+}\) immediately before harvest iterations in 2-, 3-, 6- and 18-day periods, as indicated. Fluorescence is mainly from chlorophyll-a and phycocyanin pigments. At right are width-averaged fluorescence traces of raw confocal images. Amplitudes of images and traces are normalized among hydrogels formed with the same calcium concentration and harvest
period. Images are identically contrast-enhanced for presentation. Arrows in 18-day images indicate vacancies of pigment autofluorescence that contain dead cells.

The images in Fig. 5.5 show the effects of calcium concentration and dilution on biomass yields already discussed. Images of the 1st harvested layer of hydrogels formed with 400 mM Ca\(^{2+}\) show the absence or presence of a near-surface colony layer before or after day 6, respectively. The uniformity of surface cell distribution increased with harvest iteration, as indicated in Fig. 5.5, by the general decrease in autofluorescence contrast between near-surface colony layers and the lower parts of the culture at later harvests. Since biomass yields from these harvests were uniform (Fig. 5.4(b)), near-surface colony densities decreased with additional harvests, which limits potential approaches to improve productivity by using thinner harvests.

Culture viability ultimately limits productivity and the duration of optimal production. At the final day of cultivation, SYTOX Green dead cell stain fluorescence intensity in the top 200 µm was greater in hydrogels subjected to longer harvest periods (Fig. 5.11), in all cases. In hydrogels subjected an 18-day harvest period, the greatest concentration of dead cells immediately prior to harvest was at the surface, in the vacancies indicated in Fig. 5.5 above developed near-surface colony layers. Since dead surface cells block light with no benefit to production, optimal production occurred in the shorter 2- or 3-day harvest periods that ensured top-layer culture viability while being sufficiently long to allow high-density growth (Fig. 5.4(b)). For comparison with current PSBR studies, optimal harvest periods of constant biomass productivity range from 10 to 42 days \(^{106-108}\), at which times whole culture removal is necessary to limit biomass losses in light-starved lower layers \(^{104-106}\). Culture viability limitations to PSBR-type production systems, such as the risk of biofilm sloughing due to unhealthy substrate-layer cells \(^{333}\), have not been reported. The reduced viability of near-surface cells in infrequently harvested hydrogels further validates the multi-harvest approach for optimal production.

5.3.6 Light distribution within hydrogel cultures

To evaluate the effect of different light regimes in harvested layers and lower layers in the hydrogel bulk, Fig. 5.6 shows the range of light intensities in the top two 650 µm thick layers (0 – 1300 µm) immediately prior to harvest iterations, as labelled. Calculations are based on optical density measurements and the cultivation lamp spectrum (see equation (23) and description in methods). Estimation of the lower intensities of the colored bars in Fig. 5.6 were calculated assuming uniform biomass distributions in hydrogels immediately post-harvest, which is supported by observations in
confocal images of uniform fluorescence intensity in associated hydrogel biopsies (not shown). Also shown are dashed lines, indicating light-replete, -limited and respiratory intensities.

Fig. 5.6 shows how hydrogels subjected to more frequent harvests are exposed to more light overall. In the next-to-be-harvested 650 µm – 1300 µm layers (colored bars), cells received greater light intensities and absorbed more light in hydrogels subjected to 2- and 3-day harvests (red and green bars, respectively) than in hydrogels subjected to 6- and 18-day harvests (blue and grey bars, respectively). In the case of the 18-day harvests (grey bars), light intensity falls below the respiratory regime, which helps explain lower yield performance relative to 2-day and 3-day harvests in Fig. 5.4(b). Given the low-light conditions experienced by the next-to-be-harvested layers shown in Fig. 5.6 (650 µm – 1300 µm, colored bars), a significant portion of deeper layers (1300 µm and below) experience respiration, with the deepest respiring for the longest times prior to harvest. Despite increasing respiratory biomass loss with depth in these deepest layers, no declines in later production were observed in Fig. 5.4(b). Since respiratory effects are minimized and do not hinder overall production, there is great potential for this periodic harvesting scheme to achieve longer, sustained production in thicker hydrogels.

Figure 5.6: Photosynthetic photon flux densities (400 - 700 nm) in harvested (grey bars) and adjacent lower layers (colored bars) of 650 µm thickness immediately prior to labeled harvest iterations, calculated based on optical density measurements. For example, the progression of a 4th harvested layer is marked by (*). Estimates of photosynthetic photon flux densities in adjacent lower
Conclusions

96 layers (colored bars) are calculated based on optical density measurements and assume uniform biomass density in top 650 µm immediately post-harvest. Light-based growth regimes of *S. elongatus* are shown by the labelled dashed lines.

5.3.7 Future prospects of periodically harvested hydrogel cultures

Periodic harvesting of microalgal hydrogels by partial culture removal is an entirely new approach to high-density microalgal cultivation, with a unique mix of benefits of biofilm and suspension culture formats for cost-effective production: high-density surface production for low water management costs; partial harvesting that preserves the culture for regrowth; low-density regions for deep light penetration and low-density oxygen production; and a minimal surface liquid boundary layer for enhanced gas transfer. The demonstrated trends of biomass yield with harvest iteration, combined with evaluated sub-surface light and density distributions, demonstrate the feasibility of this production scheme. In agreement with related hydrogel studies, cross-linker concentration was found to be a key optimization parameter of rigid alginate hydrogels that strongly affects growth density and distribution \(^{320}\), and limits attainable harvest thickness by shear strength. Low calcium cross-linker concentration enables both short-term production in thin, single-layer hydrogels at high productivities, and sustained production at lesser rates in hydrogels of any thicknesses, unhindered by respiration losses and oxygen accumulation at the cell-loading densities used here. In comparison, PSBR biofilms generate super-saturating oxygen concentrations in respiratory regions that strongly drive biomass consumption and limit reactor performance \(^{104-108,278}\). In these hydrogels, low-density lower layers also enabled, on average light penetration \(\sim 3\times\) deeper than typical of PSBR biofilms \(^{91,104,105,207}\). In a future commercial production facility using hydrogel cultures, although thinner, high-density harvests prolong production, more frequent harvest iterations would increase labor and/or automation cost \(^{46}\). Additionally, downstream biomass processing would benefit from the high density feedstock, but no doubt existing processes would require adjustment to deal with included hydrogel components. The biomass productivity achieved here suggests a material cost of biomass production of a few tens of cents per kg of dry mass per year (alginate-dominant), comparable with that estimated for PSBR production \(^{91}\).

5.4 Conclusions

In summary, this work demonstrates the feasibility of periodic harvesting of microalgal hydrogels by partial culture removal - a new approach to high-density microalgal cultivation. Harvested biomass densities of \(0.08 - 3.1 \times 10^9\) mL\(^{-1}\) were comparable to that of biofilms of the same species and 2 - 3
day harvest periods optimized total biomass productivity, with peak harvest productivity of $1.0 \text{ g}_{\text{DW}} \cdot \text{d}^{-1}$. Later harvests achieved more uniform biomass yields and cell distributions unaffected by bulk respiration. These results point to an opportunity for greater productivity via optimizing loading density, harvest layer thickness and alginate composition.

5.5 Supplementary Information

5.5.1 Hydrogel culture preparation

Two days prior to experiments, cells were cleaned and re-suspended in fresh media. On the initial day of experiments, cultures were adjusted to $1.5 \text{ OD}_{750}$. A solution of 6% vol/vol alginate (Sigma, A0682) balanced by media was added to the culture at a 1:2 volumetric ratio, for a final hydrogel solution of 2% alginate at $1.0 \text{ OD}_{750}$. The manufacturer’s source of alginate, *Macrocystis pyrifera*, has approximately 56% consecutive guluronic residues and 38% mannanuronic acid. Hydrogel solutions were then degassed under vacuum (51 kPa) for 1 min, and poured in 60 mm diameter petri dishes that contained solid agarose gels of 2% vol/vol balance media (Invitrogen) and additional calcium chloride dehydrate (Fig. 5.2). Agarose gels occupied all but the top 1.0, 4.0, or 4.6 mm of dishes, which defined the solidified hydrogel heights, capped by weighted lids. Hydrogel solutions were solidified (gelled) by exposure to calcium ions, as described by the “egg-box” model whereby the ions diffused from the agarose gels over 10 hours until alginate gels were solidified, resulting in equilibrium concentrations of 150, 300 or 400 mM Ca$^{2+}$. The time to reach equilibrium was estimated visually by food dye diffusion from an agarose gel into a solidified cell-free hydrogel. During the first hour of solidification, hydrogel solution was added drop-wise through holes in the lids to maintain height and bubble-free volume as the gels slightly shrunk. Hydrogels did not appreciably shrink after the first hour of solidification. The alginate protocol used herein, of 0.5:1 volumetric ratio of alginate:culture solidified by 150-400 mM Ca$^{2+}$, is similar to the optimal formulation reported of submerged 2% calcium-alginate hydrogel cultures, of *C. vulgaris* for growth and retention (0.3:1 and 500 mM, respectively) and *S. elongatus* for photosynthetic activity (250 mM).

To help maintain culture viability during solidification, hydrogels were illuminated with $\sim 15 \text{ µmol m}^{-2} \text{s}^{-1}$, which raised their initial optical densities to $1.4 \pm 0.2$ and $1.1 \pm 0.1 \text{ OD}_{750}$ for 1.0 and 4.0 mm gels, respectively, in the post-harvest growth recovery experiment, and $1.2 \pm 0.2 \text{ OD}_{750}$ in the harvesting period experiment. After solidification, solid hydrogel cultures were removed, washed with fresh media, and placed in reactors.
5.5.2 Hydrogel growth conditions in flasks

Tissue culture flasks with reclosable lids (150 cm², TPP product No. 90552) were filled with 30 mL of media, which was periodically resupplied during experiments. In each flask, four 12.6 mm agarose gel supports of 2% vol/vol balance media (13 mm height x 60 mm diameter) were partially immersed in the media bath and supported four hydrogel cultures exposed to ambient air (0.05 ± 0.01% CO₂, ~85% relative humidity, 27 ± 1 °C) in the flask headspace (Fig. 5.2).

5.5.3 Hydrogel culture density sampling scheme

The following density sampling scheme was used to simultaneously minimize the influence of enhanced growth near 2 mm diameter biopsy holes on density measurements (Fig. 5.7(a)) and operator sampling bias (Fig. 5.7(b)) in order to average-out the natural heterogeneity of the density of biopsy samples (see example in Fig. 5.7(c)). Before and after each harvest period of 2-, 3-, 6-, and 18-days, the number of biopsies removed from each gel was 8, 12, 16, and 16, respectively. Sixteen biopsies were also collected during sampling in-between 6- and 18-day harvest periods. Referring to the grid in Fig. 5.7(a), these numbers of samples were distributed in pairs of biopsies (pairs of grid numbers) taken from each sixth fraction of the surface (grid letters), starting with the outer perimeter of the hydrogel (ascending grid numbers). The distance between biopsy holes was 0.5 mm, which prevented sampling of high density regions that developed in previously sampled holes (Fig. 5.7(b))

![Figure 5.7](image)

Figure 5.7: Biopsy density sampling. (a) Biopsy grid used for sampling. The outer large circle represents the circumference of 60 mm diameter hydrogel cultures. The inner large circle contains the sampling region. The distance between them is the observed physical extent of enhanced edge growth due to extra light and gas access through the perimeter of the gel. (b) Biopsied holes made by density sampling in a hydrogel formed with 400 mM Ca²⁺ at the 2nd harvest (rightmost hole) of a 3-day harvest period, with overlaid plastic biopsy grid. A dark green ring of growth developed around
the leftmost hole made 3 days earlier. (c) Example of density heterogeneity in biopsy samples taken from a 1 mm hydrogel formed with 150 mM Ca\(^{2+}\), using the biopsy sampling grid.

5.5.4 Layout of the post-harvest growth response experiment

Six flasks were used for the post-harvest growth response experiment. For density sampling, four flasks were used. Two of these flasks contained pairs of 1.0 mm and 4.0 mm hydrogels not subjected to harvesting. The remaining two flasks contained pairs of 4 mm hydrogels subjected to one or two cycles of a 3-day harvest period, where each flask had either an equilibrium gelation concentration of 150 or 300 mM Ca\(^{2+}\). The two flasks used for confocal imaging each contained four hydrogels; one 1.0 mm gel not subjected to harvesting, and three 4.0 mm gels each subjected to none, one or two cycles of a 3-day harvest period. Hydrogels in each of these flasks were solidified by equilibrium concentrations of 150 or 300 mM Ca\(^{2+}\), differing by flask. Media bath was replaced at days 1 and 5.

5.5.5 Layout of the harvest period experiment

Six flasks were used for the harvesting period experiment. For density sampling, four flasks each contained two pairs of 4.6 mm hydrogels solidified by equilibrium concentrations of 150 and 400 mM Ca\(^{2+}\). Hydrogels within each of these flasks were subjected to the same harvesting period, differing by flask. The two flasks used for confocal imaging each contained four hydrogels, which were each subject to a different harvesting period. Hydrogels in each of these flasks were solidified by equilibrium concentrations of 150 or 400 mM Ca\(^{2+}\), differing by flask. The optical density of media baths was measured every 6 days, immediately prior to replacing baths with fresh media.

5.5.6 Cell count and dry mass calibration

Two days prior to the hydrogel harvest experiment, suspension culture from incubator flasks was used with a cell counting chamber to determine the linear relation between OD\(_{750}\) and cell number of the same species, shown in Fig. 5.8(a). The linear relation between dry mass of *Synechococcus elongatus* PCC7942 and measured optical density at 750 nm (OD\(_{750}\)), shown in Fig. 5.8(b), was previously determined using the method in reference 7.
Figure 5.8: Relation between optical density of S. elongatus WT suspension culture and (a) cell density and (b) dry weight concentration (credit: Matthew Ooms).
Figure 5.9: Photographs of hydrogel cultures on agarose supports from the harvest period experiment formed with (a) 150 and (b) 400 mM Ca$^{2+}$ undergoing the 1st harvest of 3-day harvest period, and (c) associated harvested layers. The leftmost pictured harvested layer yielded the greatest biomass of all harvests.

Figure 5.10: High-contrast fluorescence image of surface roughness due to the 4th iteration of harvesting a hydrogel formed with 400 mM Ca$^{2+}$ in a 2-day harvest period. Scale bar is 500 µm.

5.5.7 Hydrogel staining, confocal imaging, and analysis

Each image was taken from a different 8 mm biopsy extracted from hydrogels in confocal imaging flasks in the layout described above. Each biopsy was cleaved into two semicircles for redundancy and incubated in a 0.5 mM solution of SYTOX Green fluorescent dead-cell stain (2 µL) in 1 mL of media for 30 min in the dark, following the cyanobacteria staining protocol reported in reference 8. Stained biopsy halves were washed three times with fresh media and placed, rectangular-face-down, on glass slides. Water and free cells that leaked from the halves were carefully wicked away from the glass surface with a clean wipe. At a representative area of the depth profile, a z-stack of images was collected. Autofluorescence was collected primarily from chlorophyll-a, phycocyanin and allophycocyanin with Ex/Em 639 nm/663 - 738 nm and SYTOX Green fluorescence was collected with Ex/Em 468 nm/500 - 550 nm. One image from each z-stack near the glass interface was chosen to define the surface layer, having the best tradeoff between signal-to-noise and a well-defined hydrogel perimeter. Due to interaction with the glass slide, hydrogels were strained near the surface into irregular parallelograms. The irregular images were processed into rectangular images by
projecting onto a rectangle of the average length of the two sides of the parallelogram and width of the scan field, with less than 3% signal loss.

Figure 5.11: Representative profiles of the top 650 µm of hydrogel culture biopsies (left and right inset schematics) formed with 150 mM (a - d) or 400 mM (f - h) Ca²⁺ and subjected to harvesting periods (columns). Fluorescence images taken immediately before a harvest (*) or in-between
harvests are as indicated beside the day of growth (rows). Green and red colors indicate pigments (mainly chlorophyll-a and phycocyanin fluorescence) and dead cells (SYTOX Green stain fluorescence), respectively. Intensity mapping was equal for all images. Rotated traces of width-averaged, smoothed, normalized fluorescence profiles show relative changes, with later times indicated by lighter colors. Note the nonlinear time sequence of images of 18-day harvest periods.

Table 5.1: Exponential growth rates and lag times of *S. elongatus* in calcium-alginate hydrogels and suspension determined by fits to the three-phase linear model (N = 6 - 8).

<table>
<thead>
<tr>
<th>Culture</th>
<th>Gelling</th>
<th>Harvest</th>
<th>Lag</th>
<th>Specific growth rate</th>
<th>RMSE</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Height</td>
<td>[Ca²⁺]</td>
<td>N&lt;sup&gt;th&lt;/sup&gt; / period</td>
<td>hours</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hydrogel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 mm</td>
<td>150 mM</td>
<td>- / 3-day</td>
<td></td>
<td>18 ± 4</td>
<td>0.26 ± 0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>1.0 mm</td>
<td>300 mM</td>
<td>- / 3-day</td>
<td></td>
<td>7 ± 5</td>
<td>0.30 ± 0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>4.0 mm</td>
<td>150 mM</td>
<td>- / 3-day</td>
<td></td>
<td>14 ± 6</td>
<td>0.18 ± 0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>4.0 mm</td>
<td>300 mM</td>
<td>- / 3-day</td>
<td></td>
<td>6 ± 9</td>
<td>0.12 ± 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>3.4 mm</td>
<td>150 mM</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; / 3-day</td>
<td></td>
<td>80 ± 130</td>
<td>0.13 ± 0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>3.4 mm</td>
<td>300 mM</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; / 3-day</td>
<td></td>
<td>50 ± 50</td>
<td>0.10 ± 0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>2.7 mm</td>
<td>150 mM</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; / 3-day</td>
<td></td>
<td>100 ± 140</td>
<td>0.10 ± 0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>2.7 mm</td>
<td>300 mM</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; / 3-day</td>
<td></td>
<td>50 ± 130</td>
<td>0.07 ± 0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Suspension</td>
<td>4.0 mm</td>
<td>-</td>
<td></td>
<td>40 ± 40</td>
<td>0.07 ± 0.01</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Differences in specific growth rate between harvests in
were largely concealed by model-generated uncertainties.

5.5.8 Blank hydrogel densities

Blank hydrogels were subjected to identical sampling, harvesting, and measurement procedures as *S. elongatus*-loaded hydrogels in the harvest period experiment. Blank hydrogel densities were categorized by calcium concentration, harvesting period, initial height, and whether sampling was applied before or after a harvest (Fig. 5.12). Densities within these categories were averaged over identical growth periods and subtracted from the equivalent categorical optical densities measured of *S. elongatus*-loaded hydrogels. As shown in Fig. 5.12, average category densities of the blank hydrogels ranged from 0.1 to 0.3 OD$_{750}$, which was typically < 5% of *S. elongatus* optical densities.
Figure 5.12: Reference absorbance of blank hydrogels subjected to (a) 2-day, (b) 3-day, (c) 6-day, and (d) 18-day harvesting period in identical growth conditions as *S. elongatus*-loaded hydrogels. Mean absorbances before and after harvests are shown at right (points with error bars).
5.5.9 Evidence of cell leakage from hydrogels formed with 150 mM Ca\(^{2+}\)

In the flasks of the harvest period experiment, used media baths containing washing runoff and diluted calcium from blank and cell-loaded hydrogels increased from zero (fresh media) to 0.1 - 0.3 and 0.1 - 0.6 OD\(_{750}\) every 6 days before replacement, respectively. Although no pigment absorption peaks were observed, photographs of hydrogels formed with 150 mM Ca\(^{2+}\) before and after surface washes showed some differences (e.g. Fig. 5.13). Attributing the difference in final used bath optical densities to cell leakage in these hydrogels over 6 days of harvests amounts to, for example, 36% and 6% of the observed 1\(^{st}\)-to-2\(^{nd}\) harvest decreases in biomass yields among 2- and 3-day periods, respectively (Fig. 5.4).

Figure 5.13: Photographs of hydrogel cultures formed with 150 and 400 mM Ca\(^{2+}\) immediately prior to the 1\(^{st}\) harvest of a 3-day period, before and after the surface wash. Faint dark green lines present only in the top-left photograph are not due to reflected light effects.
References for Supplementary Information section 5.5


6 Conclusions

6.1 Summary

This thesis began with an overview of microalgae-based biofuel production in conventional photobioreactors, motivated by global transportation and climate change mitigation needs. Cost limitations to economic biomass generation were identified, and optimal cultivation strategies for all modes of reactor operation and types of culture product were presented. Throughout the overview, literature reports and theoretical models of conventional photobioreactor performances, indicated that reactors with short resource supply paths (i.e. high ratios of light and carbon supply areas to culture volume), low water content (high density cultivation), and cultures acclimated to local
resource levels (i.e. fixed cultures), have the greatest potential to achieve cost-effective production of low-value products, such as biofuels. This thesis then described the author's contributions toward the development of next-generation photobioreactors by developing scalable reactor architectures that enable these desired characteristics when used individually, or in combination in reactors. The proof-of-concept studies demonstrated the utility of three reactor architectures, with performance evaluation based on the growth response of *S. elongatus*.

The first developed architecture uses FTIR illumination on thin, planar glass waveguides to support and cultivate microalgal biofilms. Growth results from this study demonstrated the utility of divergent (e.g. solar) source light, and the coupling angle tunability of FTIR illumination that resulted in various surface illumination profiles; these include surface intensities of uniform profile that can support large growth areas with photoactive intensities. The second architecture was developed to avoid potential CO\(_2\) supply limitations of the first architecture upon module scale-up as stacked solid waveguides, by using instead gas-permeable planar waveguides that simultaneously supply light and carbon to cultures from one material, with demonstrated growth response. Then, to avoid productivity losses in thicker microalgal biofilms due to dark respiration, as demonstrated in literature studies, the third developed architecture uses air-exposed hydrogel cultures to achieve sustained, high-density biomass production. In these cultures, the characteristic depth-wise density stratification and high-density near-surface colony layer was exploited for production in a periodic partial culture harvesting scheme. The effects of respiratory light intensities in lower layers of the culture and hydrogel cross-linker concentration on growth were evaluated.

Results from this work provide proof-of-principle validation the three novel reactor architectures for improved microalgal production. These designs and their architecture components would provide a greater degree of control of light and carbon supply paths than found in conventional reactors, and thereby may enable improved reactor productivities with potential to improve production economics for low-value, high-demand microalgal products.

### 6.2 Future work

The economic viability of microalgal cultivation depends on balances of reactor capital and operational costs with production rate, duration, and scale (see equation 1). However, studies of the scalable reactor architectures developed in this work were limited to proof-of-concept performance evaluations based on biomass productivity of *S. elongatus* in at most 18 days of continuous cultivation coupled to bulky, laboratory-scale optical setups. To determine their cost-effectiveness for commercial applications, future work on these architectures should focus on performance
Future work

evaluations in pilot plant conditions of production longevity, scale and environment (i.e. species consortia, fluctuating temperature, and solar illumination including ultraviolet (UV) light). Specific recommendations for the focus of future studies are as follows:

- **Scalable light collection and coupling into thin waveguides.** In the waveguide-based illumination architecture studies of this work, the light sources were artificial and bulky. In contrast, planar solar concentrators are an ideal sunlight collection architecture physically compatible with scalable (stackable) waveguide-based architectures, due to their concentration factors $\geq 500 \times$, waveguide input coupling angles $\sim 30^\circ$, and internal losses $\leq 20\%$ in a contiguous optical system for light collection and distributed surface illumination $^{155,160,334-336}$. For this application, an important enabling technology will be light-reactive materials that greatly increase solar acceptance angles of planar solar concentrators (e.g. up to $40^\circ$ off-axis $^{337}$) which avoids the costs of motorized solar tracking $^{338-341}$. Future work on integrated planar solar collector/waveguide structures must evaluate biomass production performance, and fabrication and material costs.

- **Acquisition or fabrication of highly gas-permeable transparent materials.** The breathable waveguides study of this work presents in the supplementary information a short list of candidate materials for this application, which were selected from hundreds of currently commercially available but largely opaque, gas-permeable, nanoporous materials. It is highly likely that better candidate materials exist, but were not commercially/easily available due to market demands, or are available but with unknown/unreported permeabilities. Moreover, the chemical composition and fabrication methods of the identified candidate materials can strongly influence gas permeability; for example, the gas permeability of cellulose acetate butyrate is highly sensitive to butyryl content, and somewhat to acetyl and hydroxyl content $^{342-344}$. However, the CAB membranes studied in this work were the only formulation commercially available with reported gas permeabilities. Further surveying of available materials should be performed. Industrial collaborations to produce breathable waveguides for this niche application should be investigated.

- **Effect of nanopore fouling on gas permeability.** In this work, the biomass growth experiments with gas-permeable CAB waveguides were too short in duration to observe effects of biofouling and other factors that may reduce gas permeability over long operation periods (e.g. media salts) $^{345}$. These factors greatly limit the long-term performance of nanoporous membranes for water filtration, including CAB-based membranes $^{346,347}$. Future evaluation of
the effects of fouling on production, and the cost-efficacy of surface treatment approaches to mitigate fouling should be performed.

- **UV degradation of gas-permeable materials.** The growth experiments of this work used artificial visible light that lacked the UV regions of the solar spectrum. Outdoor photobioreactor materials must be resilient against solar UV degradation, since current pilot plant operations are typically one year and viable commercial operations will likely span decades. This consideration is especially important in solar concentrator and sunlight-waveguiding materials. The lifetime cost of waveguide architecture materials must be evaluated in sunlight wavelengths and waveguiding intensities.

- **Hydrogel immobilization matrices.** The hydrogel study of this work showed that rigid (high guluronic acid content) alginate limits immobilized growth, and that 1st harvested layers achieved the greatest productivities. The effect of more flexible immobilization matrices on productivity should be investigated in approaches of both periodic, partial culture harvesting and single-harvested, thin cultures.

- **Species for biomass production.** In this work, the proof-of-concept performance of the developed architectures were evaluated based on the biomass productivity of *S. elongatus*, due to its sensitive growth response to light- and carbon-limited environments (as discussed in section 2.5). Other species that exhibit comparatively greater growth rates and lipid contents and productivities must be used to evaluate architecture performances for commercial biomass production and for comparative performance evaluation to laboratory-scale reactors studied in the literature (e.g. *Halochlorella rubescens* or *Scenedesmus obliquus* for comparison with PSBR performances).

- **Air-exposed hydrogel cultivation using wastewater.** In the hydrogel study of this work, high-density production of *S. elongatus* was demonstrated in air-exposed hydrogels, partly due to surface access to CO$_2$(g). Moreover, the use of microalgae to scavenge nitrogen and phosphorous from wastewater is widely demonstrated in the literature, including in alginate hydrogel-immobilized cultures that provide protection from wastewater contaminants and easy removal of generated microalgal biomass. The potential benefits of a photobioreactor that joins these applications should be investigated; that is, evaluation of the productivity and costs of biomass generation in microagal hydrogels that diffusively supply nitrogen and phosphorous from contacting wastewater below, and are exposed to high light and CO$_2$(g) from above.
- **Operation duration.** In the hydrogel study of this work, no significant contamination was observed in air-exposed hydrogel cultures over 18 days of cultivation. The potential for contamination and its effects of production should be evaluated in longer cultivation periods (limited by the harvesting period and method). For example, in literature studies of air-exposed PSBRs no contamination has been detected in at most 80 days of cultivation, with harvests occurring in shorter periods. Operations of greater longevity will also reveal other cost factors to production.

### 6.3 Outlook

The development of photobioreactors for low-value product generation continues to be both motivated and limited by the economics of production. High up-front costs of constructing pilot plant test facilities has limited the collection of fully parametrized data at this scale. Without sufficient data, input/output models that project pilot plant performance at commercially relevant scales generate large uncertainties and must make assumptions of questionable validity. For example, a one year period of pilot plant operation is currently considered a long-term study, however life-cycle assessment models commonly assume facility lifetimes of 20 – 30 years based on this disparate data. To validate reactor designs and operational approaches, investments in pilot plant facilities must accelerate, most effectively by dramatically reducing production costs by over 50%. This is a difficult task, considering that over 40 years of conventional photobioreactor development has led to modern economically prohibitive costs of low-value products in isolated reactor operations. By coupling operations to existing markets (e.g. agriculture and wastewater treatment), or high-value co-product generation, production costs of low-value compounds can be drastically lowered. However, a reliance on coupled operations limits reactor applications and complicates design validation specifically for low-value product generation.

The recently developed subfield of biofilm-based photobioreactors acknowledges the limitations of suspension culture production in conventional photobioreactors and aims to provide a complimentary route to low-cost designs that avoid many of the operational costs of conventional reactors, while achieving greater cultivation density (with associated potential benefits to productivity, as outlined in section 2.2). Recently developed nondestructive microsensing methods and diffusion models of air-exposed biofilms revealed crucial information about production limitations in this cultivation format. A study of this work was motivated to mitigate these limitations while achieving high-density production in a compatible, air-exposed reactor format (chapter 5). Results of this study and others of air-exposed biofilm-based reactors indicate great
potential for improving cost-effective production and motivate the development of larger demonstration reactors based on high-density, fixed cultures 91.

The scalable cultivation of both fixed cultures and the most productive (thin light path) conventional suspension cultures requires stacked reactor modules. In pilot plants, the necessary dilution of sunlight is enabled by gaps between stacks of such modules on the order of 1 – 10 cm 46,80,83,94. In contrast, the waveguide-based light and carbon supply architectures developed in this work (chapters 3 and 4, respectively) rely on minimal material thickness for efficient FTIR illumination and CO₂ permeation, and so enable sub-mm gaps between culture stacks for reduced material costs and improved facility areal density.

Looking forward, to meet the immediate demands of climate change and an increasing human population, approaches toward cost-effective microalgal biofuel production must be accelerated. The emerging ‘paradigm shift’ in photobioreactor design towards biofilm cultivation is an exciting new route 91, considering the historically steady pace of driving down production costs in conventional suspension-culture reactors by incremental design improvements 22,46,54–57. Since a multidisciplinary approach is most effective, however, this author believes that novel high-density reactor designs, combined with potentially giant strides in genetic engineering approaches in the near future, will ultimately enable economically feasible industrial scale production of microalgal biofuels.
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7 Appendices

7.1 Evanescent photosynthesis: exciting cyanobacteria in a surface-confined light field

This appendix was originally published in Physical Chemistry Chemical Physics, volume 14, pages 4817-4823 (2012), and has been reproduced from reference 296 with permission from The Royal Society of Chemistry. The applicant was the third author of this work and played a supporting role in experiment design, execution, data analysis and paper writing. The efforts of the first author, Matthew D. Ooms are particularly noted, as are contributions from the other co-authors, Vincent J. Sieben, Erica E. Jung, Michael Kalontarov, David Erickson and David Sinton.

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The conversion of solar energy to chemical energy useful for maintaining cellular function in photosynthetic algae and cyanobacteria relies critically on light delivery to the microorganisms. Conventional direct irradiation of a bulk suspension leads to non-uniform light distribution within a strongly absorbing culture, and related inefficiencies. The study of small colonies of cells in controlled microenvironments would benefit from control over wavelength, intensity, and location of light energy on the scale of the microorganism. Here we demonstrate that the evanescent light field, confined near the surface of a waveguide, can be used to direct light into cyanobacteria and successfully drive photosynthesis. The method is enabled by the synergy between the penetration depth of the evanescent field and the size of the photosynthetic bacterium, both on the order of micrometres. Wild type *Synechococcus elongatus* (ATCC 33912) cells are exposed to evanescent light generated through total internal reflection of red ($\lambda = 633$ nm) light on a prism surface. Growth onset is consistently observed at intensity levels of $79 \pm 10$ W m$^{-2}$, as measured 1 μm from the surface, and $60 \pm 8$ W m$^{-2}$ as measured by a 5 μm depthwise average. These threshold values agree well with control experiments and literature values based on direct irradiation with daylight. In contrast, negligible growth is observed with evanescent light penetration depths less than the minor dimension of the rod-like bacterium (achieved at larger light incident angles). Collectively these results indicate that evanescent light waves can be used to tailor and direct light into cyanobacteria, driving photosynthesis.
7.1.1 Introduction

Since prehistoric times, photosynthetic microorganisms have played an essential role in the carbon cycle and as the primary mechanism for solar energy capture\(^{353}\) in the biosphere. In modern times, their demonstrated performance has resulted in significant interest from industry and research for the potential uses of photosynthetic microorganisms in such diverse areas as bioremediation\(^{354}\), pharmaceuticals\(^{53}\), human health\(^{355}\), and biofuel production\(^{356-358}\). Furthermore, the photosynthetic machinery of these organisms has provided clues leading to the development of improved photoelectrochemical conversion and energy capture devices\(^ {359}\). These photosynthetic microorganisms, which include a variety of bacteria species and green algae, have varied nutritional requirements but all rely on absorption of light to fuel their metabolic processes. Illuminating cells in confined environments, at high density, and with the control required by precise laboratory studies can present challenges, particularly when scales approach those of the organisms themselves.

Current methods of delivering light energy to microorganisms vary depending on scale, but generally rely on bulk illumination of suspended cultures\(^{356}\). Large scale production photobioreactors rely on direct solar illumination in either racetrack style, open air ponds\(^{360}\) or fully enclosed transparent structures\(^{361,362}\) to provide the necessary energy for photosynthesis. Solar radiation may be complimented or replaced by artificial light sources, particularly in research laboratory applications, in order to achieve more controlled lighting conditions\(^{363-365}\). Improved spatial dilution of light within the culture volume has been achieved through the addition of light-guiding elements\(^ {177,286,300,338,366}\) that help scatter light into darker regions of high density cultures. Microfluidic systems have also been identified as attractive platforms on which to study cell cultures on the cellular scale and offer advantages over larger scale devices when it comes to studying the growth dynamics and behaviour of the organisms\(^ {367-370}\). All large and small scale cultivation systems developed to date, however, rely on radiant bulk illumination. An illumination strategy that confines and delivers light on the scale of the microorganism would enable improved control and quantification.

In this paper we demonstrate the excitation of photosynthetic cyanobacteria using the evanescent light field confined to the surface of a waveguide. The method we describe is enabled by the synergy between the fundamental penetration depth of the evanescent field and the size of the photosynthetic microorganism, both on the order of micrometers. Evanescent coupling is demonstrated using a circular cross-section laser beam incident on the surface of a prism at an angle greater than critical such that the beam undergoes total internal reflection. This setup generates an elliptical evanescent field profile on the prism surface. The resulting field decays exponentially into the medium and is
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confined only to a narrow region above the prism. *Synechococcus elongatus* (ATCC 33912) is shown to grow preferentially in this field in a manner that is related to optimal radiant light levels for these bacteria. This work provides an alternative to current practices of delivering radiant light to cultures of photosynthetic bacteria by providing a means to confine and control the delivery of energy on the cellular scale.

### 7.1.2 Method and materials

#### 7.1.2.1 Bacteria cultures

In all experiments, cells of the wild type *S. elongatus* (ATCC 33912) cyanobacteria were used. Culture growth rate is strongly dependent on fluid temperature and irradiation intensity. Cells were cultured under optimal conditions of 32-36 °C and under continuous irradiation of 50-75 µE·m⁻²·s⁻¹ using fluorescent lamps, as described previously. The stock culture was kept at a constant cell density (in the exponential growth phase), by regularly diluting the culture with fresh BG11 cyanobacteria growth medium (Sigma Aldrich C3061) to maintain a constant optical density of 0.2 at 750 nm (OD₇₅₀). The OD₇₅₀ was determined using a broad spectrum halogen light source (Thorlabs OSL1) and spectrometer (Edmond brc112e) and normalized to the OD₇₅₀ of fresh BG11 growth media. Samples of this culture were used for all experiments.

#### 7.1.2.2 Experimental apparatus

Cavities to contain the bacteria culture solution were fabricated by moulding PDMS (Sylgard(R) 184 Elastomer Kit, Dow Corning) around a poly(methylmethacrylate) (PMMA) master to create cylindrical cavities 10 mm in diameter and 5 mm deep (0.4 mL). These culture cavities were bonded to the surface of a 1 mm thick BK7 glass microscope slide using oxygen plasma treatment. Once mounted to the glass plates and inoculated (dead end filling via syringe injection), the cultures were placed on the top faces of right angle BK7 prisms (Thorlabs PS908L-A), as shown in Fig. 7.1, and the cells were allowed to settle onto the surface of the glass slide. Optical contact was achieved using an index matched immersion oil (Leica 11513 859). Light was coupled to the chamber from a helium neon laser (633 nm Thorlabs HRR020) directed toward the prism by reflecting it off a broadband dielectric mirror (Thorlabs CM1-4E) mounted to a precision rotation mount (Thorlabs CRM1P). The incident angle at the glass media interface was adjusted by changing the angle of the mirror in the rotation mount. The prism/culture assembly was mounted to a sliding stage, which allowed the laser beam to be maintained in the centre of the culture chamber as the angle of incidence was varied. The prism assembly was aligned such that the reflection of the beam leaving the prism
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did not pass through the culture. This ensured that optical excitation of the bacteria was solely due to the evanescent field where the beam was totally internally reflected at the glass-bacteria culture interface.

Fig. 7.1 Exciting photosynthetic bacteria with the evanescent light field. (A) Schematic showing evanescent coupling of a photosynthetic bacterium on the surface of a waveguide. The characteristic decay of the red light intensity, as plotted at right, is on the order of the cell size (B) Experimental setup to generate the evanescent wave at the surface of the glass slide. The input beam is Gaussian and the evanescent field resulting from total internal reflection is elliptical in shape, as shown.

Laser beam power into and out of the prism was measured using a photodiode power sensor (Thorlabs S120C) and measured once at the beginning of the experiment and once at the end. The entire experimental apparatus was optically isolated in enclosures made from 5 mm thick hardboard (Thorlabs TB4). These chambers were kept at a constant temperature of 32-36 °C for optimal cell growth rates for the duration of the evanescent growth experiments using a 950 W enclosure fan heaters (CR030599, OMEGA Engineering Inc., USA).
7.1.2.3 Measurement of growth rings

Growth patterns were imaged using standard bright field microscopy techniques. Images were collected using an SLR camera (Nikon D60) fitted to an upright microscope (Leica DMLM/P). The images were capture at 5x magnification using identical camera settings (exposure, colour parameters, etc.). Multiple images were required to capture the full spatial extent of the growth rings at this magnification. The individual images were then compiled in an automated fashion to create a composite image of each growth ring. The composites were then processed in Matlab employing colour thresholding to identify pixels with Green/Red and Green/Blue ratios larger than 1. A normalized histogram was created for pixels that satisfied these criteria, based on their radial distance from the centre of the beam profile (the elliptical profiles were converted to circular profiles using the experimental angles and Snell’s law).

7.1.3 Results and discussion

The evanescent excitation approach is shown in Fig. 7.1(A). A bacterium on the surface of the waveguide receives light from the evanescent light field which decays rapidly with distance from the surface. The prism-based experimental setup used to generate the evanescent field is shown in Fig. 7.1(B). The evanescent field develops at the interface between the liquid and the glass where the light is totally internally reflected $^{163,375}$. Total internal reflection, and the corresponding evanescent field, result when light is incident at glass-media interface at angles greater than the critical angle, $\theta_\text{i} > \theta_c$. Reflecting a circular cross-section input beam develops an elliptical evanescent field at the point of reflection, as shown. While there are many ways to generate an evanescent field, this approach is simple and importantly, provides an evanescent light field distribution that can be reliably described with theory in all three dimensions.

Cell cultures were first placed under direct laser light exposure, to establish the effectiveness of using monochromatic red ($\lambda = 633$ nm) at growing *S. elongatus*, and measure cell response to direct radiation light. The beam from a Helium-Neon (HeNe) laser was passed through a culture cavity perpendicular to the bottom glass slide and the culture was left to grow for 72 hours (under conditions described in the methods section). This type of direct irradiation experiment was done for various laser powers, yielding consistent results to those shown in Fig. 7.2. Fig. 7.2(A) shows a typical growth ring pattern, where the effect on growth from the three distinct intensity regions is evident. There is a bleached (yellowish orange) region in the centre, a growth region (green) and a negligible-growth outer region. To quantify growth in a radial profile, the image was filtered for green intensity and integrated in circumference (details in Methods). The resulting radial growth profile is
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plotted with the laser intensity profile in Fig. 7.2(B). The threshold electric field intensities (low and high) between regions were determined from the intersection of the full width at half the maximum (FWHM) growth locations and the incident light power profile. The resulting threshold values of 66 W m\(^{-2}\) and 12 W m\(^{-2}\) (shown in Fig. 7.2(B) by the rectangle within the growth peak), indicate the productive growth intensities of \(S. \text{elongatus}\) under direct irradiation at \(\lambda = 633\) nm.

Fig. 7.2 Growing photosynthetic bacteria using direct irradiation. (A) Image of cyanobacteria growth pattern resulting from direct irradiation, showing distinct regions of photoinhibition (centre), growth, surrounded by negligible growth. (B) Plot correlating radial growth intensity to laser light intensity. Outlying peaks beyond 1.2 mm are artefacts of the imaging setup and do not correspond to growth. FWHM thresholds on the growth region correspond to radiant light intensities of 66 W m\(^{-2}\) and 12 W m\(^{-2}\), shown as upper and lower bounds, respectively.

Relating these direct irradiation experiment results to known growth characteristics of \(S. \text{elongatus}\) requires estimating the daylight equivalent power of red light at \(\lambda = 633\) nm. To do so, we compared
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Radiometric measurements of daylight to optimal intensity ranges published in literature. At high light intensities, the rate of radiation induced damage to the cell’s photosystems exceeds the cell’s ability to repair itself and the result is a sharp decrease in photosynthetic activity, or photoinhibition. Photoinhibition can further lead to excessive destruction of light absorbing pigments, primarily chlorophyll a within the cell’s photocenters, in a process known as photobleaching. High light conditions that approach saturating intensities are reported as a Photosynthetic Photon Flux Density (PPFD) on the order of 150 µE·m⁻²·s⁻¹ to 500 µE·m⁻²·s⁻¹ in the Photosynthetically Active Radiation (PAR) wavelength range (400-700nm), or 10%-25% of full daylight. To convert the photosynthetic photon flux density to radiometric units (i.e. W m⁻²), the optical power of full daylight was measured at 635 nm to be 1.37 W m⁻² (48°25′43″N 123°21′56″W). The spectral power distribution of normal daylight was then calculated from this set point and the relative spectral power distribution defined by CIE Standard Illuminant D65, as shown in Fig. 7.3. Total full daylight irradiance of photosynthetically active radiation was calculated to be 472 W m⁻², which corresponds to a photosynthetic photon flux density of 2137 µE·m⁻²·s⁻¹. This value was independently confirmed by a QSR-2100 (Biospherical Instruments Inc.) light meter measurement of 2100-2300 µE·m⁻²·s⁻¹.

Also shown in Fig. 7.3, is the absorption spectrum for the cell cultures used for these experiments. Absorption peaks at λ=435 nm, λ=625 nm, and λ=677 nm are typical for *S. elongatus* and represent absorption by the light harvesting pigments Chl a (λ=435 nm and λ=677 nm) and the phycobilisomes of the photosystem II antenna structures – primarily phycocyanin (625 nm).

![Fig. 7.3](image)

**Fig. 7.3** Spectral power distribution of daylight and normalised absorption spectrum of *S. elongatus* culture. The photoinhibition threshold at λ 633nm measured from experiments in Fig. 7.2 (66 W m⁻²) correlates to established photoinhibition intensities of white light exposure, when related through the absorption spectrum shown.
Under normal daylight conditions, the effective photon flux seen by *S. elongatus* given its varied absorption ability for different wavelengths of light is 1044 µE·m⁻²·s⁻¹ of photosynthetically active radiation (400 nm < λ < 700 nm). This is determined by weighing the spectral power distribution for daylight by the normalized absorption spectrum of the cell culture and integrating across the photosynthetically active region. It should be noted that this value represents a best case maximum for absorbed photosynthetic energy and assumes that all energy absorbed by the bacteria is funnelled into the photosynthetic infrastructure of the cell. To determine a corresponding irradiance for monochromatic light at λ=633 nm that would simulate these daylight conditions, this broadband photon flux is multiplied by the energy contained in 1 µE of photons with λ=633 nm (i.e. 0.189 J·µE⁻¹ determined from the Planck–Einstein equation) and divided by the normalized absorption of the cell culture at 633 nm (i.e. 0.65). This results in an irradiance of 301 W m⁻² of λ=633 nm light that is required to simulate the photosynthetic photon flux of normal daylight. Since the threshold for the onset of photobleaching measured in the direct irradiation experiments was 66 W m⁻², it can be inferred that ~22% of full daylight is the upper limit for our cultures before severe photobleaching occurs. This value agrees well with the upper bounds of what are considered high-light conditions in literature. Two key assumptions made here are that 1) photons of all energies within the photosynthetically active region have an equal potential to contribute to photobleaching and 2) the absorption of light by the cell is primarily due to pigments associated with light harvesting and energy transfer to the photosynthetic reaction centers. Given that the quantum efficiency of photosynthesis is known to be high and that the daylight equivalent power for red light calculated here agrees generally with the onset of photobleaching both under red light conditions determined experimentally in this study and those under daylight conditions published in literature, these assumptions are considered justified for an order of magnitude estimate.

The light intensity distribution in an evanescent light field varies both in the plane of the surface and depthwise into the media. Established theory was applied to describe the evanescent electric field intensity and used to correlate field strength to experimental growth results (model details in supporting information, SI). Fig. 7.4(A) shows the penetration depth of the evanescent light field as a function of incident angle. Here, the penetration depth is quantified as the location where the field intensity drops e⁻², or 87%, of the peak intensity at the surface. The geometry of *S. elongatus* is shown inset in Fig. 7.4(A) for reference, and the dashed line indicates a penetration depth of 1 µm which occurs at an angle of incidence of θ₂ = θc + 0.074°. As shown, the penetration depth of the evanescent field is a strong function of incident angle, with values corresponding to the inherent lengthscale of
the bacterium occurring only near the critical angle (below 0.5° past critical). Because of this narrow envelope, only cells settling onto the surface will be within range of the evanescent field. While the extent of the evanescent field, and the size of the bacterium are well established (as shown together in Fig. 7.4), the method does not enable resolution on the scale of individual thylakoid membranes to determine the interaction between the evanescent field and specific photocenters within the cell. Growth of cells is instead attributed to excitation of photocenters assumed to be distributed around the cell where the average evanescent intensity across the cell dimension falls within the range of acceptable intensities.

Fig. 7.4 Theoretical light intensity distribution in the evanescent light field, and corresponding predicted growth patterns. (A) Plot of the penetration depth as a function of incident laser angle for a glass-media interface. Penetration depth is defined as the location where field intensity drops $e^{-2}$, or 87%, from that at the surface. The dashed line indicates a penetration depth of 1 µm occurring at $\theta_d = \theta_c + 0.074^\circ$, and the geometry of *S. elongatus* is shown inset for reference. (B) Surface plot of evanescent field, 1 µm from the surface, with power intensity plotted to indicate the photoinhibited, growth, and negligible-growth regions, based on thresholds measured for radiant light. Based on
these values an elliptical ring pattern of growth is predicted, as shown by the useful portion of the power spectrum shown in green shading (region 2). The vertical line plot indicates the useful light intensity decay with distance.

Fig. 7.4(B) shows the predicted evanescent field intensity in the plane, and the characteristic oval shape for an incident 0.5-mm diameter Gaussian beam at $\lambda = 633$ nm. The intensity values indicated correspond to the evanescent light intensity at 1 µm from the glass-media interface, with an incident angle of $62^0$ ($\theta_{12} = \theta_c + 0.5^0$) and penetration depth of 400 nm. Based on the above-determined threshold light intensity for the red light employed here (66 W m$^{-2}$, at 633 nm), the expected growth regions can be predicted based on the calculated evanescent field intensity. As shown in Fig. 7.4(B), in region 1 the evanescent field intensity exceeds the red component of 10% daylight and would be expected to lead to photoinhibition in a radiant light system. This analysis would predict an elliptical ring pattern of growth, as shown by the useful portion of the power spectrum shown in green shading (region 2). The vertical line plot indicates the useful light intensity decay with distance. Relatively intense growth is expected near the inside boundary where useful light intensities are high, and growth rates would decay with the light intensity outward. Although the sharpness of the inside edge of the growth profile is an artefact of the threshold boundary condition, the model provides the predicted pattern of growth for a photosynthetic microorganism cultured in this evanescent field.

Evanescent light based excitation of the culture was performed using the experimental setup shown in Fig. 7.1(B). Three laser powers were employed (1 mW, 0.5 mW, 0.25 mW) with incident laser angles of $62^0$ ($\theta_{12} = \theta_c + 0.5^0$), and total internal reflection was ensured by measuring the output intensity. Each experiment was performed in triplicate and the cultures were exposed to the evanescent field for 72 hours. Fig. 7.5(A-C) show substantial bacteria growth in response to the evanescent light field at the surface of the glass-media interface. The growth patterns showed the elliptical shape mirroring the evanescent light field intensity, and delineate the three characteristic regions (photoinhibition, growth, negligible-growth), providing data on the onset of growth under evanescent light. As the laser power was reduced (Fig. 7.5(A-C) the radial distribution moved inward, consistent with the change in the light intensity profile. To relate the observed growth to the evanescent field intensity, the images were filtered for green intensity, scaled along the axis of the beam, and integrated to provide growth profiles. Fig. 7.5(D-F) shows the growth profiles for each light power with the corresponding evanescent field intensities plotted at the surface, 1 µm above the surface, and as a 5-µm average. Due to rapidly decaying nature of the evanescent field, the surface intensity is much higher than that at 1 µm above the surface, which is also similar to the average intensity over the first 5 µm (both 1 µm and 5 µm are relevant lengthscales of this rod-shaped
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bacteria). The power range determined from the direct radiation experiments is shown by the red band for reference. The onset of growth occurs at a radial location where the evanescent light intensity – as measured at 1 µm and as a 5-µm average – drops to a value corresponding to the threshold of 66 W m⁻², established from direct radiation experiments. As the total intensity of light is decreased (Fig. 7.5(A-C) and (D-F) the location of the onset intensity moves inward, and remains consistent with the predicted power curves. Specifically, the full-width at half maximum indicating growth onset is observed at 1-µm intensity levels of \(79 \pm 10\) W m⁻², and observed at \(60 \pm 8\) W m⁻² for the 5-µm average light intensity. Importantly, these results both demonstrate growth of photosynthetic bacteria using evanescent light, and provide metrics for their successful cultivation within this unique light field.
Fig. 7.5 Growing photosynthetic bacteria using evanescent light. (A-C) Images of cyanobacteria growth patterns resulting from evanescent excitation at the glass-media interface for incident light powers of 1 mW, 0.5 mW, and 0.25 mW, respectively. The elliptical growth patterns correspond to the evanescent field geometry, and show distinct regions of photoinhibition (centre), and growth, surrounded by negligible growth. (D-F) Corresponding growth profiles for each light power with the corresponding evanescent field intensities plotted at the surface, 1 µm above the surface, and as a 5-µm average. The power range determined from the direct radiation experiments (Fig. 7.2) is shown by the red band for reference. The full-width at half maximum indicating growth onset is
observed at 1-µm intensity levels of 79 ± 10 W m⁻², and observed at 60 ± 8 W m⁻² for the 5-µm average light intensity. These values bracket the 66 W m⁻² threshold determined for radiant light at this wavelength.

The growth patterns shown in Fig. 7.5, and additional experiments (Fig. 7.7), show some downbeam bias, that is, growth intensity increases with distance from the laser source. When the cells interact with the evanescent field near the surface, some of the light is absorbed and utilized, while some of the light is scattered. The light will be scattered preferentially in the direction of the beam. With the present experimental setup, this scattered light would contribute to higher growth rates, and thicker biofilms, on the downbeam side of the ring pattern. This effect was noticed in most cases with downbeam growth biases of 1%, 8% and 15% for the 0.25 mW, 0.50 mW, 1.0 mW cases plotted in Fig. 7.5. Although the extent of this bias varied between trials, and some trials showed negligible, and even a small upbeam bias (details in Fig. 7.8), the effect was in general small and in all cases less than 15%. While it is likely that downbeam bias and secondary scattering effects influence growth, the relative symmetry of the growth patterns indicates that the downbeam scattering effect is minor.

The additional effect of light penetration depth was investigated using incident light at larger angles past critical (θ_C < θ_i < θ_C + 5°). At angles greater than 0.5° over critical (as plotted in Fig. 7.5), however, only faint growth rings were observed. We attribute the lack of growth at larger angles to the change in penetration depth which diminishes rapidly with increasing incident angle, as shown in Fig. 7.4(A). Specifically, the penetration depth corresponds to the minor-dimension of the rod-shaped bacterium (1 µm) only at angles less than θ_C + 0.074°. These results are thus consistent with the observed evanescent growth patterns in that the penetration depth approached the cell diameter only at small angles away from critical.

### 7.1.4 Conclusion

We have demonstrated that evanescent fields can be utilised to grow photosynthetic bacteria. Cultures of *S. elongatus* were exposed to direct laser illumination (633 nm) and grew optimally with light intensities that ranged from 12-66 W m⁻². When excited with an evanescent field, this optimal growth band occurred between surface intensities of 30-650 W m⁻². This difference was attributed to the exponential decay of evanescent fields into the culture media, and at heights of 1-2 µm from the surface, approximately the width of an individual cell, the intensity falls within the 3-60 W m⁻² ideal. This narrowly confined region for growth enables targeted light delivery to individual bacteria by tapping energy from the evanescent field. This work provides an alternative to current practices
of delivering radiant light to cultures of photosynthetic bacteria. While direct illumination of a thin biofilm of cells may also be a viable approach to achieve controlled illumination, it presents no significant advantage over direct illumination of a bulk culture. In contrast the confinement inherent to the evanescent approach enables select illumination of surface cells and improved control, particularly in regards to the spatial distribution of light. Furthermore, the cell-scale limited extent of the light field provides a tool to probe microorganism response which is not possible with direct illumination of thin microbial biofilms and provides a platform for improved understanding of photosynthetic processes in microorganisms.

7.1.5 Supplementary Information

7.1.5.1 Model Details

A unpolarized Helium-Neon laser with a circular cross-section beam profile can be modelled as a Gaussian using the following equation (TEM00)\(^1\):

\[
I_{n1}(r, a) = I_0(a) \ e^{-2(r^2)/w(a)^2}
\]

where \(r\) is the radius from the beam center, \(a\) is the distance from the beam waist, \(w(a)\) is the beam width and \(I_0(a)\) is the peak intensity, both at distance \(a\). After determining the intensity profile for the apparatus shown in Fig. 7.1 of the paper at the air-to-prism interface (\(n_1\) to \(n_2\) - before the beam enters the prism), the Fresnel transmission coefficients for both s- and p-polarizations (\(t_1^s\) and \(t_1^p\), respectively) were used to determine the intensity profile of the beam in the prism:

\[
I_{0,n2} = \begin{cases} 
|t_1^s|^2 \cdot I_{0,n1}, & s - \text{pol} \\
|t_1^p|^2 \cdot I_{0,n1}, & p - \text{pol} 
\end{cases}
\]

\[
t_1^s = \frac{2n_1 \cos \theta_{i1}}{n_1 \cos \theta_{i1} + n_2 \cos \theta_{t1}}
\]

\[
t_1^p = \frac{2n_1 \cos \theta_{i1}}{n_1 \cos \theta_{t1} + n_2 \cos \theta_{i1}}
\]

where, \(\theta_{i1}\) is the incident angle (\(\theta_{i1} = 45^\circ - \theta_{\text{laser}}\)) and \(\theta_{t1}\) is the transmitted angle (calculated from Snell’s law) at the air-prism interface. The evanescent intensity profile at the prism-sample interface was calculated for s-polarized and p-polarized light using the matrix formalization as described in references \(^2\)\(^-\)\(^6\). The matrix method is useful because it can be used to account for multi-layered systems (i.e. index matching fluid, different slide and prism materials, etc.) \(^4\)\(^7\). The formulae for this
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Method are:

\[
I_{0,n3} = \begin{cases} 
|t_2^s|^2 \cdot I_{0,n2}, & s - \text{pol} \\
C_p \cdot |t_2^p|^2 \cdot I_{0,n2}, & p - \text{pol}
\end{cases}
\]  

(5)

where \( t_2^s = \frac{1}{M_{11}^{s,p}} \) and \( C_p = |\cos \theta_{ts}|^2 + |\sin \theta_{ts}|^2 \)

(6)

and \( \begin{pmatrix} E_i \\ E_r \\ 0 \end{pmatrix} = M^{s,p} \begin{pmatrix} E_t \\ 0 \end{pmatrix} \)  

(7)

where \( M^{s,p} \) is the matrix of the stack that links the incident, reflected and transmitted electric fields \((E_i, E_r, E_t)\) for s- or p-polarised light, \( C_p \) is the magnitude factor that takes into account the two field components of p-polarised light for total internal reflection as in Axelrod et al.\(^5\) and \( \theta_{ts} \) is the final transmitted angle (imaginary, calculated from Snell’s law) at the sample interface. The overall matrix can be calculated for \( N \) layers \((l = 0, 1, 2, ..., s)\) from the dynamical and the propagation matrices \((D_l\) and \( P_l\)) as:

\[
M^{s,p} = \begin{pmatrix} M_{11} & M_{12} \\ M_{21} & M_{22} \end{pmatrix} = D_0^{-1} \left[ \prod_{l=1}^{N} D_l P_l D_{l-1}^{-1} \right] D_s
\]

(8)

\[
D_1 = \begin{pmatrix} 1 & 1 \\ n_l \cos \theta_l & -n_l \cos \theta_l \end{pmatrix}, \text{ for } s - \text{pol} \\
\begin{pmatrix} \cos \theta_l & \cos \theta_l \\ n_l & -n_l \end{pmatrix}, \text{ for } p - \text{pol}
\]

(9)

\[
P_l = \begin{pmatrix} e^{-ik_l d_l} & 0 \\ 0 & e^{ik_l d_l} \end{pmatrix}, \text{ where } k_l = n_l \frac{w}{c} \cos \theta_l
\]

(10)

where for each layer: \( n_l \) is the complex index of refraction, \( \theta_l \) is the complex angle of propagation (to the normal, determined by Snell’s law), \( k_l \) is the component of the wave vector along the direction of propagation, \( d_l \) is the thickness of the thin film and \( w \) is the angular frequency of light. The evanescent intensity at any position perpendicular to the TIR interface decays exponentially with \( z \) as \(^5\):

\[
I_{n3}(z) = I_{0,n3} e^{-z/d}
\]

(11)

where, \( d_{\text{pen}} = \frac{\lambda_0}{4\pi} \left[ n_1^2 \sin^2 \theta_{i2} - n_3^2 \right]^{-1/2} \)

(12)

where \( \lambda_0 \) is the light wavelength in vacuum, \( \theta_{i2} = 45^\circ + \theta_{t1} \) is the incident angle with reference to
the top of the prism and \( n_3 \) is the index of refraction in the liquid sample. In this work the model was applied to the two-layer system of glass and media.

The evanescent intensity amplitudes were spatially mapped from the initial laser-profile (circular) to the prism-sample interface (elliptical). Since the interface surfaces are normal to the plane of incidence, the spatial profile will be distorted only in one dimension, calculated using the cosine of the incident angle.

7.1.5.2 Supplementary Experimental Results

Fig. 7.6 Results from secondary evanescent light based growth experiments collected using the methods described in the paper. (A-C) Images of cyanobacteria growth patterns resulting from evanescent excitation at the glass-media interface for incident light powers of 1mW, 0.5mW, and 0.25mW, respectively. The elliptical growth patterns correspond to the evanescent field geometry, and shows distinct regions of photoinhibition (centre), and growth, surrounded by negligible growth. (D-F) Corresponding growth profiles for each light power with the corresponding evanescent field intensities plotted at the surface, 1 µm above the surface, and as a 5-µm average. The power range determined from the direct radiation experiments (Fig. 7.2) is shown by the red
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band for reference. (G-I) Quantification of the upbeam/downbeam growth bias for each respective ring pattern indicating the relative degree of growth intensity relative to the center of the beam profile in either the left (downbeam) or right (upbeam) direction.

Fig. 7.7 Results from tertiary evanescent light based growth experiments collected using the methods described in the paper, and plotted as in Fig. 7.6. (A-C) Images of cyanobacteria growth patterns resulting from evanescent excitation at the glass-media interface for incident light powers of 1mW, 0.5mW, and 0.25mW, respectively. (D-F) Corresponding growth profiles for each light power with the corresponding evanescent field intensities plotted at the surface, 1 µm above the surface, and as a 5-µm average. (G-I) Quantification of the upbeam/downbeam growth bias for each respective ring pattern indicating the relative degree of growth intensity relative to the center of the beam profile in either the left (downbeam) or right (upbeam) direction.
Fig. 7.8 Quantification of the upbeam/downbeam growth bias for the growth patterns presented in Fig. 7.5A-C indicating the degree of growth intensity relative to the center of the beam profile in either the left (downbeam) or right (upbeam) direction. Graphs correspond to laser powers of (A) 1.0 mW, (B) 0.5 mW, and (C) 0.25 mW.

References for Supplementary Information section 7.1.5


