Multiplexed Microfluidics for the Study of Photosynthetic Microorganisms

by

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Abstract

The ability of large datasets of simple metrics (i.e. growth rate, lipid accumulation) to contribute to the understanding of microalgal photosynthesis is explored in this thesis. Specifically, three tools were developed, (i) a microfluidic multiplexed irradiance assay, which was composed of a microfluidic chip and a liquid crystal display; (ii) a second order mathematical model which can predict the growth under fluctuating light for periods ranging from a day to one under second and; (iii) a combinatorial assay for studying the mixed effect of light and nitrogen on growth and lipid accumulation. The multiplexed microfluidic irradiance assay (Microalgae on display) was capable of producing a large number of independent light treatments (200+), while avoiding the self-shading typical of flask-scale experiments. A set of linear ordinary differential equations were capable of capturing a second order behavior of photosynthetic growth, specifically a penalty at intermediate frequencies. This behavior lies outside the prevailing understanding of a high and low frequency limit. Lastly, the interplay between irradiance intensity and ammonium was resolved, showing that ammonium concentration plays a role at high light intensities only.
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Chapter 1.

1.1 Motivation

The transformation of solar irradiance into chemical energy, performed by photosynthetic organisms, is critical to the ecosystem. Unfortunately, the rising concentration of greenhouse gases in the environment is substantially changing the climate, jeopardizing the health of primary producers (photosynthetic organisms) and the integrity of the ecosystem. However, photosynthetic organisms can be used to alleviate this by serving as a sink for CO\textsubscript{2}. Industrially this is possible by integrating a photobioreactor to point CO\textsubscript{2} sources. In nature increased primary productivity could, in principle, offset increased CO\textsubscript{2}, however this ability to consume CO\textsubscript{2} is dependent on multiple environmental parameters.

The successful implementation of photosynthetic microorganisms as tools for carbon capture, or to produce biofuels or bioproducts requires a strong understanding of photosynthetic growth. This strong understanding can then be used to develop design practices to produce effective photobioreactors. Two classes of tools can serve the design of photobioreactors, multiplexed experimental platforms cable of acquiring large amounts of data, and models simple enough to be not computationally expensive, yet detailed enough to be able to predict reactor performance. Moreover, both tools can help serve to understand the effects of climate change on ecosystems in general, by providing data or predictive power.

1.2 Thesis Overview

This section outlines both core contributions of my thesis as well as additional contributions made through the course of my PhD studies. The core contributions are included as chapters of the thesis, while supplementary contributions are included as appendices.

1.2.1 Core Contributions

Chapter 2 provides an overview of photosynthesis to give background information to the reader.

Chapter 3 is a section of an invited review paper under review with *Sustainable Energy and Fuels*, where I am an equally contributing first author: Microfluidic methods for informing biofuel production are discussed. (SE-REV-05-2017-000236).
Chapter 4 presents the development of a microfluidic screening platform for studying the effect of irradiance conditions on microalgal growth. The work entailed coupling a commercially available LCD to an array of microfluidic chamber. This work was published in *Lab on a Chip*\(^1\) and featured on the front cover of Issue 15 in 2015.

Chapter 5 presents a frequency dependent penalty on photosynthetic growth, which is caused by regulatory mechanisms of photosynthesis. This work entailed both a high-throughput experimental study and the development of a simplified mathematical model. The manuscript has been submitted to *Scientific Reports* (SREP-17-21855).

Chapter 6 presents a coupled nutrient and light screening platform. This device was a collaborative effort, where I am the second author. The work was published in *Lab on a chip* in 2016.\(^2\)

Chapter 7 summarizes the main contributions and presents a brief outlook.

### 1.2.2 Supplementary Contributions

Section 8.1 presents the manuscript “High-throughput assessments of the ecological impacts of global change,” this collaborative effort that builds upon the core contributions presented in chapters three and five. This work shows the use of an aerogel-based gradient generator to perform full-factorial experiments on organisms of various scales. My contribution to this work relates to early guidance, project objective, and manuscript editing. This manuscript has been submitted to *Science Advances* (ScienceAdvances-D-17-01189).

Section 8.2 consists of the publication “Predominance of sperm motion in corners” a study quantifying the degree to which sperm accumulate in microchannel corners. This work demonstrates that a corner causes an accumulation effect stronger than the superposition of two walls. In this work, I contributed to running experiments, data analysis and manuscript preparation. This paper was published in *Scientific Reports* on May 13 2016.\(^3\)

Section 8.3 includes the manuscript “Nanomorphology-enhanced gas-evolution intensifies CO\(_2\) reduction electrochemistry”, in *ACS Sustainable Chemistry and Engineering*, where I am second author. My contribution includes both manuscript editing and advice regarding formulating the mathematical model describing multispecies transport such that it can be solved using MATLAB. The skillset to contribute to this manuscript was developed during the process of the manuscript of the penalty of photosynthetic growth.
1.2.3 Publications not included in thesis

In addition to the publications previously discussed, I contributed to two additional publications. The first, a review article “Microfluidics for Male Fertility,” which was conditionally accepted in *Nature Reviews Urology* where I am equally contributing first author. Though I contributed throughout, my main contribution was the sections related to the fundamental understanding of rheotaxis and boundary following behavior. Lastly, I contributed to the manuscript “Light dilution via wavelength management for efficient high-density photobioreactors” which has been published in *Biotechnology and Bioengineering* on 14 March 2017.
Chapter 2. Introduction

This chapter presents an overview of photosynthesis to give background information for the reader. Specifically, the light reactions, dark reaction and regulation of the Calvin cycle are presented.

2.1 Photosynthesis

Photosynthesis is a process which uses light energy to reduce \( \text{CO}_2 \). Globally, 250 billion tons of \( \text{CO}_2 \) are reduced via photosynthesis per year.\(^{4,5}\) There are two major sets of reactions in photosynthesis, the light reactions which capture light energy to produce ATP and NADPH, and the dark reactions where ATP and NADPH are used to fix and reduce carbon dioxide.\(^{6,7}\) Photosynthesis occurs in both micro and macro scale organisms as well as terrestrial and aquatic organisms.\(^ {4-7}\)

2.1.1 Light reactions

The light reactions of a photosynthetic organism (Figure 2-1) occur along the photosynthetic electron transport chain. Briefly, light is captured by both photosystems I and II.\(^ {8-10}\) Photons captured by photosystem II are used to remove electrons from water, producing protons and oxygen. This electron is then shuttled along the electron transport chain, where its energy is used to

![Image of photosynthesis](image.png)

Figure 2-1: Photosynthesis: the light reactions (Reproduced and modified from Wikimedia Commons, credit: User Somepics.)
move a proton across the thylakoid membrane (the thylakoid membrane is the membrane housing the photosynthetic electron transport chain). Using the light it absorbed, Photosystem I e-energizes this electron and uses it to reduce NADP+ to NADPH. 8-10 Lastly, the energy associated with the proton gradient is used to produce ATP using ATP synthase.

2.1.2 Dark reactions, the Calvin-Benson-Bassham Cycle

The Calvin-Benson-Bassham (CBB) cycle (Figure 2-2) is a sequence of biochemical redox reactions, which collectively convert three CO2 molecules into one molecule of glyceraldehyde-3-phosphate. Glucose is then produced from two molecules of glyceraldehyde-3-phosphate.\textsuperscript{6,11-13} Generally, there are three main phases, (i) carbon fixation, where RuBisCO catalyzes the reaction between three molecules of CO2 three molecules of Ribulose-1,5-bisphosphate (RuBP, \(C_5H_{12}O_{11}P_2\)) to produce six molecules of glycerate 3-phosphate (3-PGA, \(6C_3H_7O_7P\)), (ii) carbon reduction, where 3-PGA is reduced to glyceraldehyde 3-phosphate (GA3P, \(C_3H_7O_6P\)) using ATP and NADPH and (iii) RuBP regeneration where five out of six molecules of GA3P are converted back into RuBP, to continue the cycle. The molecule of glyceraldehyde-3-phosphate which is not recycled into RuBP is used to produce glucose.

The phases of the Calvin Cycle can be summarized by the following three reactions. Note that to balance the equations one must consider the additional oxygen and hydrogen present in ATP as opposed to ADP.

Phase 1, Carbon Fixation

\[3CO_2 + 3H_2O + 3C_5H_{12}O_{11}P_2 \rightarrow 6C_3H_7O_7P\]

Phase 2, Reduction

\[6C_3H_7O_7P + 6ATP + 6NADPH + 6H^+ \rightarrow 6C_3H_7O_6P + 6ADP + NADP^+ + 6PO_4^{3-} + 18H^+\]

Phase 3, RuBP regeneration

\[5C_3H_7O_6P + 2H_2O + 3ATP \rightarrow 3C_5H_{12}O_{11}P_2 + 3ADP + 2PO_4^{3-} + 6H^+\]

Giving the following overall reaction

\[3CO_2 + 5H_2O + 9ATP + 6NADPH + 6H^+ \rightarrow C_3H_7O_6P + 9ADP + 6NADP^+ + 8PO_4^{3-} + 24H^+\]
2.1.3 Regulation of the Calvin cycle

The Calvin cycle is active when the cell is receiving light, and for seconds to minutes afterwards.\textsuperscript{13-16} During dark phases, the Calvin cycle is deactivated so that RuBP does not become depleted, which would cause the cycle to collapse.\textsuperscript{13} In addition, inhibitors of RuBisCO activity can protect RuBisCO from degradation in the dark\textsuperscript{17} and when under environmental stress.\textsuperscript{18} Irradiance controls the degree of activation of the Calvin cycle by indirectly activating Rubisco activase by increasing the amount of ATP and by promoting RuBP regeneration via redox sensing of the photosynthetic electron transport chain.\textsuperscript{19} The role of Rubisco activase is to aid in the removal of competitive inhibitors from the active site of RuBisCO through conformational changes (Figure 2-3).\textsuperscript{4,5,20} RuBisCO can be inhibited both when bound and when not bound to CO\textsubscript{2}.

Precise values for rate constants are sparse, since the details of the regulation of the Calvin cycle is an ongoing research topic.\textsuperscript{5,11} Activation and deactivation kinetics vary across species\textsuperscript{21-23} and the rate constants are typically extracted by fitting complex models to CO\textsubscript{2} assimilation experiments.\textsuperscript{24-26} Deactivation rates for RuBP regeneration are not explicitly available experimentally, but can be inferred to be a factor of 1-4 times slower than the activation rate based on various models fit to data.\textsuperscript{24,25,27} The regulation of RuBisCo activity is typically one to two orders of magnitude slower than RuBP regeneration with an activation rate constant 5-10 times higher than the deactivation rate constant.\textsuperscript{23-25,27,28}
2.2 Photosynthesis in fluctuating light

To discuss how fluctuating irradiance affects photosynthetic growth, some discussion on growth under continuous irradiance is required. A photosynthesis (or growth) versus irradiance curve has four regions with respect to intensity: (1) a dark region where light intensity is not sufficient to drive photosynthesis, (2) a low light region of where increasing illumination increases photosynthetic output, (3) a saturated region where photosynthetic output is constant with increasing light intensity and (4) a photo-inhibited region where increasing light decreases photosynthetic output.\textsuperscript{29,30} Under light saturated conditions, photosynthesis is limited typically by carbon uptake, but could also be limited by either nitrogen or phosphorus availability.

Multiple timescales are associated with the reactions of a photosynthetic organism.\textsuperscript{10} The absorption of photons occurs rapidly, on the order of femtoseconds. Followed by photochemical reactions which occur within nanoseconds. It then takes microseconds for electrons to be transported along the electron transport chain to perform biochemical reactions which take milliseconds to seconds to occur. These biochemical reactions are then regulated on the order of seconds to hours.\textsuperscript{10} This mismatch between reactions rates highlight the importance of
understanding how light fluctuations and time varying irradiance affects photosynthesis and the growth of photosynthetic organisms.

Typically, the frequency response of photosynthetic growth under fluctuating light can be characterized by two extremes, a high frequency limit where growth matches the growth possible at the same time-average light intensity and a low frequency limit where growth aligns to the average of the growth rates. Under sufficiently light frequencies (>1 Hz) photosynthetic growth begins to exceed the average growth rate and increase with increasing frequency. This behaviour is attributed to a mismatch in reaction rates, which allows for continued carbon fixation during a portion of the dark phase of the light fluctuation. Specifically, the slow carbon fixation continues due to the presence of ATP and NADPH from the dark reactions. As the frequency increases, the dark phase shortens and the section of the dark phase where carbon assimilation becomes increasingly important, allowing growth to approach the high-frequency limit.
Chapter 3. Informing microalgal production

This chapter is a section of an invited review paper, currently under review in *Sustainable Energy and Fuels* (SE-REV-05-2017-000236). I am one of five equally contributing first authors. The scope of the review encompasses technology to inform microalgal production, design photobioreactors and extract products from microalgae. In addition to editing the manuscript throughout, I drafted the section titled Informing microalgal production, included as this chapter. Additional authors for this work include Scott Pierobon, Xiang Cheng, Brian Nguyen, Evan Karakolis and David Sinton. Reproduced with permission from The Royal Society of Chemistry.

### 3.1 The complexity of microalgal photosynthesis

Microalgal productivity is dependent on a variety of parameters, namely temperature, the light characteristics, the aqueous environment, the gaseous environment and the interactions between these variables. High throughput screening devices, particularly microfluidic devices, present and opportunity to rapidly explore the parameter space associated with microalgal productivity. Microfluidic devices for multiplexed studies of cell biology have already been developed and reviewed extensively. However, such devices are aimed at studying the role of the chemical environment, typically drugs. In contrast, platforms capable of studying photosynthetic organisms should be capable of multiplexing notably different variables, light, temperature and dissolved gas concentrations. Thus, new screening platforms have been developed over recent years.

Moreover, implementing this data can involve either direct operating conditions, or predictive models to be used with full scale modeling of the reactor.

Aspects of the light environment to be studied include the intensity, spectral distribution and time variance. Briefly, increasing light intensity increases growth until a saturating intensity, where other nutrients are the limiting factor and additional light does not increase growth. A further increase in irradiance can become harmful to microalgae due to excess reactive oxygen species production. The light spectrum plays a role, predominantly dictated by the pigments present in a microalgal cell. Depending on a photon’s wavelength it can either drive photosynthetic growth, if absorbed by pigments associated with the photosynthetic apparatus, or induce signal transduction. Lastly, the time variance of light plays an important role, where under high frequency fluctuations, photosynthesis occurs at the same rate it would under the equivalent time-
averaged irradiance, due to a mismatch in reaction rates.\textsuperscript{5,31–39} In short, multiple aspects of the light and chemical environment require comprehensive study and there is a growing need for the associated experimental tools.

### 3.2 Multiplexed screening of microalgae

#### 3.2.1 Irradiance screening

Devices capable of multiplexing the light environment have been recently developed.\textsuperscript{1,55–58} These devices multiplex light environments using a diverse set of methods including individual light sources,\textsuperscript{30} fluid handling to act as neutral density filters,\textsuperscript{59,60} liquid crystal displays\textsuperscript{1,2} and recently, photonic cavities\textsuperscript{57} (Figure 3-1). The methods typically involve adapting an optical device to a microfluidic cell culture chamber. The short optical path of the cell culture chamber allows for a relatively constant light intensity through the depth, improving the accuracy of results. In contrast, flask-scale (10-1000 mL) studies of microalgal growth are obscured due to gradients of light intensity stemming from long optical paths, particularly at higher cell densities.

However, none of these techniques provide the ability to simultaneously control the spectrum, irradiance and time variance of the light environment. LED arrays and LCDs provide good control over irradiance and time variance with the ability to adjust the relative intensity of three sections of the spectrum (corresponding to the red, green and blue sections of the visible spectrum). Fluid handling techniques of dyes (Figure 3-1 e and f)\textsuperscript{59,60} can in principle be used to control the spectrum, provided the dye or combination of dyes are available, however these methods are likely complex and expensive to fabricate. Moreover, they are also subject to issues of stability related to maintaining well balanced fluid flow of dyes when making gradients and have a slow response time. For example Kim et al.\textsuperscript{59} designed a device which could switch from light to dark in two minutes at best, which is much too slow to mimic mixing effects. A more robust set of light intensities using dyes was shown by Lou et al.\textsuperscript{60} where channels of various heights are used to create multiple irradiance intensities, the benefit of robustness in this approach comes at the price of flexibility.

The use of plasmonics\textsuperscript{57} provides an academically interesting technique to control local irradiance intensity, but does not have any practical advantages over the use of dyes, LEDs or LCDs. Moreover, coping with the difficulty of characterizing the highly localized light environment in a plasmonic device is likely to be intimidating and not of interest to biologists. One possible exception is those that are interested in illuminating only a portion of a cell. However, this is more easily accomplished adapting current microscopy techniques (e.g. Fluorescence Recovery after photobleaching)\textsuperscript{50,61}. 
Phototaxis experiments have been performed by integrating projectors (specifically spatial light modulators) into the optical path of a microscope. Devices of a similar design may be useful for high throughput heavily controlled screening, by integrating multiple light sources, similar to a laser scanning confocal microscope.

3.2.2 Carbon dioxide screening

Photosynthetic organisms use CO₂ for growth, and thus can serve as a means of carbon capture. However, the CO₂ response of microalgae is complex, due to its role as a source of growth, acidification and the presence of the carbon concentrating mechanics, therefore multiplexing the CO₂ concentration would be of value, particularly in conjunction with the light environment. A key area where multiplexing is lacking is in gas concentrations. Multiplexing the gas environment is distinct from the chemical environment, since the chemical environment can easily be adjusted by pipetting, including the use of automated pipetting systems. In addition, diffusion-based gradient generators have primarily developed for aqueous chemicals, and some gas diffusion devices have also been realized. However, these devices have been limited to under ten levels. A large-scale gas multiplexing apparatus has yet to be demonstrated and would be greatly beneficial.
3.2.3 High Value Products

Optimizing the production of high-value bioproducts is key to strengthening the business case for microalgae cultivation.\textsuperscript{66} In addition, biomanufacturing can be seen as a means to lower the capital cost of chemical synthesis, which can have a disruptive market effect.\textsuperscript{67} Typically, the accumulation of desired bioproducts depends on environmental conditions. Specifically, stressful conditions such as excess light, high salinity, and nutrient stress often lead to the hyperaccumulation of valuable bioproducts. Maximizing productivity is a balance between biomass generation, bioproduct accumulation, and cell viability.\textsuperscript{68,69} These complicating parameters mean that techniques to maximize experimental throughput are essential to mapping out the entire parameter space and identifying optimum productivity conditions. The previously mentioned screening platforms can be used to provide a diverse set of environmental parameters, however the readout is typically limited to cell density, or at best a fluorescent signal associated with lipid production. Unfortunately, the low sample volumes of microfluidic systems present an obstacle since most analytical chemical devices require volumes on the order of milliliters, whereas a microfluidic photobioreactor chamber rarely exceeds 100 nL. This presents an opportunity for improving on-chip label free interrogation such as IR spectroscopy (Raman scattering or absorption) and integrated sample prep possible with centrifugal microfluidics or similar micro total analysis systems.\textsuperscript{70}

3.3 Scaling up knowledge

Transferring the light and chemical information from multiplexed studies to the design of a photobioreactor is more complex than matching the parameters of the reactor to the lab data, due to the heterogeneity of conditions within the reactor - specifically gradients in light intensity and chemical concentration. Our recent work highlights the possible disconnect between small scale information and reactor scale data, by demonstrating that poorly absorbed green light is more effective than well absorbed red light at intensities when running a high density photobioreactor.\textsuperscript{71} The improved performance occurs due to a more uniform light distribution through the reactor. Being more representative of the light environment of a given microalgal cell, data from short optical path reactors (reactors where cells receive a similar irradiance intensity) should be used in conjunction with details regarding the large-scale gradients in full-scale photobioreactors.
Due to the combination of light gradients and mixing, microalgae in a large scale photobioreactor, receive a time varied irradiance. As mentioned earlier, the growth of microalgae is dependent on the time history of the light it receives. (Figure 3-2 a). Detailed design of a photobioreactor would require a model that is not too heavy computationally, and could be used in conjunction with a CFD code to track macroalgal trajectories. To address this, multiple simple two or three state models have been developed (Figure 3-2 b and c). Generally these models simplify the major photosynthetic reactions into a fast set of reaction and a slow set of reactions. Due to the mismatch in rates, there is a buildup of intermediate products to allow growth in the dark, and such dynamic light effects can be captured through suitable models.

The bulk of the models simplify photosynthesis by considering the light harvesting apparatus and the electron transport chain as a single Photosynthetic Unit (PSU), (Figure 3-2 b). Light drives the excitation of the photosynthetic unit, where the excited state corresponds to the cell having an increased concentration of ATP and NADPH. The ATP and NADPH are then used during the deexcitation process which represents the Calvin cycle. Moreover, these models have also captured the effect of photoinhibition. This simplified model has been used to capture growth in flashing light, where at a sufficiently high frequency (>1 Hz) the growth rate can reach the growth rate possible under the equivalent time-average irradiance. A photosynthetic unit can also become
inhibited should it receive an excessively high irradiance for a sustained period. In contrast to the PSU style model, models from Zarmi and colleagues\textsuperscript{48, 79–81} (Figure 3-2 c and d) relate to the mismatch between reactions along the photosynthetic electron transport chain. Though progress has been made in developing useable models, integrating all the relevant timescales associated with photosynthetic growth and induction timescales is required.

### 3.4 Genetic engineering and strain selection

Genetic engineering and strain selection can benefit from the fluid handling and automation associated with microfluidic techniques (Figure 3-3). Digital microfluidics has shown promise in the reduction of labor associated with genetic engineering as well as savings on costly reagents\textsuperscript{82, 83} Strain selection methods have been improved using droplet microfluidics extensively\textsuperscript{84} In addition, recent devices have emerged that leverage correlations between buoyancy and lipid content\textsuperscript{85} as well as phototaxis and photosynthetic growth\textsuperscript{86} Tailoring strain selection and genetic engineering tools to be effective for microalgal screening would accelerate microalgal implementation.

![Figure 3-3](image-url)

Figure 3-3: (a) Strain selection based on improved phototactic response, (b) Gene manipulation using droplet microfluidics. Reprinted with permission from Shih et al.\textsuperscript{83} Copyright 2015 American Chemical Society (c) Selection of high lipid producing yeast using buoyancy, Reprinted from Metabolic Engineering, 29, Leqian Liu, Anny Pan, Caitlin Spofford, Nijia Zhou, Hal S. Alper, An evolutionary metabolic engineering approach for enhancing lipogenesis in Yarrowia lipolytica, 36-45, Copyright 2015, with permission from Elsevier
Chapter 4. Microalgae on display: A microfluidic pixel-based irradiance assay for photosynthetic growth

This chapter presents the development of a microfluidic screening platform for studying the effect of irradiance conditions on microalgal growth. I am the only first author on the publication. This work was published in *Lab on a Chip*¹ and featured on the front cover of Issue 15 in 2015. Additional authors for this work include Jason Riordon and David Sinton. Reproduced from Ref. 1 with permission from The Royal Society of Chemistry.

Abstract

Microalgal biofuel is an emerging sustainable energy resource. Photosynthetic growth is heavily dependent on irradiance, therefore photobioreactor design optimization requires comprehensive screening of irradiance variables, such as intensity, time variance and spectral composition. Here we present a microfluidic irradiance assay which leverages liquid crystal display technology to provide multiplexed screening of irradiance conditions on growth. An array of 238 microreactors are operated in parallel with identical chemical environments. The approach is demonstrated by performing three irradiance assays. The first assay evaluates the effect of intensity on growth, quantifying saturating intensity. The second assay quantifies the influence of time-varied intensity and the threshold frequency for growth. Lastly, the coupled influence of red-blue spectral composition and intensity is assessed. Each multiplexed assay is completed within three days. In contrast, completing the same number of experiments using conventional incubation flasks would require several years. Not only does our approach enable more rapid screening, but the short optical path avoids self-shading issues inherent to flask based systems.

4.1 Introduction

Biofuel technology is garnering significant research interest due to its potential to significantly reduce CO₂ emissions and displace fossil fuels.⁸⁷–⁸⁹ Specifically, photosynthetic organisms are adept at both solar energy capture and CO₂ reduction. Microalgae produce a higher yield per unit area than plant-based biofuels.⁸⁷,⁸⁸ To further improve productivity and land use, microalgae can be cultivated in photobioreactors instead of open ponds,⁹⁰ leading to reduced contamination, better control of culture conditions and most importantly improved light usage. Better light usage can be accomplished by architectures that improve light distribution, such as tubular photobioreactors or waveguide-based photobioreactors.⁹¹,⁹² Furthermore, designs aimed at tailoring the spectral
distribution within the photobioreactor have been proposed, which leverage the high efficiency of light emitting diodes (LEDs),\textsuperscript{93} wavelength selective reflectors\textsuperscript{94} and fluorescent dyes.\textsuperscript{95} A well designed photobioreactor, particularly one leveraging advanced lighting schemes, requires detailed knowledge of microalgal growth. There are a large number of factors that influence microalgal growth, including irradiance parameters such as intensity, frequency, duty cycle and spectral composition, and chemical parameters, such as CO\textsubscript{2} and O\textsubscript{2} concentrations. If one assumes that at least 10 settings per parameter are required, combined screening of these 6 parameters would require a daunting 10\textsuperscript{6} experiments, per microalgal strain of interest.

Irradiance screening is of particular importance since microalgal growth is heavily dependent on light properties,\textsuperscript{6} namely, the intensity, time variance (frequency and duty cycle) and spectral composition. From an intensity perspective, an ideal photobioreactor would operate at a level sufficient to drive growth, but below the saturation point - the point at which additional irradiance does not improve growth rate. At even higher levels, additional light decreases growth rate through photoinhibition.\textsuperscript{29} Maintaining the optimum illumination throughout a photobioreactor is limited by self-shading, where cells closest to the light source receive excess illumination at the detriment of cells deeper within the culture, which are less illuminated.\textsuperscript{80,81,96,97} This effect can be mitigated by continuously mixing, causing cells to travel between high and low intensity regions of the photobioreactor, such that they receive the optimal time-averaged intensity. Bioreactors have shown increased performance with increased mixing due to improved chemical transport and better light utilization.\textsuperscript{48,80,81,96} Another important irradiance parameter is spectral composition. Microalgal growth is dependent on wavelength by virtue of cell pigmentation, which varies largely between species.\textsuperscript{49} Light absorbed by pigments is transferred to neighbouring pigments, drives photosynthetic electron transport or induces signal transduction.\textsuperscript{50–54} In short, the optimal growth conditions of microalgae within a photobioreactor are dependent on several independent irradiance variables.

The current method to screen microalgal growth requires culture flasks fitted with individual light sources, which are monitored over several days.\textsuperscript{59,68} However, the associated cost in performing a large number of these time-intensive experiments is prohibitive,\textsuperscript{59,68} and the quality of the data is limited as light levels vary within the relatively large flasks.\textsuperscript{98} Miniaturized versions of irradiance assays that use multi-well plates and multiple light-emitting diodes have been developed.\textsuperscript{30,68,99} Microfluidic cell culture platforms are particularly well-suited for multiplexed biological assays, where many experiments can be performed in parallel in finely tuned chemical environments.\textsuperscript{100,101}
Recent microfluidic advances directed at algal cultures include microfluidic reactors that study microalgal growth in static droplet arrays and multiplexed chemical environments. Recently, Shih et al. demonstrated how the effect of irradiance conditions on the growth of algae could be studied within a digital microfluidic platform. Eight cultures were studied simultaneously, each grown using a separate LED light source. Further optical multiplexing was achieved by Kim et al. who used microfluidic handling of dyes to filter light from a single source into an array of 64 photobioreactors. Specifically, a microfluidic gradient of dye was used to control irradiance intensity and create light-dark cycles on the order of hours. Collectively, these efforts demonstrate the tremendous potential for microalgal screening with microfluidics. Methods to date, however, cannot provide simultaneous local control over the three critical light variables – intensity, time variance and spectral composition.

Here we report a multiplexed pixel-based irradiance platform, shown in Figure 4-1, capable of rapidly screening the growth response of photosynthetic organisms to irradiance variables. We leverage highly-developed and now ubiquitous liquid crystal display (LCD) technology for local control of all irradiance variables – intensity, time variance and spectral composition – in 238 parallel microreactors. Similarly, we leverage the multiplexing capabilities of microfluidics to

Figure 4-1: Microalgal irradiance assay. a) Schematic of the multiplexed pixel-based irradiance platform, consisting of a PDMS-on-glass cell culture chip, a programmable LCD screen and an LED array backlight. b) Pixels directly below each incubation microreactor are individually controlled to project the desired irradiance. The irradiance intensity, time variance and spectral composition are each tuned based on experimental requirements. The PDMS is illustrated as transparent for clarity; in all experiments it is cast black (opaque) by adding graphite.
provide continuous chemical control during cell incubation. We demonstrate our pixel-based platform’s versatility by performing three irradiance assays targeted at intensity, time variance and spectral composition, on the cyanobacterium *Synechococcus elongatus*.

**4.2 Materials and Methods**

**4.2.1 Microalgal screening platform design and fabrication**

The microalgal screening platform included a microfluidic cell culture chip, an LCD (eDIPTFT32-A, Electronic Assembly, Gilching, Germany) and a backlight (Figure 4-1). The backlight consists of a series of 10 LED strips (VL-H03W5501080D20, Super Bright LEDs, Saint Louis, MO, USA) attached to an aluminum heat sink plate using thermally conductive tape. The LEDs were powered using constant current modules (A009-D-V-1400, Super Bright LEDs, Saint Louis, MO, USA) and a 24 V power supply (RSP-150-24, Mean Well, Fremont, CA, USA). To improve spatial uniformity, the irradiance from the backlight was sent through a diffuser fabricated from Poly(methyl methacrylate) (PMMA). An LCD screen was modified by removing the attached, relatively weak, backlight. The LCD was epoxied to the bottom of a custom PMMA basin, and positioned 75 mm directly above the backlight. A microfluidic cell culture chip was aligned, and fastened directly above the LCD. Chip hydration during experiments was maintained by filling the basin with deionized water, which was continuously cycled using peristaltic pumps (100.070.012.030/4, Williamson Manufacturing Company, East Sussex, UK) at a rate of 15 mL/min through a water bath held at a constant temperature of 30 °C. The entire assembly was wrapped in parafilm for the duration of each experiment, and aliquots of 5 mL were periodically added to top-off the basin as necessary. The irradiance conditions of each individual microreactor were controlled by setting the Red-Green-Blue (RGB) values of the corresponding pixels, as illustrated in Figure 4-1 b. The LCD was interfaced to a computer via USB and programmed using Electronic Assembly LCD Tools software. A photograph of the experimental setup is included in Supplementary Information (Figure S1).

**4.2.2 Microfluidic chip design and fabrication**

Two chip designs were prepared in this work. The first design was a dead-end-filled microreactor array with either a 8 × 15 array or 14 × 20 array of microreactors (Figure 4-1). In practice, a 14 × 17 array (instead of a 14 × 20 array) of microreactors was used because of edge shading issues for a total of 238 microreactors. In this microreactor array design, a serpentine channel connected the chambers of the microreactor in order to continually replenish nutrients and remove waste products, similar to designs presented elsewhere. Our microreactors were 600 μm × 600 μm in size, which exactly matched the dimensions of a 3 × 3 array of pixels. Furthermore, these
chambers were appropriately sized to continually monitor the growth of colonies of (initially) a few hundred cyanobacteria cells over several days.

The second design was a single 28 mm × 45 mm reactor that encompassed most of the LCD screen, and enabled preliminary culturing tests to be performed over a wide area. To prevent the chamber from collapsing, the design featured an array of posts 150 μm in diameter, arranged in a square grid with a pitch of 500 μm. A schematic of both designs is included in Supplemental Information (Figure S2).

Both cell culture chips were fabricated using soft lithography using SU8-2050 (Microchem, Westborough, MA, USA) producing masters 75 μm tall. Poly(dimethylsiloxane) (PDMS) (Silgards 184: Dow Corning, MI, USA) was made opaque by adding graphite at a concentration of 10 wt%. After curing, the PDMS chips were attached to a 1 mm × 40 mm × 60 mm glass microscope slide using a corona treater (BD-20AC, Electro-Technic Products Inc., IL, USA), and bonded overnight.107

4.2.3 Screen Characterization
The irradiance associated with RGB settings was first quantified using a power meter (SKP 200 and SKP 215, Skye Instruments, Llandrindod Wells, UK) placed directly above the LCD screen. In this characterization, all pixels were set to identical RGB values, and the irradiance intensity of the screen as a whole was measured for various RGB values. Similarly, spectral distributions of RGB values were characterized using an integrating sphere (RSA-00-FO, Ocean Optics, Dunedin, FL, USA)
and spectrometer (BRC-112E-USB-VIS/NIR, Edmond Optics Barrington, NJ, USA). This data was later used to precisely control lighting conditions in multiplexed irradiance experiments.

Irradiance temporal uniformity was characterized by recording a sequence of images using an inverted microscope (CKX41, Olympus) with a 20 × objective and a CCD camera (Coolsnap MYO, Photometrics, Tucson, AZ, USA) set at an exposure time of 0.5 ms. As for all LCD screens, pixel RGB values are controlled by periodically applying a voltage at each pixel coordinate. The pixel state will relax between voltage cycles, causing a small drift in intensity. In our case, as neighbouring pixels were operating on offset voltage cycles, this effect was mitigated and led to a temporal fluctuation of 8 % at an RGB value of 155 within the microreactors at a rate of roughly 60 Hz. This fluctuation is dependent on voltage settings. Full data relating to temporal uniformity is presented as Supplementary Information (Figure S3).

Irradiance spatial uniformity was characterized across the entire multiplexed irradiance chip using a 2.5 × objective. The variability was found to be less than 10 % of the mean value. The irradiance spatial variability within individual microreactors was characterized using a 40 × objective, and the standard deviation was found to be less than 12 % of the mean. Data relating to spatial uniformity is presented as Supplementary Information (Figure S4 and Figure S5). Lastly, light leakage from adjacent reactors was found to be insignificant (Figure S6).

### 4.2.4 Cell proliferation measurements

Bacterial growth inside each microreactor was characterized using a Leica DMI6000B inverted fluorescent microscope (Leica, Heerbrugg Switzerland) with a 10 X objective (PL Fluotar, Leica) and a TX2 filtercube (Ex 560/40, Di 595, Em 630/30). This spectral range coincides with the fluorescence of Phycocyanin, a major pigment within *S. Elongatus*, which allowed high-contrast fluorescent imaging of cell proliferation. The average fluorescence intensity of each microreactor was quantified using MATLAB software. Each image was corrected using a dark and light reference. In the case of the design featuring a single large reactor, a Nikon A1 confocal microscope was used with a 4 × objective. A 14 × 20 grid of images was stitched together using NIS Elements software for full chip imaging.

Lighting conditions were observed as having minimal effect on fluorescence emission (Figure S7). Thus, the total fluorescence intensity of a microreactor as a whole was taken to be proportional to the number of cells within the microreactor. Assessing cell growth using total fluorescence is preferred over metrics such as are coverage and cell number, since clearly defining cell boundaries
at high cell densities is not practical. This relationship allowed the time-evolution of fluorescence signal to be taken as an approximation of cell growth.

4.2.5 Microfluidic Cell Culture

*Synechococcus elongatus* PCC7942 T2SEΩ was provided by Professor Rakefet Schwarz (Bar-Ilan University, Israel). This microalgal strain is biofilm-forming, non-motile and resistant to Kanamycin.¹¹⁰ *S. elongatus* was chosen since it is well studied, fully sequenced and readily genetically modified for generating diverse bioproducts.¹¹¹–¹¹³ Recent genetic modifications have allowed for improved carbon capture, ethanol production and isobutanol production.¹¹¹–¹¹³ From a practical standpoint, this modified strain made experimental procedures simpler, since it is antibiotic resistant and non-motile. Cells were cultured to an optical density of 0.15 over several days in BG-11 media supplemented with 50 μg/mL of the antibiotic Kanamycin within an incubator operating at 30 °C.

For experiments featuring the multiplexed irradiance design the chip was initially filled by immersion into a 100 mL cell suspension and applying a vacuum of -80 kPa (gauge pressure) for 20 min. The chip was then immersed in deionized water bath at 30 °C while the main serpentine channel was rinsed with BG 11 media at 20 μL/min for 2 hrs. The chip was imaged as previously described, and then mounted to the LCD screen. Alignment between the cell culture chip and LCD screen was facilitated by a set of three 1 mm diameter through holes in the PDMS and three corresponding markers on the display. Microreactors have an area equivalent to a 3 × 3 pixel grid, and were illuminated by a 7 × 7 pixel grid. This larger illumination grid provided a good alignment tolerance (400 μm tolerance) and mitigated edge lighting effects. While each microreactor was centered within the corresponding illuminated pixel grid, the boundaries of the microreactors did not necessarily align to the boundaries of the inner 3 × 3 pixel grid. During cell culture incubation, fresh media was flown through the chip at a rate of 20 μL/min.

In the case of the single large reactor chip experiment, media was loaded into the chip before injecting the microalgal suspension. Both the media and microalgal cell suspension were injected at a rate of 10 μL/min for over 30 minutes. Inlet and outlet ports were plugged. The chip was fixed onto the screen as described previously.

4.3 Results and Discussion

To demonstrate spatial control of microalgal growth using the LCD platform, *S. elongatus* was grown for 3 days within a single microfluidic chamber, with the screen displaying the University of Toronto
Figure 4-3: Effect of irradiance intensity on cell growth. a) Typical fluorescence images of microalgal proliferation over 3 days of growth for all fifteen irradiance conditions. Images are inverted for clarity. b) Time evolution of average fluorescence intensity over 3 days of growth for 5 different irradiance conditions, normalized to initial fluorescence, average value shown for clarity, error bar represents standard deviation of eight replicates. c) Average fluorescence of all microreactors after 1, 2 and 3 days of growth, normalized to initial fluorescence. d) Photosynthetic growth rate as a function of irradiance based on changes in fluorescence over 1 and 2 days. Scale bar is 500 µm.

crest, as shown in Figure 4-2. The microfluidic chip is described in Materials and Methods and detailed schematics can be found in Supplemental Figure 4-2 d and e. The white and black sections correspond to irradiance intensities of 147 µmol·m⁻²·s⁻¹ and 5 µmol·m⁻²·s⁻¹, respectively (values bracketing the productive light intensity range of this organism). The platform induced growth within the lit areas, resulting in a grown replica. The similarity between both images highlights the large area, high resolution, control provided in this approach. The blurring occurring at some edges as well as the loss of some smaller features are attributed to cells being displaced by fluid flow over three days within the very large aspect ratio microfluidic chamber used in this case (an issue resolved by using isolated microreactors as below). Notably, the lighting approach presented here is lens free, requiring no expensive optical system, in contrast to platforms which involve integrating a projector into a microscope.
4.3.1 Effect of light intensity on cell growth

Irradiance intensity is the raw amount of energy supplied to a photosynthetic cell. Quantifying the effect of irradiance intensity on growth rate is fundamental to photobioreactor design, particularly since irradiance has a non-linear effect on growth rate. The effect of irradiance intensity on cell growth was quantified using a multiplexed reactor chip featuring an array of 120 microreactor chambers. Dead-end compartmentalization within the microreactors shielded cells from flow, and provided a constant nutrient supply through diffusion from the main channel. Figure 4-3 a shows representative fluorescence microscopy images of microalgae cells over three days for 15 irradiance intensities, each replicated 8 times, for a total of 120 simultaneous growth experiments. The full chip was imaged daily, over three days. Uniform loading of the chambers was confirmed after loading, and no substantial spatial bias in cell distribution was observed over time.

The intensity assay growth results are quantified in Figure 4-3 b where the average fluorescence of all eight replicates is plotted over time for the five (of 15) irradiance conditions shown in Figure 4-3 a (imaging details in Methods). At the lowest intensity, 5 µmol·m⁻²·s⁻¹, the cell density did not increase significantly during incubation, indicating little or no growth under these ultra-low light conditions, seen in both fluorescence images in Figure 4-3 a and quantified in Figure 4-3 b and c. Over the first two days, higher irradiance increases cell growth with a decreasing marginal return, as observed in comparing the growth within microreactors irradiated with 23 µmol·m⁻²·s⁻¹, 50 µmol·m⁻²·s⁻¹ and 148 µmol·m⁻²·s⁻¹. Cells that were cultured at an irradiance of 23 µmol·m⁻²·s⁻¹ increased in concentration by a factor of 2.8 ± 0.6 over two days. In comparison, microalgae irradiated with approximately double the light intensity, 50 µmol·m⁻²·s⁻¹, were approximately twice as concentrated (6.5 ± 0.8) after two days of growth. However, a further increase in irradiance to 148 µmol·m⁻²·s⁻¹ showed only a 36% increase in cell concentration (8.8 ± 2.2), indicating that additional irradiance did not contribute significantly to further cell growth. This decreasing marginal return is further shown in Figure 4-3 c, which plots the increase in fluorescence signal for all 120 experiments after one, two and three days fitted to exponential functions. Figure 4-3 c further highlights how microalgal growth responds little to incremental irradiance increases above 50 µmol·m⁻²·s⁻¹.

The common benchmark in assessing the effect of irradiance on photosynthetic productivity is the photosynthesis-irradiance (PI) curve. Photosynthesis-irradiance can be quantified using a variety of techniques, including measuring the oxygen production rate, measuring the inorganic carbon consumption rate, tracking radioactively labelled carbon or measuring the photosynthetic
electron transport using fluorimetry. Figure 4-3 shows photosynthesis-irradiance measured over one and two days of growth, as quantified through the relative fluorescence increase of each microreactor. In accordance with common practices, our data is fitted to the exponential function:

\[ u = u_{\text{max}} \left( 1 - e^{-\frac{\alpha I}{u_{\text{max}}}} \right) \]

where \( u \) is the growth rate, \( u_{\text{max}} \) the maximum growth rate, \( \alpha \) the maximum quantum photosynthetic yield and \( I \), the irradiance intensity. An \( R^2 \) value of 0.86 confirms the strong fit.

The maximum growth rate was \( 1.19 \pm 0.02 \text{ day}^{-1} \), well within the range of 1-3 day\(^{-1} \) reported in literature. The most comparable conditions to our case were recently used by Kuan et al. who reported a growth rate of 1.25 day\(^{-1} \) for an irradiance of 120 \( \mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1} \) at a temperature of 33 °C and an atmospheric CO\(_2\) concentration. Achieving higher growth rates was achieved in other works using elevated CO\(_2\) concentrations.
The onset of saturation, defined as the ratio between maximum rate of photosynthesis and maximum quantum photosynthetic yield (0.029 ± 0.001), was 41 ± 2 µmol·m⁻²·s⁻¹. This saturation onset is lower than previously reported values for flask-based studies, which range from 50 µmol·m⁻²·s⁻¹ to 90 µmol·m⁻²·s⁻¹, based on either oxygen evolution, electron transport or carbon uptake. The discrepancy between our microfluidic method and bulk flask-based studies is likely the result of self-shading in flasks. For example, if the incident light on a bulk culture is sufficient to saturate growth, only the near-surface cells will be saturated, and further irradiance will promote growth of cells deeper in the culture, thus increasing the perceived saturation irradiance in flask-based studies. Therefore, our platform presents an opportunity to precisely control irradiance intensity at the cellular level for entire microreactors, since the thickness of the reactor (75 µm) is three orders of magnitude less than regular flasks. Furthermore, microalgae grow mostly as a monolayer, or in cases of prolonged culture at high irradiances as roughly three layers, thus providing effectively no self-shading. In addition, our chip provides a two orders of magnitude improvement in parallelization over bulk approaches, allowing for a finer detailed study of the role of irradiance intensity on photosynthetic growth.

4.3.2 Effect of time-varied irradiance on cell growth

Studying the effect of time-varied irradiance on photosynthetic growth informs the amount of mixing required in a photobioreactor to optimally utilize incident light. As a reactor is mixed, cells travel throughout the reactor from darker regions to brighter regions, experiencing a time-varied irradiance. The effect of time-varied irradiance, specifically frequency and duty cycle, was evaluated here using our multiplexed platform. In total, 120 separate experiments were performed simultaneously. Figure 4-4 shows how cell growth is influenced by both irradiance frequency and duty cycle, with total irradiance kept constant. In all cases, microreactors received an identical time-averaged intensity of 40 µmol·m⁻²·s⁻¹. In time-varied cases, lighting oscillated stepwise between 147 µmol·m⁻²·s⁻¹ and 5 µmol·m⁻²·s⁻¹ with a 25% duty cycle, or between 76 µmol·m⁻²·s⁻¹ and 5 µmol·m⁻²·s⁻¹ with a 50% duty cycle. Frequencies were varied between 0.025 Hz and 5 Hz. Also shown in Figure 4-4 is the growth rate measured under continuous illumination at 40 µmol·m⁻²·s⁻¹.

As shown in Figure 4-4, every 50% duty cycle case maintains a higher growth rate than the corresponding 25% duty cycle cases of the same frequency, and the difference becomes larger at lower frequencies. This result is expected based on the nonlinearity observed between irradiance intensity and cell growth, as observed in Figure 4-3. Specifically, the peak irradiance values exceed
saturation, thus increased irradiance intensity has minimal impact on growth in comparison to an increased duty cycle.

Frequency was also found to have a strong impact on growth. Growth rates at frequencies above 0.5 Hz were found to match that of the continuous irradiance experiment (0.7 day\(^{-1}\)). Light reactions, which include charge separation, photosynthetic electron transport and formation of ATP and NADPH, occur more rapidly than dark reactions, namely CO\(_2\) reduction by the Calvin cycle.\(^{38,30,96}\) Due to this mismatch between rates, dark periods allow for excess products from the rapid light reactions to be consumed by the slower dark reactions. Effectively, cells are incubated within a regime where growth rate is dictated by the time-averaged irradiance. At frequencies lower than 0.5 Hz, growth rate decreases. This can be attributed to lost cell productivity during the latter stage of the dark periods, where products from the light reaction are depleted. In addition, at lower frequencies, prolonged exposure to high irradiance causes accumulation of reactive oxygen species, which are a source of oxidative stress on organisms.\(^{96}\) This effect was likely present at very low frequencies, such as at a frequency of 0.025 Hz, where growth was minimal. These results suggest that mixing within a photobioreactor should occur at a frequency above 0.5 Hz for optimal productivity.

Our growth vs irradiance frequency results are in keeping with previous studies which have examined time variance by flickering incident light on a bulk culture.\(^{33-35}\) However, in bulk experiments, self-shading within shaken incubation flasks leads to additional intensity fluctuations (i.e. combined mixing and flickering frequency) for individual cells. An individual cell will thus experience light fluctuations on two time scales, obfuscating specific frequency-based growth effects. In our microfluidic chip, all cells within a given microreactor are exposed to identical irradiance conditions. We postulate that the growth vs frequency results shown in Figure 4-3 are more accurate than previous bulk studies, since self-shading is not an issue.

**4.3.3 Effect of spectral composition on cell growth**

The irradiance spectral composition affects photosynthetic organisms differently since cell pigmentation, which controls light absorption, varies between species.\(^{49,54}\) Further, emergent artificial lighting strategies (LEDs\(^ {93}\), wavelength selective reflectors\(^ {94}\), and fluorescent dyes\(^ {95}\)) require spectral screening to inform photobioreactor design. The role of spectral composition on growth was studied by varying the red hue for 6 irradiance intensities ranging from 12 µmol·m\(^{-2}\)·s\(^{-1}\) to 42 µmol·m\(^{-2}\)·s\(^{-1}\). Hue is defined as the ratio of spectral contributions from wavelengths above 575 nm (red) to that of the full spectrum (red + blue). These spectra were obtained by adjusting
red and blue levels of the RGB settings on the LCD screen, while keeping the green level at zero. Details regarding spectra are included in Supplementary Information (Figure S8).

Figure 4-5a shows how this variation in spectral composition and intensity impacts cell growth, as measured through the relative increase in cell fluorescence after three days of growth. Figure 4-5 covers a total of 222 total experiments, with 5-7 duplicate experiments being performed for each irradiance condition. A second order polynomial is fit through the data set, with a strong correlation coefficient \(R^2 = 0.91\). Figure 4-5a shows that cell growth is increased with both higher irradiance intensity and higher red hue. This general trend is consistent with results from other studies,\textsuperscript{54,124} and can be attributed to the absorption of red light by photosynthetic antennae complexes, known as phycobilisomes. The phycobilisomes capture and distribute light energy to photosynthetic reaction centers.\textsuperscript{125,126}

Figure 4-5b shows a subset of the data in Figure 4-5a to further quantify how cell growth responds more strongly to red light than blue light. Fit lines were added to highlight the role of increasing red hue for various intensities. As irradiance increases from 12 \(\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\) to 42 \(\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\), the slopes of these curves increased from 0.0035 \((R^2 = 0.073)\) to 0.022 \((R^2 = 0.77)\), respectively. This result suggests that irradiance intensity influences the role of spectral composition. Interestingly, \textit{S. elongatus} effectively demonstrate a stronger preference for red light over blue light at higher
intensities, an effect uniquely accessible using our highly multiplexed approach.

4.4 Conclusions

In summary, we developed a multiplexed pixel-based irradiance platform, capable of rapidly screening the effect of various illumination conditions on cell growth. Our platform combines the inherent multiplexed optical control of LCDs with microfluidic cell handling and chemical control. Over 200 experiments in an area of approximately 24 cm² were performed simultaneously, yielding roughly 10 experiments per square centimeter. The effect of three major irradiance parameters on microalgae growth were studied: intensity, time variance and spectral composition. All three of these assays informed photobioreactor design and culturing operations: quantification of the saturation intensity; quantification of the threshold frequency for growth; and elucidating the mixed effect of spectral composition and irradiance intensity. Furthermore, all cells within each microreactor were grown under effectively identical irradiance conditions, avoiding the issues of self-shading light attenuation inherent to bulk flask-based cultures. The platform is well suited to assess the role of irradiation parameters on other key metrics of biofuel production such as intracellular lipid accumulation. Such studies would likely necessitate alternative materials, since the lipophilic probes typically used are readily absorbed by PDMS.¹²⁷,¹²⁸

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4.5 Supplementary Information

Microalgae on display: A microfluidic pixel-based irradiance assay for photosynthetic growth

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Figure S1: Experimental setup for microfluidic pixel-based irradiance platform. A transparent PDMS chip filled with dye is used here for clarity (all chips used in experiments are opaque).
Figure S2: Detailed schematics of microfluidic chips used in this work. a) Large format 14 x 20 microreactor array. b) Small format 8 x 15 microreactor array. Both microreactor style chips have three alignment markers. c) Key dimensions of microreactors used for array designs. d) Post array chip, used to produce the University of Toronto crest replica. e) Enlarged view of post array chip, showing a comb structure used to homogenize flow and aid in uniform cell seeding.
Figure S3: Temporal uniformity of irradiance within an area of a microreactor corresponding to the size of a pixel for different pixel levels of (a) red, (b) green or (c) blue. The error bars represent min-max values of the 60Hz light signal.

Figure S4: Spatial uniformity of irradiance across a microreactor. Intensity within the microreactor is plotted as a function of position, where the positive direction in the plot corresponds to downwards along the microreactor, as shown in the inset. Lighting conditions correspond to a blue pixel level of 255, with red and green at a level of 0. The plot is obtained by combining intensity measurements from 8 images spanning the height of the microreactor. The standard deviation between the 1000 and 5000 pixel positions is 12% of the mean.
Figure S5: Spatial uniformity of irradiance across the large format 14 x 20 microreactor array. a) The 14 x 20 microreactor array, with row and column numbering. b) Relative intensity along columns 1, 4 and 10.

Figure S6: Light Leakage between adjacent microreactors, characterised by placing a photomask against the LCD. a) Microscope image of two adjacent microreactors both set to and RGB value of (0,0,0). b) Microscope image of two adjacent microreactors, where the left reactor is set to (255,255,255) and the right reactor is set to (0,0,0). c) Intensity plot corresponding to a) and b), the inset highlights the intensity in the reactor kept at (0,0,0) for both cases.
Figure S7: Fluorescence signal measured across microalgal cells after two days growth, for three different incubation irradiance intensities, presented as a box and whisker plot. Fluorescence intensity was measured across the width of 25-35 microalgal cells for each irradiance. The box encompasses data from the 25th to 75th percentile and the whiskers capture the 10th and 90th percentiles. The average value is denoted by an X. Measured fluorescence intensity does not change significantly based on incubation irradiance conditions.
Figure S8: Sample irradiance spectra of LCD screen operating under red, blue and purple settings. Corresponding RGB values are indicated in the legend.
Chapter 5. A penalty on photosynthetic growth in fluctuating light

This chapter presents a frequency dependent penalty on photosynthetic growth, which is caused by regulatory mechanisms of photosynthesis. The manuscript has been submitted to *Scientific Reports* (SREP-17-21855). I am the sole first author on the publication. Additional authors for this work include, Brian Nguyen, Thomas Burdyny, David Sinton.

**Abstract**

Fluctuating light is the norm for photosynthetic organisms, with a wide range of frequencies (0.00001 to 10 Hz) owing to diurnal cycles, cloud cover, canopy shifting and mixing; with broad implications for climate change, agriculture and bioproduct production. Photosynthetic growth in fluctuating light is generally considered to improve with increasing fluctuation frequency. Here we demonstrate that the regulation of photosynthesis imposes a penalty on growth in fluctuating light for frequencies in the range of 0.01 to 0.1 Hz. We provide a comprehensive sweep of frequencies and duty cycles. In addition, we develop a 2nd order model that identifies the source of the penalty to be the regulation of the Calvin cycle – present at all frequencies but compensated at high frequencies by slow kinetics of RuBisCO.

**5.1 Introduction**

In nature, solar flux is discontinuous, fluctuating due to the diurnal cycle, varying cloud cover, canopy shifting and circulation in bodies of water, photosynthetic organisms are therefore exposed to a diverse set of fluctuating light profiles ranging from 0.00001 to 10 Hz. Terrestrial plants, typically experience fluctuations on the order of 0.001 to 1 Hz due to canopy shifting and varying cloud cover. In the case of aquatic microorganisms, cyanobacteria and eukaryotic algae, fluctuating irradiance is driven by a combination of mixing and spatial light heterogeneity. Specifically, light is most intense at the surface of a body of water, then decreases with depth due to turbidity. Mixing causes cells to travel upwards from areas of low light towards the photic zone, where light is sufficient to drive growth. In estuaries the resulting frequency is on the order of 0.02 to 0.0002 Hz. In contrast marine organisms may experience much slower variations, where crossing from the dark region to the photic zone may take days.

Since light drives primary productivity, the ability of photosynthetic organisms to capture fluctuating light, will impact its ability to fulfill its ecological role. As such, environmental
parameters such as wind, circulation due to temperature gradients and turbidity can impact primary productivity. In terms of industrial applications, these fluctuating irradiance conditions are common in agriculture, as well as in photobioreactors. In agriculture, fluctuating light occurs due to variations in cloud cover and plant displacement. Fluctuating light in photobioreactors is due to mixing in and out of the photic zone, similar in nature to shallow estuaries. Depending on reactor geometry, mixing can induce a variety of fluctuating light profiles, with frequencies ranging from 0.05-5 Hz, where frequencies over 1 Hz can improve reactor performance.39,46,48,29,81,89,133–135

Photosynthetic growth in fluctuating light is generally considered to be bound by two extremes, a high frequency limit where the growth rate is dictated by the time-averaged light intensity (full-light-integration), and a low frequency limit where photosynthetic growth is equivalent to sum of the growth rate in each light condition (no-light-integration).31–37 At high frequencies (>1 Hz), photosynthetic growth occurs during both the light and the dark phase. High frequency fluctuations allow for continuous growth through a mismatch of reaction rates, specifically rapid light harvesting reactions and slower enzymatic reactions.5,38,39 The mismatch between rates causes an accumulation of intermediate products during the light phase which can then be consumed during the dark phase, allowing for nearly continuous growth. As frequency decreases, the fraction of the dark period where growth continues decreases, and the resulting growth rate decays to the low frequency (no-light-integration) case, where there is no flashing light benefit. Specifically, the mismatch is between (i) the rapid light-driven generation of ATP and NADPH by the photosynthetic electron transport chain and (ii) the slower Calvin cycle. 5,38,39,72 In the case of very high frequencies, (> 100 Hz), mismatches along the photosynthetic electron transport chain can also contribute to the flashing light effect.81,136,137

Motivated by the complexity and importance of photosynthetic growth under fluctuating light, a variety of models have been proposed, all of which predict growth rates between the established high and low frequency bounds.31–38,77,138 These models range in complexity, but all contain a light dependent excitation step (capturing the formation of ATP and NADPH), and a light independent step (capturing the consumption of ATP and NADPH by the Calvin cycle), which allow them to capture the behaviour between the high and low frequency bounds. However, photosynthesis is a tightly regulated process which does not respond instantaneously to changing irradiance levels,19,129,139–144 suggesting dynamics outside the high and low frequency bounds are likely.
Here we demonstrate a flashing light penalty where the photosynthetic growth is below the low frequency limit associated with no-light-integration. Specifically, growth is severely hampered for frequencies in the range of approximately 0.1 to 0.01 Hz, an effect not captured with current models. The penalty presented here is heavily dependent on duty cycle: small duty cycles have a substantial penalty, whereas large duty cycles are shown to have nearly no penalty. This penalty was present under photoautotrophic growth for both *Synechococcus elongatus* and *Chlamydomonas reinhardtii*. We attribute this penalty to the regulation of Calvin cycle by irradiance, where after a sufficiently long period in the dark, the Calvin cycle is inactive. When exposed to light the Calvin cycle is activated, albeit with a time lag that is significant at frequencies in the range 0.1 to 0.01 Hz – a range that is germane to natural, agricultural and industrial systems. By expanding an existing mechanistic model, we demonstrate that the partial activation state of the Calvin cycle is responsible for the frequency dependent penalty affecting photosynthetic organisms.

### 5.2 Results and Discussion

We investigate the effect of fluctuating light on photosynthetic growth through a combined experimental and analytical approach. Specifically, we investigate the effects of frequency (light-dark cycles per second) and duty cycle (fraction of a cycle where the light is at maximum intensity) on photosynthetic growth. Two organisms were chosen, *Synechococcus elongatus* and *Chlamydomonas reinhardtii*. *Synechococcus elongatus* is frequently used in biotechnology since it can be transformed for chemical production or increased growth. Moreover the *Synechococcus* genus is present in a wide range of marine, freshwater and terrestrial environments, across a broad temperature range, thus an important contributor to primary productivity. *Chlamydomonas reinhardtii* is a model organism where mutated strains are readily available and it typically resides in freshwater and soils. In addition, *Chlamydomonas reinhardtii*, as a mixotroph, is capable of heterotrophic, photoheterotrophic and photoautotrophic growth. To sweep the large parameter space associated with the entire frequency spectrum relevant to photosynthetic growth and distinct duty cycles we used projection to provide independent control of light conditions in each chamber of a 384-well plate (Figure 5-1 a), with full details included in methods.
Photosynthetic growth is composed of two major sets of reactions, the light dependent reactions and the light independent reactions. The light dependent reactions include the generation of ATP and NADPH from light and oxygen evolution. These reactions occur along a set of membrane bound proteins, which capture light energy and use it to split water, evolve oxygen and make ATP and NADPH. The light independent reactions use the products from the light dependent reaction (ATP and NADPH) to reduce carbon via the Calvin cycle.

To gain further insight into the behaviour of photosynthetic growth in fluctuating light, we developed a mathematical model which accounts for the regulation of the Calvin cycle by irradiance (Figure 5-1 b). Specifically, we adapted the model presented by Camacho Rubio et al. to include the effect of irradiance on the degree of activation of the Calvin cycle. The degree of activation of the
Calvin Cycle represents the instantaneous reaction rate relative to the rate that would be obtained under continuous irradiance. Both the RuBP regeneration and RuBisCO activation contribute to the degree of activation of the Calvin Cycle. In the context of the model, the degree of activation of the Calvin cycle controls the rate at which activated photosynthetic units are converted into biomass and light harvesting systems are returned to their resting state. This regulation of the reaction aligns with the role of the Calvin cycle, specifically, using energy captured by the photosynthetic electron transport to reduce carbon. The full details of the model and MATLAB scripts are included as supplementary information.

In Figure 5-2 both experimental and modelling results are presented, showing the effect of frequency and duty cycle for square wave light profiles fluctuating between an irradiance of 215 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) and \( \approx 0 \ \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \). The frequency ranges from 0.00001 to 1 Hz for duty cycles of 0.25 (b), 0.375 (c), 0.5 (d), 0.625 (e) and 0.75 (f). The growth vs irradiance curve obtained under continuous irradiance (Figure 5-2 a), enables interpretation of the effect of flashing light frequency, by (i) providing the growth rate corresponding to the time averaged intensities for the five flashing light cases, (ii) demonstrating that the irradiance chosen is saturating and (iii) providing data to extract parameters to be used in the model. The growth-irradiance curve shows a saturating relationship where growth increases with irradiance up until an intensity of 150 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \), with the maximum growth rate aligning with literature values.\(^{1,115,121}\) The growth under continuous light was obtained on the same well plate as all of the fluctuating cases. Growth curves are included as supplemental information, to demonstrate continued exponential growth over the two days (Supplemental Figure 5-2).
The Calvin cycle imposes a penalty on photosynthetic growth in fluctuating light. Each frequency response plot (Figure 5-2 b-f) is annotated with two reference lines, (i) growth with full-light-integration, where growth would be dictated by the time-average irradiance (dashed lines in Figure 5-2 b-f) and (ii) growth with no-light-integration, where growth is equivalent to the sum of the
growth rate in each light condition (dotted lines in Figure 5-2b-f). At the extreme frequencies, our data for all duty cycles agrees with literature data. Specifically, at high frequencies, growth approaches the full-light-integration limit. Likewise at low frequencies, growth approaches the no-light-integration limit with decreasing frequency. \(^{38,39}\) However, our data shows that at frequencies
ranging from 0.01 to 0.1 Hz, growth is significantly lower (two-sided t-test, $P < 10^{-5}$ for duty cycles of 0.25 and 0.375) than the low frequency case – indicating a penalty. Specifically, the minimum growth rate is as low as 50 percent of the no-light-integration case for the 0.25 duty cycle.

The presence of a penalty suggests behaviour outside the mismatch between photochemical and enzymatic reactions. We hypothesize that this penalty is caused by the regulation of the Calvin cycle by light,$^{19,24}$ which is strongly supported by the agreement between our model and experimental data. The parameters used in the model align with the range of rate constants presented in literature for both the activation and deactivation rates of (i) RuBP regeneration ($k_{cal1on} = 0.05$, $k_{cal1off} = 0.025$) and (ii) RuBisCO activation ($k_{cal2on} = 0.001$, $k_{cal2off} = 0.0001$),$^{14,19,22-28,150-152}$ justifying the role of Calvin cycle regulation in the observed penalty. A full discussion regarding the parameters employed here is provided as supplemental information.

The severity of the penalty decreases with increasing duty cycle (Figure 5-3). Specifically, the minimum growth rate is approximately 50, 60, 70, 80 and 90 percent of the no-light-integration case, for duty cycles of 0.25, 0.375, 0.5, 0.625 and 0.75, respectively. In addition, results from the

![Figure 5-3: Effect of duty cycle on relative minimum growth rate. The minimum growth rate is expressed as a percentage of the no-light-integration growth rate. The data represents the mean and standard deviation of three well plates each with four technical replicates per condition, for a total of twelve data points, except for the 0.625 duty cycle case, which is limited to two well plates of four replicates.](image)
model are included to extend the range of duty cycles studied. The model trend is non-linear, where the penalty becomes increasingly severe with decreasing duty cycle.

Mixotrophy tempers the fluctuating light penalty. In addition to photoautotrophic growth, where organisms use light energy to reduce CO₂ into organic forms, some photosynthetic organisms can grow photoheterotrophically, where light is used in conjunction with an organic carbon source, or heterotrophically where an organic carbon source is used as energy. *Chlamydomonas reinhardtii* wild type was used since it can grow mixotrophically, having the ability to choose between the three previously mentioned growth modes. To study the effect of photoheterotrophy on the fluctuating light penalty, we grew *Chlamydomonas* in Sueoka's high salt medium with (HSA) and without acetate (HS). In the presence of acetate (mixotrophic), the penalty is substantially decreased for a 0.25 duty cycle (Figure 5-4), where the growth rate is 20% the no-light-integration case under photoautotrophic growth and 85% under photoheterotrophic growth ($P \approx 10^{-5}$). In comparison with *Synechococcus* (Figure 5-3), *Chlamydomonas* exhibits a slightly more severe penalty for a 0.25 duty cycle, but a comparable penalty at larger duty cycles.

![Figure 5-4: Effect of mixotrophic growth on the relative minimum growth rate for the organism Chlamydomonas Reinhardtii. The minimum growth rate is expressed as a percentage of the no-light-integration growth rate. Data represents the mean and standard deviation of two experiments, each with four technical replicates.](image-url)
To gain insight into the transient behaviour of photosynthesis in fluctuating light, we apply the model to track the instantaneous growth (Figure 5-5 a, b, c), the RuBP regeneration activation factor (d, e, f) and the RuBisCO activation factor (g, h, i) over one period. These activations factors represent the relative activity of the two light regulated aspects of the Calvin cycle (RuBP

Figure 5-5: Effect of duty cycle and frequency on the transient behaviour of photosynthetic growth, based on mathematical modeling. Instantaneous growth rates (a, b, c), RuBP regeneration activation factor (d, e, f) and RuBisCO activation factor (g, h, i) and for duty cycles of 0.25 (a, d, g), 0.5 (b, e, h), and 0.75 (c, f, i), for frequencies matching continuous light, frequency which incurred a penalty and low frequency matching the growth integration.
regeneration and RuBisCO activity). In continuous darkness, the factors are 0, and under continuous light the factors are given by $k_{on}/(k_{on} + k_{off})$. These variables are shown for duty cycles of 0.25, 0.5 and 0.75 at high frequency, where growth is above the no-light-integration case, moderate frequency where growth is below the no-light-integration case and at low frequency, where growth matches the no-light-integration case. The response for instantaneous growth and RuBP regeneration follow a square wave for the lowest frequency, since the rates are multiple orders of magnitude faster than the fluctuation frequency. Similarly, the RuBisCO activation factor increases nearly instantaneously in the light phase, then gradually deactivates in the dark phase.

For intermediate frequencies, corresponding to cases below the growth integration line, the instantaneous growth does not reach the growth achieved under the low frequency case, thus for the bulk of the light phase photosynthetic growth is impaired, to some degree. The decreased instantaneous growth rate is caused by the regulation of the Calvin cycle, particularly RuBP regeneration, being partially active for most, if not all, of the period (Figure 5-5 d-e-f).

Similar to the case of the intermediate frequencies, under high frequency flashing light, the instantaneous growth rate does not reach the growth achieved under peak irradiance value. However, the presence of growth during the dark phase compensates for the slower growth during the light phase, allowing for full-light-integration. Both the RuBisCO activation factor at frequencies of 0.025 and 2 Hz and the RuBP regeneration at frequencies of 2 Hz are relatively constant over a period, since the light fluctuations are over two orders of magnitude faster than the rate constants. However, the activation factors are below the equilibrium activation factors (0.666 for RuBP regeneration and 0.91 for RuBisCO activation, ( Figure 5-5 - d-e-f-g-h-i blue lines corresponding to 0.00001 Hz)), suggesting that the activation kinetics still impose a penalty at higher frequencies.

The mismatch between reaction rates compensates for the regulation of the Calvin cycle at high frequencies. The partial activation state of the Calvin cycle at high frequencies motivates reconsidering the role of the conventional flashing light effect, i.e. growth bound between a high and a low frequency limit. In Figure 5-6a, the full model presented here is deconstructed into two special cases: a penalty-free case and a penalty-only case, for a 0.25 duty cycle. The penalty-free case shown is the conventional two-state model which assumes that the Calvin cycle responds instantaneously to a change in irradiance. In contrast, the penalty-only case assumes that there is no mismatch in reaction rates and by extension no growth during the dark phase, but includes the lag times for the Calvin cycle.
The penalty-free case predicts a higher growth rate than both the experimental data and the complete model for all frequencies. This discrepancy suggests that the regulation of the Calvin cycle decreases growth for all frequencies above a certain threshold, or in other words acts like a low-pass filter. Moreover, the penalty-only case shows decreasing growth with increasing frequency, affirming that growth is increasingly penalized with increasing light fluctuation frequency. The full model generally follows the penalty-only case until a frequency of roughly 0.01 Hz, where the full model begins to increase. Similarly, the penalty-free case begins to increase above the no-light-integration line at 0.01 Hz. This comparison between the full model and special cases (Figure 5-6a) indicates that the slow kinetics of RuBisCO compensates for the light dependent regulation of the Calvin cycle at high frequencies.

RuBP regeneration regulation is the dominant source of the penalty. The relative contributions of RuBisCO regulation and RuBP regeneration are shown by comparing the full model to the special cases which are penalized by either RuBisCO activation or RuBP regeneration, as opposed to both (Figure 5-6b). The 0.25 duty cycle is shown as an example, since it is the case where the penalty is strongest. RuBisCO activation is the dominant source of the penalty for frequencies below 0.004 Hz.

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**Figure 5-6:** Relative contributions of (i) the conventional flashing light effect, (ii) RuBisCO regulation and (iii) RuBP regeneration on photosynthetic growth under fluctuating light, interpreted using two special cases of the mathematical model. Data corresponds to a 0.25 duty cycle. (a) Full model and experimental data plotted with two special cases: (i) a model free of the flashing light penalty and (ii) a model which only has the flashing light penalty and no benefit of the flashing light effect (b) Full model and two special cases which each have only one regulatory mechanism, either RuBisCO activation or RuBP regeneration.
whereas RuBP regeneration imposes a penalty for frequencies above 0.004 Hz. The penalty associated with RuBisCO activation is weak, since the activation rate for RuBisCO is roughly an order of magnitude higher than the deactivation rate. In contrast, RuBP regeneration imposes a severe penalty since the activation rate is only double the deactivation rate. In addition, a shoulder is present at a frequency of roughly 0.004 Hz, where the RuBP regeneration penalty begins to increase with increasing frequency. The presence of a dip, shoulder and gradual increase is due to the combination of the two distinct regulatory mechanisms.

### 5.3 Conclusion

This work presents evidence that the regulation of photosynthesis in fluctuating light has an adverse effect on photoautotrophic growth, particularly strong in a range around 0.01-0.1 Hz - a range that is typical of nature, agriculture, and industry. We postulate that the light dependent regulation of the Calvin cycle is the biological process responsible for impeding growth. As such, under photoheterotrophic conditions, the penalty is substantially reduced, highlighting the role of the Calvin cycle. The ability of photoheterotrophic growth to reduce the penalty attests to the role of the regulation of the Calvin Cycle in penalizing photosynthetic growth. Under photoheterotrophic growth, organic carbon is acquired instead of CO2, reducing the need for the Calvin cycle.\textsuperscript{154,155}

The penalty is demonstrated here for organisms that use the Calvin cycle for carbon fixation. We expect that similar penalties may exist in other organisms where light regulation plays a role, for instance in regulating the reverse Krebs cycle of green sulfur bacteria. Purple non-sulfur bacteria, which only have a single photosystem \textsuperscript{156,157} likely exhibit a penalty since the Calvin cycle can be indirectly regulated by light through the redox state of the cell. Moreover, accumulation of RuBP can increase transcription of proteins relevant to the regulation of the Calvin cycle \textsuperscript{158}. Considering that the Calvin cycle is tightly regulated in multiple organisms, it is likely that a similar penalty will exist in purple non-sulfur bacteria.

Two facets of the Calvin cycle are presented as a source of the penalty, (i) regeneration of RuBP and (ii) the activation state of RuBisCO, as shown by our mathematical model. There is a distinct penalty strongest at approximately 0.02 Hz, attributed to RuBP regeneration regulation, while at lower frequencies ranging from 0.001 to 0.0002 Hz there is a subtle penalty due to RuBisCO regulation. Though Synechococcus elongatus and Chlamydomonas reinhardtii may not express RuBisCO activase specifically, the catalytic state of RuBisCO is maintained in the light by enzymes which
operate in a similar fashion. Moreover, our numerical model clarifies the transient nature of photosynthesis in fluctuating light. Specifically, the Calvin cycle is not fully active during the light phase, which in turn penalizes growth in fluctuating light. Furthermore, we expand the scope of the traditional flashing light effect from enabling improved growth above the no-light-integration case to allowing for the organism to compensate for the regulation of the Calvin cycle. This is noteworthy, in that RuBisCO’s slow kinetics are considered to be a disadvantage, however, our results suggest that these slow kinetics are in some sense desirable since they allow a photosynthetic organism to compensate for delayed activation of the Calvin cycle.

Our data suggests that the causes of light fluctuations could be indirectly considered as stressors, should they force fluctuations into frequencies in the penalized range. Specifically, changes in the physical environment such variations in wind and natural convection driven mixing coupled to increases in turbidity could reduce photoautotrophic growth. Regarding climate change, increases in temperature are having an impact on circulation in bodies of water, and therefore the second order behaviour of photoautotrophic growth should be considered when forecasting the impacts of climate change. Furthermore, the majority of controlled experiments have been performed under steady state irradiance conditions, and do not capture any interactive effects between environmental drivers and fluctuating light. Since fluctuating light imparts a penalty on growth, experiments with continuous irradiance could be underestimating the harm imposed by environmental stressors, such as CO$_2$ acidification stress or nutrient limitation. First, CO$_2$ acidification stress can be more severe under a higher intensity light in marine species. Second, light and CO$_2$ can be seen as aiding resources, due to their role in supplying energy to the cell, an organism in fluctuating light may be more sensitive to nutrient limitation. Causes of fluctuating irradiance should be considered when assessing ecosystems, either due to a direct penalty or in combination with various stressors.

The penalty is present in both a cyanobacteria and a eukaryotic algae under photoautotrophic growth. However, under mixotrophic conditions (the ability to adopt either photoautotrophic, photoheterotrophic or chemoheterotrophic growth), *Chlamydomonas* exhibits little to no penalty compared with the no-light-integration case and as duty cycles are changed. The ability of mixotrophy to alleviate the flashing light penalty broadens the role of mixotrophy from the ability to thrive in low light and CO$_2$ to the ability to thrive where light is intermittent. It is important to note that alternating between photoautotrophic, photoheterotrophic and heterotrophic growth requires some adjustment in metabolism and transcription, which could incur an additional, small
penalty. Mixotrophy is common in organisms evolved to live in lower light or CO₂. Our results indicate a broadened role of mixotrophy, enabling growth where light is intermittent.

Lastly, these results are pertinent to biotechnology. The presence of a penalty in Chlamydomonas suggests a similar behaviour may exist in higher plants (by extension agriculture), since they share similar carbon fixation machinery. To improve agricultural yields, researchers often suggest re-engineering RuBisCO to improve crop performance. Though re-engineering of RuBisCO could be effective, our data highlight that increasing the activation rate of the Calvin cycle would improve growth through better light utilization. Lastly, current models for predicting photobioreactor performance are bound between the full-light-integration and no-light-integration behaviours. As such these models do not capture the photosynthetic penalty associated with fluctuating light that is inherent to most operations and particularly at high densities.

5.4 Methods
5.4.1 Cell culture conditions
Synechococcus elongatus PCC7942 T2SEΩ (provided by Professor Rakefet Schwarz, Bar-Ilan University, Israel) was cultured at 28 °C under continuous irradiance of 45 µmol·m⁻²·s⁻¹ and room CO₂ (≈600 ppm) for long term storage. Approximately 48 hours prior to experimentation, cells were transferred to a 1% CO₂ environment. Cells were cultured in a double concentration BG-11 media (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA), which was pH buffered to 7.5 with 20mM HEPES (Sigma Aldrich, Oakville, Ontario Canada). Cell culture media was sterilized using 0.22 µm vacuum filtration units. This modified strain is resistant to kanamycin.

Wild-type Chlamydomonas reinhardtii (CC-124), was obtained from the Chlamydomonas Resource Centre (University of Minnesota, St. Paul, Minnesota). The organism was culture with either Sueoka’s high salt medium with (HSA) and without acetate (HS), pH buffered to 7 with a 20mM concentration of MOPS. Chlamydomonas reinhardtii cells were maintained at 25 °C under a continuous irradiance of µmol·m⁻²·s⁻¹ and room CO₂ (≈600 ppm), for long term storage. Approximately 48 hours prior to experimentation, cells were transferred to a 1% CO₂ environment.

5.4.2 Experimental apparatus
Spatial control of irradiance across a 384-well plate was achieved using an LCD projector (EPSON EX3220 SVGA 3LCD), shown in Figure 5-1a and a sample of the resulting irradiance pattern shown in Figure 5-1b. The various irradiances cases were achieved by creating a 24 hour video file using a MATLAB script (included as supplemental). The resulting video was then played using VLC media.
player (VideoLAN, Paris, France). The frequency and duty cycle layouts are shown in Figure S1, respectively. In addition, the spectral profile is included as Figure S3. The video file was manually aligned to the well plate, and continued alignment was ensured by mounting both the projector and well plate to an optical bread board. The well plate was incubated at 1% CO₂ by enclosing the plate in a custom made transparent acrylic chamber, which was fed with a 1% CO₂ gas mixture. Temperature was maintained at 28 C for *Synechococcus elongatus* and 25 C for *Chlamydomonas reinhardtii* using resistive heaters and a thermostat.

5.4.3 Experimental protocol

Approximately 48 hours prior to being transferred to the 384-well plate, *S. elongatus* was re-suspended to an optical density of 0.05 (measured in a 1cm cuvette) and cultured at 1% CO₂ under an irradiance of 80 µmol·m²·s⁻¹, supplied by the projector used during the experiment. The CO₂ was monitored using a NIR CO₂ sensor (CO2meter). This was done to acclimate the cells to both the CO₂ concentration and irradiance spectral profile. A 1% CO₂ was chosen increase the transport into the chambers (details in SI).

For the purposes of screening the effect of irradiance frequency on growth, microalgae were diluted to an optical density of 0.05 (measured in a 1cm cuvette), then loaded into a 384-well plate. Each chamber of an opaque 384-well plate received 50 µL of cell suspension. During culturing, the plate was sealed using a breathable film (Double Skin, 4titude). The well plate was mounted to an optical bread board located below a projector and aligned to a video file to create the various light conditions and to ensure continued alignment during the experiment.

During measurement, the breathable film was replaced with an optical grade plate sealing film (VIEWseal, Greiner). Optical density in the 384-well plate was measured using a BMG PheraStar plate reader. Prior to measurement, the plates were vortex mixed then centrifuged briefly (30 s) at approximately 130 g's to ensure well dispersed cells and to eliminate bubbles. After the measurement phase, the optical film was replaced with a new breathable film and the well plate was re-mounted to the experimental setup.

5.4.4 Growth under continuous irradiance

The continuous irradiance data was included on the same well-plate as the growth under fluctuating irradiance. For *Synechococcus elongatus*, exponential growth was maintained over the course of the two day experiment as shown by the high light case included in Figure S2. For *Chlamydomonas reinhardtii*, experiments were run for three days, however growth rates over
either two or three days were used, due to the vastly different growth rates for the various conditions.

5.5 Nomenclature for the mathematical model

I: Irradiance intensity
PSU: Resting Photosynthetic Unit
PSU*: Activated Photosynthetic Unit
CAL1*: Inactive Calvin cycle for RuBP regeneration
CAL1: Active Calvin cycle for RuBP regeneration
CAL2*: Inactive Calvin cycle for RuBisCO
CAL2: Active Calvin cycle for RuBisCO
kcal1ON: activation rate of RuBP regeneration
kcal1OFF: deactivation rate of RuBP regeneration
kcal2ON: activation rate of RuBisCO
kcal2OFF: deactivation rate of RuBisCO
x*: Fraction of activated PSUs
y*: Fraction of active RuBP regeneration
z*: Fraction of active RuBisCO
xss*: Fraction of activated PSUs at steady state
yss*: Fraction of active RuBP regeneration at steady state
zss*: Fraction of active RuBisCO at steady state
R: maximum rate of deactivation of activated photosynthetic units
K: half saturation constant
Pm: maximum growth rate
ka: light harvesting constant

5.6 Supplemental information

Supplementary information for “A Penalty on photosynthetic growth in fluctuating light”
Figure S1: (a) Layout of frequencies and (b) duty cycles used, the colours indicate a log scale in the case of frequency and linear for duty cycle, grayscale colouring indicates continuous irradiance cases and light blue indicates empty chambers used as blanks.

Figure S2: Continued exponential growth over two days for the fastest growing case of 215 μmol·m⁻²·s⁻¹ and the intermediate irradiance of 80 μmol·m⁻²·s⁻¹.

### 5.6.1 Carbon dioxide concentration in the microwells

The carbon dioxide concentration around the well plate was maintained at 1% during the course of the experiment by enclosing the well plate in a clear acrylic box and flushing the box with a 1% CO₂-in-air gas mixture. This concentration was used to ensure adequate CO₂ transport through the breathable film and into the culture. Transport through both the breathable film and the cell suspension can be understood through Fick’s Law.

\[
J = D \frac{\partial c}{\partial x}
\]

Where \( J \) is the flux, \( D \) the diffusivity, \( c \) the concentration and \( x \) distance. We can calculate the flux required based on (i) the CO₂ rate of a cell (roughly \( 5 \times 10^{-18} \) mol/cell·s)\(^{169} \) and (ii) assuming a relatively dense cell suspension of \( 10^7 \) cells/ml with (iii) 50 μL of cell suspension per well and each well has a surface of (9mm²). The flux is then roughly \( 3 \times 10^{-7} \) mol/m²·s. To achieve such a flux, the concentration gradient across the membrane (\( dc/dx \)), assuming a diffusivity through the membrane of \( 5 \times 10^{-8} \) m²/s\(^{170} \), would need to be roughly \( 10^{-2} \) mol/m³ which would reduce the CO₂ concentration by less than 10%. Moreover, continued exponential growth suggests that the algae
are not CO$_2$ limited, since growth did not decrease substantially as the transport requirement increased.

### 5.6.2 Irradiance Spectral Distribution

A large portion of the irradiance spectrum (figure S3) is in the range of 500-600nm, where the absorption of *Synechoccus elongatus* ranges from 40-60%. This may suggest that light is not well absorbed by *Synechoccus elongatus*, however, light is sufficiently absorbed to drive growth, as seen by the saturating relationship between light intensity and growth rate in Figure 2a. Specifically, the cells are no longer light-limited for irradiances over 150 µmol·m$^{-2}$·s$^{-1}$, seen by the levelling off in Figure 2a. Both photosystems II and I are excited under this irradiance spectrum, since the phycobilisomes absorb light in the 550-650nm range and transfer this light energy to either photosystem.$^{125,126}$

![Figure S3: Irradiance Spectrum of the light source used here and the absorption spectrum of *Synechococcus elongatus*. Measured with an integrating sphere and spectrometer.](image-url)
Figure S4: computational model used. The figure from the main text is repeated for convenience and the associated equations are derived in full.

5.6.3 Details of Mathematical Model

For the PSUs

\[
\frac{dPSU^0}{dt} = -k_a I PSU^0 + \frac{CAL_1^*}{CAL_1^* + CAL_1^0} \frac{CAL_2^*}{CAL_2^* + CAL_2^0} R PSU^* \quad (1)
\]

\[
\frac{dPSU^*}{dt} = k_a I PSU^0 - \frac{CAL_1^*}{CAL_1^* + CAL_1^0} \frac{CAL_2^*}{CAL_2^* + CAL_2^0} R PSU^* \quad (2)
\]

For the degree of Calvin cycle activation,
\[
\frac{dCAL_1^0}{dt} = -k_{cal1ON}CAL^0 + k_{cal1OFF}CAL^* \quad (3)
\]
\[
\frac{dCAL_1^*}{dt} = k_{cal1ON}CAL^0 - k_{cal1OFF}CAL^* \quad (4)
\]
\[
\frac{dCAL_2^0}{dt} = -k_{cal2ON}CAL^0 + k_{cal2OFF}CAL^* \quad (5)
\]
\[
\frac{dCAL_2^*}{dt} = k_{cal2ON}CAL^0 - k_{cal2OFF}CAL^* \quad (6)
\]

Where \( k_{cal1ON} \) and \( k_{cal2ON} \) are 0 in the dark.

Taking into account that the sum of the active and inactive Calvin cycle should remain constant, we non-dimensionalize the concentration of active calvin cycle molecules as,

\[
\frac{dy^*}{dt} = k_{cal1ON} (1 - y^*) - k_{cal1OFF}y^* \quad (7)
\]

Where,

\[
y^* = \frac{CAL_1^*}{CAL_1^* + CAL_1^0}
\]

\[
\frac{dz^*}{dt} = k_{cal2ON} (1 - z^*) - k_{cal2OFF}z^* \quad (8)
\]

Where,

\[
z^* = \frac{CAL_2^*}{CAL_2^* + CAL_2^0}
\]

Under continuous irradiance the activity of the Calvin cycle can be determined from the steady state solution of equations (5) and (6).

\[
0 = k_{cal1ON} (1 - y_{ss}^*) - k_{cal1OFF}y_{ss}^*
\]

\[
0 = k_{cal2ON} (1 - z_{ss}^*) - k_{cal2OFF}z_{ss}^*
\]
Giving the following for the active fraction

\[ y_{ss}^* = \frac{K_{Calvin1}}{K_{Calvin1} + 1} \]  
(9)

\[ z_{ss}^* = \frac{K_{Calvin2}}{K_{Calvin2} + 1} \]  
(10)

Where,

\[ K_{Calvin1} = \frac{k_{cal1ON}}{k_{cal1OFF}} \]

\[ K_{Calvin2} = \frac{k_{cal2ON}}{k_{cal2OFF}} \]

Taking into account that the sum of the active and inactive photosynthetic units should remain constant, we non-dimensionlalize the concentration of active photosynthetic units as,

\[ \frac{dx^*}{dt} = \beta \left( \frac{1}{\alpha} \left( \frac{K_{CAL1}}{K_{CAL1} + 1} \right) \left( \frac{K_{CAL2}}{K_{CAL2} + 1} \right) (1 - x^*) - \frac{y^* z^* x^*}{k + x^*} \right) \]  
(11)

Where,

\[ \beta = \frac{R}{PSU^0 + PSU^*}, \quad \alpha = \frac{R}{k_a(PSU^0 + PSU^*)} K_{CAL1} K_{CAL2}, \quad \kappa = \frac{K_S}{PSU^0 + PSU^*}, \quad x^* = \frac{PSU^*}{PSU^0 + PSU^*} \]

At steady state conditions we get,

\[ 0 = \beta \left( \frac{1}{\alpha} \left( \frac{K_{CAL1}}{K_{CAL1} + 1} \right) \left( \frac{K_{CAL2}}{K_{CAL2} + 1} \right) (1 - x_{ss}^*) - y_{ss}^* z_{ss}^* \frac{x_{ss}^*}{k + x_{ss}^*} \right) \]

Substituting the previously determined steady state values \( y_{ss} \) and \( z_{ss} \) we obtain,
0 = \beta \left( \frac{l}{\alpha} \left( \frac{K_{CAL1}}{K_{CAL1} + 1} \right) \left( \frac{K_{CAL2}}{K_{CAL2} + 1} \right) (1 - x_{ss}^*) - \left( \frac{K_{CAL1}}{K_{CAL1} + 1} \right) \left( \frac{K_{CAL2}}{K_{CAL2} + 1} \right) \frac{x_{ss}^*}{\kappa + x_{ss}^*} \right)

Which simplifies to

0 = \frac{l}{\alpha} (1 - x_{ss}^*) - \frac{x_{ss}^*}{\kappa + x_{ss}^*}

Solving for $x_{ss}^*$,

$0 = x_{ss}^{*2} + \left( \frac{\alpha}{l} + \kappa - 1 \right) x_{ss}^* - \kappa$

$x_{ss}^* = \frac{\left(1 - \kappa - \frac{\alpha}{l} \right) \pm \sqrt{\left( 1 - \kappa - \frac{\alpha}{l} \right)^2 - 4\kappa}}{2}$

The rate of biomass accumulation is proportional to the rate at which PSUs decay from excited to unexcited,

$\frac{P}{P_m} = y^* z^* \frac{x^*}{\kappa + x^*}$

Where the product, $P_m y^*$, is the maximum growth rate

At steady state we obtain,

$\frac{P}{P_m} = \frac{l}{\alpha} \left( \frac{K_{CAL1}}{K_{CAL1} + 1} \right) \left( \frac{K_{CAL2}}{K_{CAL2} + 1} \right) (1 - x_{ss}^*)$

Substituting the expression for $x_{ss}^*$ we obtain,

$P = P_m \frac{l}{2\alpha} \left( \frac{K_{CAL1}}{K_{CAL1} + 1} \right) \left( \frac{K_{CAL2}}{K_{CAL2} + 1} \right) \left( 2 - \left( 1 - \kappa - \frac{\alpha}{l} \right) - \sqrt{\left( 1 - \kappa - \frac{\alpha}{l} \right)^2 - 4\kappa} \right)$

(12)

Equation (12) is used to extract parameters $P_m$, \( \kappa \) and \( \alpha \), which are used to solve equations (7), (8) and (11) simultaneously.

$\frac{dy^*}{dt} = k_{cal1ON} (1 - y^*) - k_{cal1OFF} y^*$

(7)
\[
\frac{dz^*}{dt} = k_{cal2ON} (1 - z^*) - k_{cal2OFF} z^*
\]  
(8)

\[
\frac{dx^*}{dt} = \beta \left( \frac{I}{\alpha \left( \frac{K_{CAL1}}{K_{CAL1}+1} \right)} \left( \frac{K_{CAL2}}{K_{CAL2}+1} \right) (1 - x^*) - y^* z^* \frac{x^*}{\kappa + x^*} \right)
\]  
(11)

### 5.6.4 Details regarding parameters

The continuous light data gave values of 141, 0.04357 and 1.425 for parameters \( \alpha, \kappa \) and \( P_m \), respectively (\( R^2 = 0.997 \)). With regards to parameters relevant to fluctuating light, the parameter \( \beta \), chosen here to be 2, represents the maximum rate of de-excitation of active photosynthetic units relative to the total amount of photosynthetic units in a cell. This parameter relates to the frequency required to reach full light integration, which is not the focus of this work. Values between 2-15 will have been shown to fit previous experimental data.\(^{38,73}\) An approximation of \( \beta \), could be the rate at which a cell can reduce carbon per photosystem. This can be approximated by considering the RuBisCO catalytic rate (3-10 s\(^{-1}\)),\(^{5,171}\) the number of RubisCO active sites per carboxysome (2000-8000),\(^{171,172}\) and the number of carboxysomes per cell (~4)\(^{173}\) giving values of \( \beta \) of roughly 0.2-3, which is reasonable in comparison to the fitted parameters. In short, the value of the parameter \( \beta \) agrees with previous works and is in general agreement with what would be expected based on the constituents of a photosynthetic organism.

Irradiance controls the degree of activation of the Calvin cycle by indirectly activating Rubisco Activase by increasing the amount of ATP and by promoting RuBP regeneration via redox sensing of the photosynthetic electron transport chain.\(^{19}\) Precise values for relevant rate constants are sparse, since the details of the regulation of the Calvin cycle is an ongoing research topic,\(^{5,11}\) activation and deactivation kinetics vary across species\(^{21-23}\) and the rate constants are typically extracted by fitting complex models to CO\(_2\) assimilation experiments.\(^{24-26}\) The activation rates used in our model align well with experimental values measured for CO\(_2\) assimilation in leaves with active RuBisCO (0.2-0.01 s\(^{-1}\)).\(^{14,22,24,25,27,150}\) Deactivation rates for RuBP regeneration are not explicitly available experimentally, but can be inferred to be a factor of 1-4 times slower than the activation rate based on various models fit to data.\(^{24,25,27}\) The regulation of RuBisCo activity is typically one to two orders of magnitude slower than RuBP regeneration with an activation rate constant 5-10 times higher than the deactivation rate constant.\(^{23-25,27,28}\) Data for six marine diatoms and a cyanobacterial species show RuBisCO activation rates in the range of 0.01 - 0.001 s\(^{-1}\).\(^{22,23,152}\) Highlighting the kinetics related to the catalytic rate of RuBisCO in microalgae. Our fitted experimental values are in agreement with the values reported in literature.
Chapter 6. Dual gradients of light intensity and nutrient concentration for full-factorial mapping of photosynthetic productivity

This chapter presents the development of a microfluidic screening platform for studying the effect of irradiance conditions and ammonium concentration on microalgal growth and lipid accumulation. I assisted in defining the project, performing experiments and editing the manuscript. Additional authors for this work include Brian Nguyen and David Sinton. Reproduced from Ref. 2 with permission from The Royal Society of Chemistry.

Abstract
Optimizing bioproduct generation from microalgae is complicated by the myriad of coupled parameters affecting photosynthetic productivity. Quantifying the effect of multiple coupled parameters in full-factorial fashion requires a prohibitively high number of experiments. We present a simple hydrogel-based platform for the rapid, full-factorial mapping of light and nutrient availability on the growth and lipid accumulation of microalgae. We accomplish this without microfabrication using thin sheets of cell-laden hydrogels. By immobilizing the algae in a hydrogel matrix we are able to take full advantage of the continuous spatial chemical gradient produced by a diffusion-based gradient generator while eliminating the need for chambers. We map the effect of light intensities between 0 µmol m$^{-2}$s$^{-1}$ and 130 µmol m$^{-2}$s$^{-1}$ (~28 W/m$^2$) coupled with ammonium concentrations between 0 mM and 7 mM on Chlamydomonas reinhardtii. Our data set, verified with bulk experiments, clarifies the role of ammonium availability on the photosynthetic productivity Chlamydomonas reinhardtii, demonstrating the dependance of ammonium inhibition on light intensity. Specifically, a sharp optimal growth peak emerges at approximately 2mM only for light intensities between 80 and 100 µmol m$^{-2}$s$^{-1}$ – suggesting that ammonium inhibition is insignificant at lower light intensities. We speculate that this phenomenon is due to the regulation of the high affinity ammonium transport system in Chlamydomonas reinhardtii as well as free ammonia toxicity. The complexity of this photosynthetic biological response highlights the importance of full-factorial data sets as enabled here.

6.1 Introduction
Climate change due to fossil fuel usage is among the most pressing problems of our time. Algae-based biofuels promise to provide an alternative to high energy density liquid fuels without
competing for arable land.\textsuperscript{175,176} There is also potential to optimize photosynthetic productivity of algae in controlled environments either in suspension or immobilized in hydrogel or a biofilm.\textsuperscript{1,176–182} However, much of this potential remains untapped due to complex and often coupled effects of multiple environmental parameters on algae growth and lipid accumulation. These coupled effects are best represented by full-factorial data sets, whereby every combination of each variable is independently assessed.\textsuperscript{162,183} However, full-factorial testing requires hundreds or even thousands of unique experimental conditions which is infeasible with conventional, flask-based techniques.\textsuperscript{162,183}

Two important parameters for algae growth and lipid accumulation are irradiance and nitrogen availability.\textsuperscript{1,90,184–186} Optimizing both parameters is not straightforward. First in the context of nutrients, nitrogen in the form of nitrates, nitrites, or ammonium, is essential for algal growth. However, nitrogen replete conditions, typical of standard culture media, do not necessarily result in optimal growth conditions.\textsuperscript{64} Further, excess ammonium can have inhibitory effects on algal growth.\textsuperscript{64,187,188} In addition, nitrogen depleted conditions induce desirable lipid accumulation, at the expense of growth.\textsuperscript{184,186,189,190} Second, in the context of light, irradiance is not only essential for growth, it regulates nitrogen uptake systems in algae.\textsuperscript{191} The complexity of the nitrogen dependence is thus compounded by light variable dependence. With these coupled sensitivities, standard culture media and irradiance intensities are typically suboptimal.

Lab-on-a-chip methods are well-positioned to provide both the control and massive parallelization required for full-factorial testing of coupled parameters with hundreds, if not thousands of simultaneous experiments.\textsuperscript{1,192,193} Microfluidic devices have been developed to study the influence of chemical species on algal growth.\textsuperscript{64,194} These microfluidic devices couple a cell culture array to a microfluidic gradient generators. The most common form of gradient generator for this application is the “Christmas tree” gradient generator.\textsuperscript{194–196} Recently, Kim et. al\textsuperscript{64} took advantage of the simplicity, and flow-free nature of a diffusion-based gradient generator to study the effect of nitrogen concentration on algal growth – observing the effect multiple levels of nitrogen concentration in a high-throughput manner.\textsuperscript{64} A key advantage of these lab-on-a-chip devices over traditional bulk cultures is the ability to maintain chemostatic conditions. Chemostats allow the quantification of algae growth under controlled specific chemical conditions. Irradiance control devices, including our recently reported LCD-based platform,\textsuperscript{1} have also been coupled to cell culture arrays to study the effect of light variables on algal growth. With our previous platform we are able to observe the effect of different levels of light intensity. In addition to our platform, LEDs\textsuperscript{30,68} and
dyes have been used to control light in microreactors. All current microfluidic algae screening approaches, however, lack the multi-parameter integration required for detailed full-factorial studies due to a lack of: (i) independent control over both light and chemical variables; and (ii) parameter resolution over the range of interest.

Here we a report a multiplexed combinatorial screening platform for quantifying the effect of irradiance and nitrogen on the model algae \textit{Chlamydomonas reinhardtii}. Our approach consists of a diffusion-based gradient generator and pixel-based irradiance control surrounding a hydrogel laden with photosynthetic microorganisms. The result is a high resolution full-factorial screening method capable of quantifying the effect of irradiance and nitrogen concentration on the growth and lipid productivity. By immobilizing the algae in a hydrogel matrix we are able to take full advantage of the continuous spatial chemical gradient produced by a diffusion-based gradient generator while eliminating the need to load cell culture chambers. Moreover, we eliminate the need for microfabrication, and the use of PDMS which interferes with lipophilic probes readout due to absorption and fluorescence. Lastly, unlike with cell traps - the existing technique of immobilizing algal cells on lab-on-a-chip devices - our technique is shear-free. Our full-factorial data set reveals an interactive effect light and ammonium on growth kinetics, verified with bulk flask cultures.

6.2 Materials and Methods
The algae screening device is illustrated in Figure 6-1. The screening device consists of a 1 mm thick cell-laden hydrogel secured between a 4.5 mm thick gradient generating layer and an irradiance control layer. The gradient generating layer, consisting of two parallel source-sink channels, 30mm in length and 20mm apart with a 3mm square cross-section, was fabricated by pouring a 4% w/v volume agarose (Invitrogen UltraPure™) solution into a Poly(methyl methacrylate) (PMMA) mould. A glass microscope slide was placed on top of the mould to ensure a flat surface. As previously reported, this configuration generates linear a concentration gradient between the source and sink channel.\textsuperscript{64,198–200} We verified this by using fluorescein as a tracer compound (Figure S1).

The cell-laden hydrogel was prepared by cross-linking an alginate-cell solution using a calcium rich
agarose hydrogel block as shown in Figure 6-2. First, an alginate-cell solution was created by mixing a suspension of 0.2 optical density cell suspension with a 4% alginate (Sigma W201502) solution in equal volumes, which yielded an alginate cell suspension with a final effective optical density of 0.1 and an alginate concentration of 2%. A calcium-rich agarose block was fabricated in a PMMA mould by adding 150mM CaCl$_2$ to a 4% w/v volume agarose solution and pouring into a PMMA mould. The alginate-cell solution was poured on the calcium-rich agarose layer and allowed to crosslink to form a cell-laden alginate hydrogel layer. The crosslinked alginate is inert to *Chlamydomonas reinhardtii* $^{179,201,202}$ This process allows the fabrication of thin, smooth, and uniform films of algae-laden hydrogel under gentle$^{178}$ conditions, ideal for housing algal cells for irradiance and chemical screening. Because the algae are immobilized in the hydrogel matrix they are spatially isolated and held in place without the need for any micro-fabricated features. Further, the algae-laden hydrogel can be recovered from our screening platform to perform analysis that would be difficult in microfluidic chip, such as staining for fluorescent quantification of lipids.

The pixel based irradiance control platform was adapted from our prior work.$^1$ The irradiance screening platform included an LCD with the included backlight removed (eDIPTFT32-A, Electronic Assembly, Gilching, Germany) and a backlight comprised of 10 LED strips (VL-H03W5501080D20, Super Bright LEDs, Saint Louis, MO, USA) powered by constant current modules coupled to a 24V power supply. The LCD was positioned 75mm above the backlight, at the bottom of a custom PMMA basin filled with water.
*Chlamydomonas reinhardtii* strain CC-86 was obtained from the *Chlamydomonas* Resource Centre (University of Minnesota, St. Paul, Minnesota) and cultured in an incubator at 25 degrees Celsius in Tris-Acetate-Phosphate (TAP) Media supplemented with 50 µg/mL of the antibiotic Kanamycin. The stock cultures were subject to continuous illumination at approximately 40 µmol m\(^{-2}\)s\(^{-1}\). This strain of *Chlamydomonas reinhardtii* is resistant to Kanamycin.

For full-factorial screening experiments, TAP media was pumped through the source channel of the gradient generating layer and TAP-N (TAP without ammonium) was pumped through the sink channel of the gradient generating layer continuously using peristaltic pumps for 28 hours to allow the gradient to reach equilibrium according to Fick's laws of diffusion. These settings allow us to resolve the effect of millimolar ranges of ammonium concentrations of common interest. Alternatively, by reducing the concentration of the source channel the device could provide resolution at the micromolar level, albeit within a smaller total concentration range. The cell-laden hydrogel was then placed into the assembly. The chemical screening assembly was placed on top of the LCD in the PMMA basin in a water bath kept at 25 degrees Celsius. A 2-cm light gradient was applied using the LCD by fitting pixel value to light intensity (Figure S2). After 48 hours of growth the cell-laden layer was removed for staining and imaging.

For the flask-based validation experiments, *Chlamydomonas reinhardtii* was suspended in media concentrations 25% (1.75mM), 50% (3.5mM), and 100% (7.0 mM) that of typical ammonium concentration in TAP media with other media components identical, respectively at an optical density of 0.05 (6 replicates each). The cultures were maintained at 25 C. Sets of flasks containing the different concentrations of ammonium were exposed to white light intensities of 40 and 90 µmol m\(^{-2}\)s\(^{-1}\). Flask experiments started at a low optical density and were conducted over a relatively short period of time to minimize perturbation of the ammonium concentration in the flask as a result of uptake by cells.

The cell-laden hydrogel was removed from the assembly and immersed into a 5 µM staining solution of BODIPY 493/503 (ThermoFisher Scientific) in TAP media for 60 min. The solution was made by adding 50 µL of a 5 mM stock solution (BODIPY dissolved in DMSO) to 50 mL of TAP media. The stained hydrogel placed on a microscope slide for imaging with a Nikon Eclipse Ti laser scanning confocal inverted microscope equipped with a motorized stage. A 640nm laser was used as an excitation source to observe growth. A 488nm laser was used to excite the BODIPY-stained intracellular lipid droplets. The area of interest on the cell-laden hydrogel was scanned. The images
were analysed in ImageJ by taking the background-subtracted fluorescence intensities with the MicroArray Profile plugin. While a confocal microscope was used due to availability, widefield imaging is also suitable. We are able to obtain data about growth and lipid accumulation by observing chlorophyll autofluorescence and BODIPY stained lipids respectively.

### 6.3 Results and Discussion

Figure 6-3 maps growth and lipid response to irradiance intensity and ammonium concentration, with sample fluorescence images shown to the right of the colour bar. The full-factorial data set shows that the growth of *Chlamydomonas reinhardtii* is relatively unaffected by ammonium concentrations in the millimolar range at irradiances below about 80 µmol m⁻²s⁻¹. However, at 80 to 100 µmol m⁻²s⁻¹ a distinct peak emerges at around 1-2 mM suggesting that ammonium becomes inhibiting to growth above 2 mM.

This approach clarifies the role of ammonium influence by including the coupled effect of irradiance. Specifically, ammonium inhibition at 1-2 mM only significantly occurs at relatively high...
irradiances (80 to 100 μmol photons m\(^{-2}\) s\(^{-1}\)). Many studies suggest that an ammonium concentration on the order of 1 mM is sufficient and there is little benefit or penalty in the 1 – 10 mM range, consistent with the Monod model.\(^{205-207}\) Our results are consistent with these studies which were conducted at 70 μmol m\(^{-2}\) s\(^{-1}\) or below in bulk cultures, which are prone to self-shading.\(^{205-207}\) However, in contrast to these studies, Kim et al.\(^{64}\) recently found that ammonium was inhibiting at 1-2 mM under their light conditions, approximately 80 μmol m\(^{-2}\) s\(^{-1}\). In short, this detailed full-factorial data set reconciles a discrepancy in literature regarding the effect of ammonium on photosynthetic productivity.

We confirm our observations from the cell-laden hydrogel by growing traditional bulk cultures at select conditions (Figure 6-4). In agreement with the full-factorial platform, the bulk cultures show no clear ammonium inhibition at low irradiance (40 μmol m\(^{-2}\) s\(^{-1}\)), but exhibit inhibition at high irradiance (90 μmol m\(^{-2}\) s\(^{-1}\)). Specifically, a clear optimal concentration of ammonium only emerges in the high irradiance case. Figure 6-4 shows that at high irradiances, a maximal growth of 2.0 day\(^{-1}\) occurred at a starting ammonium concentration of 1.75 mM (0.25 standard media). As ammonium concentration increases to 3.5mM and 7mM, growth rate decreases to 1.92 day\(^{-1}\) and 1.85 day\(^{-1}\) respectively. In contrast, at low irradiances, growth at 1.75 mM and 3.5 mM starting ammonium concentrations where similar at approximately 1.7 day\(^{-1}\). Growth at 7 mM starting ammonium concentrations was lower with a growth rate of 1.53 day\(^{-1}\). The growth signal from our full-factorial screening platform at 1.75 mM ammonium and 90 μmol m\(^{-2}\) s\(^{-1}\) was approximately 1.45 times higher than the growth signal at 7mM and 90 μmol m\(^{-2}\) s\(^{-1}\). This ratio is comparable to that derived from the flask experiments, approximately 1.35 – validating that our approach of screening immobilized cells is relevant to bulk suspension cultures. Note that since the flasks are not chemostatic (they deviate from initial conditions), a smaller ratio as observed here is to be expected.

The strong coupled dependence of ammonium inhibition of growth and irradiance can potentially be explained by the multi-parameter regulation of ammonium transport in *Chlamydomonas reinhardtii*. Ammonium transport in *Chlamydomonas reinhardtii* is regulated by both ammonium concentration and irradiance.\(^{191}\) *Chlamydomonas reinhardtii* has two known sets of ammonium transporters, a high affinity transport system and a low affinity transport system.\(^{191}\) The high affinity system excels at ammonium transport at low ammonium concentration, and is down-regulated at high ammonium concentration.\(^{191}\) The high affinity system is also up-regulated at higher irradiances.\(^{191}\) As a result, a combination of high irradiance and relatively low ammonium
concentration are likely required for optimal functioning of the ammonium transport system. However, high irradiances increase free ammonia toxicity\textsuperscript{187,188} – an effect that could explain diminishing growth past a saturating light intensity of approximately 100 µmol m\(^{-2}\)s\(^{-1}\). While there is a single point of high cell density at approximately 6.5mM and 125 µmol m\(^{-2}\)s\(^{-1}\) this point is not significant with local averaging, (Figure S3) and is considered here to be without biological significance. The complexity of these and other photosynthetic biological responses highlight the need for full-factorial quantification as enabled here.

### 6.4 Conclusion

We have developed a microfabrication-free, full-factorial screening platform capable of continuous mapping of growth and lipid response to nutrient and irradiance conditions. This platform provides the unprecedented ability to resolve detailed full-factorial maps of the effects of coupled parameters on photosynthetic productivity. A thin and smooth cell-laden hydrogel allowed us to combine a diffusion-based concentration gradient generator with a pixel-based irradiance-control platform to achieve full spatial chemical and light control. Moreover, the hydrogel-based nature of our platform, in contrast to PDMS-based devices, allows lipid quantification. We quantified the irradiance dependence of ammonium inhibition of *Chlamydomonas reinhardtii* growth. Our data shows a sharply defined optimal condition between 80-100 µmol m\(^{-2}\)s\(^{-1}\) and 2 mM ammonium
concentration. The full-factorial results – verified by select bulk cultures – reconciled an irregularity in the literature. We speculate that the light intensity dependence of ammonium response is due to a combination the regulation of the high affinity transport system as well as free ammonia toxicity. The complexity of this photosynthetic biological response highlights the importance of full-factorial data sets as enabled here. More broadly, this approach is amenable to assessing a wide range of parameters on photosynthetic microorganisms, both for enhanced bioproduct generation and assessing environmental stressors.

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6.5 Supplementary Information
Figure S1: Fluorescein characterization of gradient generator at approximately 100hrs. Note that the diffusion coefficient of fluorescein is approximately a quarter of that of ammonium.

Figure S2: Light Intensity dependence of pixel value. This curve is used to select the appropriate pixel value to obtain the desired light intensity.
Calculations for diffusion time:

\[
t \approx \frac{x^2}{2D} = \frac{(20\text{cm})^2}{2 \left(2 \text{cm}^2 \times 10^{-5} \text{s}^{-1}\right)} \approx 28 \text{ hours}
\]

Figure S3: Smoothed growth data.
Chapter 7. Conclusions

7.1 Summary
The core of this work includes three demonstrations, a microfluidic LCD based irradiance assay shown in Chapter 4; a frequency dependent penalty on photosynthetic growth which was understood by both a high-throughput irradiance screening assay and a simplified model based on first order kinetics in Chapter 5; and a coupled irradiance-nitrogen screening platform which demonstrated a mixed effect of light and nitrogen in Chapter 6.

The irradiance assay presented in Chapter 4 is composed of a microfluidic chamber and a liquid crystal display. The microfluidic chip was used to contain the microalgae during cultivation, while the liquid crystal display controlled the light environment of the micro photobioreactors. In contrast to typical culture-flask scale studies, the short optical path enables more uniform light at high cell densities. Similarly, nutrient availability and gas transport can be enhanced thanks to the high surface area to volume and ability to constantly supply nutrients. However, these can also be addressed in bulk scale systems with mixing, bubbling and dilution.

Chapter 5 includes experimental and theoretical data detailing a frequency dependent penalty on photosynthetic growth in fluctuating light. This penalty was attributed to the activation kinetics of carbon assimilation. The model developed both gives insight into what occurs during photosynthesis and could be used as a simple model to predict bioreactor productivity, ecosystem dynamics and agricultural yields – all aspects where light is variable.

In Chapter 6 both light and nutrients are simultaneously studied using a multiplexed assay. The assay is characterized by coupling a hydrogel diffusion-based gradient generator to the LCD based multiplexing system from Chapter 4. Though also interesting, it falls victim to the typical microfluidic issues of being difficult to use. This was addressed by the device presented as an appendix in section 8.1, which uses a multiwell plate in conjunction with typical fluid handling to create multiple chemical environments, while a projector was used for light control and an aerogel was used for long range gas control.

7.2 Outlook
The ability to produce large amounts of data regarding the effects of light and chemical variables on photosynthetic organisms can allow for a deeper understanding of the inner working of photosynthetic growth. This understanding can take the form of an interesting synergistic effect, for instance, the interplay between light and nitrogen shown in Chapter 6, or as shown in Chapter 5, a frequency dependent penalty which helped unveil the second order behavior of photosynthesis and can be used to calibrate a model. The
domains which can benefit from this increased understanding include biotechnology, in the form of either making biofuels, agriculture and environmental understanding, as it pertains to understanding the response of organisms to local and global stressors. Moreover, with a strong understanding of the interaction between multiple parameters with the ability to understanding the dynamic effects, detailed models are possible. These models could be used to predict agriculture performance, ecosystem behavior and reactor productivity.

Regarding the frequency dependant penalty from chapter 5, there are two possible research avenues to pursue; either gaining a deeper understanding of the penalty by performing time resolved experiments with additional measurements, or studying how the penalty is affected by other stressors. Time resolved experiments of interest could be to directly measure RuBisCO’s activation state, oxygen evolution, or CO₂ uptake. These measurements can give further insight into the regulation of photosynthesis by irradiance. Studying the role of pH, nutrient limitation or temperature would be of interest as these could play a role in activation and deactivation kinetics of RuBisCO. Thus, measuring the dynamic effects of light can show that certain stressors may be stronger than expected in the natural environment where light is typically varying.

More broadly, there are many opportunities to build further on this work, and that of the supplementary contributions. The ability to perform a multiplexed study on the frequency response of living organisms can help unravel their complex internal behavior, similar to what was shown in chapter 5 of this thesis, and in some recent works. Examples of studying the frequency response are ubiquitous in engineering and physics, namely the rheological measurements of Non-Newtonian fluids, characterizing electrical circuits and mechanical vibrational systems. Developing a technique for biology which is analogous to a function generator and oscilloscope, will be an important path to understanding the complexity of life.
Chapter 8. Appendices

8.1 High-throughput assessments of the ecological impacts of global change

This chapter presents the development of an aerogel-based screening platform capable of assessing the synergistic effects of climate change and local pollutants on microalgae, plants and zooplankton. I am second author on the publication currently under review at Science Advances. Additional authors for this work include Brian Nguyen, Chelsea M Rochmam and David Sinton.

Abstract

As a result of the incredible progress of our own species, we expose the others to a range of global and local stressors. For example, aquatic organisms are under stress from global climate change and point-source chemical and/or nutrient pollution. Still, most experiments measure impacts from only one stressor because the complexity of measuring responses to multiple stressors is prohibitive. Here, we developed an approach to multiplex treatments in microcosms to test the impacts of multiple stressors, including parameters relevant to climate change and point source pollutants. Our approach leverages (1) the high rate of purely diffusive gas transport in aerogels to produce well-defined centimeter-scale gas concentration gradients, (2) spatial light control and (3) established automated liquid handling. Using a five-parameter full factorial study, we measured biological effects in over 700 treatments using the microalgae *Chlamydomonas reinhardtii*. Our results indicate that temperature stress decreases with increasing CO$_2$ concentration. Moreover, CO$_2$ can alleviate the stress from multi-resource limitation by acting as an aiding resource. We also demonstrate the ability to assess the consequences of multiple stressors on biota and communities, measuring effects from common surfactants and nanoparticles coupled with a range of future climate scenarios on model flora (*Lemna gibba*) and fauna (*Artemia salina*). Overall, we demonstrate the ability to study multiple stressors in complex experiments using individual or multiple organisms.

Aquatic communities are under stress from a diverse combination of stressors that act together to alter biotic systems at all levels of biological organization, including at ecological levels (e.g., effects on population size and community structure). These include the stresses of climate change, exploitation of resources and a diversity of chemical and nutrient pollutants. Although
biological communities are never exposed to just one stressor at a time, most studies focus on effects from one stressor \(^{213}\). Globally, communities are coping with warming in combination with increased CO\(_2\) levels, occurring concurrently with local environmental stressors from synthetic chemicals \(^{214}\). Specifically, the fundamental challenge of predicting such responses stems from needing to measure impacts from multiple variables at once, including changes in aqueous nutrients and toxins, gasses, light, and temperature \(^{162,183,215–217}\). Microcosms and mesocosms allow the control necessary to provide mechanistic and predictive insights with a high degree of control over parameters. \(^{218,219}\) Unfortunately, with current microcosm-based techniques, full factorial experiments involving multiple variables (e.g., temperature, light, gas, chemical, and nanomaterial pollutants) are considered practically impossible once the number of unique conditions reaches the 100’s – typical of multi-parameter studies \(^{162,183}\). As result, most studies only focus on a single parameter \(^{213}\).

While local stressors (e.g., point source pollution, resource extraction) vary geographically, stress associated with elevated CO\(_2\) is global. Still, we generally measure impacts from anthropogenic stressors in isolation. While elevated atmospheric CO\(_2\) is ubiquitous and a particularly important environmental driver that can have outsized influence on plants, microbes, and ultimately ecosystems \(^{162,220}\), understanding biotic responses to the continually increasing CO\(_2\) levels, in combination with other drivers \(^{162,215,221}\), is fundamental to understanding any ecological impacts of global change. In addition, it helps more realistically predict future atmospheric CO\(_2\) concentrations – as effected by primary productivity feedback \(^{164,222}\). At the organism level, responses to elevated CO\(_2\) concentrations are multi-faceted, especially when combined with other environmental parameters \(^{162,164}\). At the community level, this complexity is compounded by trophic interactions\(^{223}\). Unfortunately, multiplexing CO\(_2\) concentrations in experiments is not feasible with conventional techniques, since traditional multiplex gas handling requires costly and elaborate equipment even for a few distinct concentration levels \(^{183}\).

In addition to CO\(_2\), there are several key parameters to consider relevant to climate change alone. These include nitrogen, phosphate, temperature and light \(^{162}\). Moreover, an experimenter may want to measure impacts from climate change in concert with other anthropogenic stressors, such as a chemicals or nanomaterials. A full factorial sweep of 32 levels of CO\(_2\), representing future climate scenarios with CO\(_2\) typical supersaturation in lakes \(^{224,225}\) and soils \(^{226}\), in concert with two levels of each of the other aforementioned parameters with 3 replicates would require over 1,500 experimental units. To avoid the challenge, models and experimental design optimization techniques have been developed to predict responses using partial or collapsed factorial data sets.
Although models can expand predictive power from smaller data sets, they may overlook interactive effects and thus provide limited mechanistic insight. In the context of the complex environmental questions, all approaches benefit from the ability to acquire larger experimental data sets.

Microdevices have shown promise in overcoming the throughput barriers owing to their exceptional liquid handling capabilities. While microdevices provide a route to meeting the high-throughput requirements, current microdevices lack the capability, versatility, usability, and scalability required to address the throughput challenge presented by multi-stressor global change. Diffusion based microfluidic gradient generators can provide a range of concentrations by leveraging the ability of small molecules to diffuse between a source and a sink through a bulk material, typically PDMS or hydrogel. However, current gradient generators are limited in size due to the relatively small rate of diffusion in PDMS and hydrogels. As a result, these platforms can accommodate only a few (< 10) distinct chemical environments in small chambers and can only accommodate very small culture volumes. Thus, in their current state, existing platforms are incapable of producing large-scale gas gradients required to produce sufficient distinct levels needed to resolve the impacts of CO2 concentration on cells (much less multicellular biota) and allow simultaneous control of multiple variables. Moreover, microdevices currently face practical barriers to widespread adoption. Specifically, scaling microfabrication remains a challenge, a limitation compounded by the single-use nature of microdevices and the need for specialized equipment to operate.

Here, we developed a technique that is high-throughput and uses microcosms to measure impacts to individuals and biological communities from CO2 in combination with multiple stressors (e.g., temperature, light, nutrient availability, chemical pollutants, particulate pollutants). We leverage the uniquely high rate of diffusion-dominated mass transport in aerogels to create a gradient generator to sweep multiple CO2 concentrations. We combine our aerogel-based gradient generator with established automated liquid handling and LCD projection to produce a platform that enables detailed climate change-relevant full factorial studies involving microorganisms, as well as small multicellular flora and fauna. We apply this approach to multi-parameter experiments with three kinds of model organisms. With the model microorganism *Chlamydomonas reinhardtii*, we demonstrate that our platform can obtain a full-factorial dataset consisting of 5 environmental parameters, 768 treatments, entailing ~3000 experimental units. With model flora, *Lemna gibba*, we performed experiments with 60 parallel experimental units, demonstrating the ability to measure the combined effects of chemical pollution and elevated CO2 levels on growth. With a
model community, *Artemia salina* and *Duniealla salina*, 48 parallel experimental units demonstrate the ability to test simple model ecosystems in multiplex. This approach uniquely enables the study of the sensitivity of organisms to climate change in concert with other environmental stressors and measure the compound ecological impacts to model flora and fauna from global change.

**Results**

We demonstrate the capability of our platform to measure impacts to individual organisms and biological communities from a combination of elevated CO$_2$ and a variety of local stressors. The capabilities of the experimentation platform are described first, followed by applications to assessing effects related to the multiple facets of climate change.

**Multiplex climate change experimentation platform.**

*Aerogel Gradient Generator.* Fig. 1 shows our aerogel-based gas concentration gradient generator. Aerogels are open-cell, nanoporous foams, with extremely high porosity. The open-cell structure and high porosity of aerogels enables the rate of diffusion in aerogels to be about one-tenth of that in open air ~1000 times higher than the rate of diffusion in PDMS or water$^{237}$. Furthermore, the nanoporosity of aerogels allows mass transport to be consistently dominated by diffusion with negligible advective transport, even in the gas phase allowing predictable equilibrium concentration profiles (details in supplementary)$^{238}$.

The source-sink configuration (Fig. 1a) of our device is designed to create linear gas concentration gradients across a well plate-sized area (approximately 7 cm by 10 cm), between the source and sink channels. To confirm the linearity and equilibrium time of a gas concentration gradient (Fig. 1c), 4 sampling points were measured across a generated oxygen gradient (Fig. S1). The response time of our gradient generator is approximately three orders of magnitude faster (Fig. 1d) than existing microfluidic gradient generators as a direct consequence of the high diffusivity in aerogels$^{238}$. This rapid response time enables the creation of centimeter-scale concentration gradients on convenient experiment setup timescales (~ 10 min). In contrast, such a gradient would take ~7 days with hydrogel or PDMS-based gradient generators. The ability to generate CO$_2$ gradients over well plates was confirmed using a bromothymol blue-based pH indicator solution in 96-well plates (Fig. 1b). The gradient can be applied across a standard well plate, with different organisms and liquid-phase compositions (Fig. S2).
The high rate of diffusion has the added benefit of being robust against perturbation by cell culture. In contrast to slower diffusion-based gradient generators, our numerical simulations show that the gradient in the aerogel device is not significantly perturbed (<2.5%) even if a 1536-well plate was filled with dense cultures all drawing CO₂ at maximal rates (Fig. S3 and Supplementary Table 1). In contrast, a modest culture would perturb a traditional diffusion-based gradient generator, even if the set-up time could be tolerated (Fig. S3 and Supplementary Table 1). Further, as the aerogel gradient generator works solely by gas-phase diffusion, we expect it to be compatible with the full range of biologically relevant gas streams without modification. As a result, our platform can, in principle, be broadly applied to other life science or chemical research where gas concentrations are relevant including: small animal and cellular chemotaxis and stem cell culture conditions in addition to the application we demonstrate here. An added benefit is that the aerogel gradient generator, unlike with current microdevices, is decoupled from the culture vessels and has an indefinite shelf life. Consequently, the gradient generator can be used for an indefinite number of experimental runs and only the well plate needs to be replaced. Additionally, the approach is widely compatible with established liquid handling techniques.

**Figure 1:** (a) Expanded schematic of the aerogel-based gas gradient generating method with insets showing model organisms compatible with the setup, from left to right: microalgae, macrophytes, and crustaceans (b) pH-indicator visualization of the CO₂ gradient in a clear 96-well plate, at increasing concentrations of CO₂ the bromothymol blue-based pH indicator solution turns from blue to yellow due to increasing CO₂-induced acidity (c) Measurement of oxygen concentration at equilibrium at points along an oxygen gradient, markers represent measurements on two separate devices of identical design. The linearity of the gradient and the temporal diffusion profile confirm that the mass transport of gas in the aerogel can be accurately described solely using Fick's laws. (d) Measured concentration of a sample point over time after applying gas concentrations showing ~10min to reach equilibrium, consistent with a diffusion coefficient of approximately 2 x 10⁻² cm²/s or one-tenth of that of open air.
**Spatial Light Control.** Light conditions are key to microalgal growth\(^{241}\). As such, to multiplex light conditions, we used LCD projection to spatially control light intensity (Fig. S4). Aligning a projected image to a well plate coupled to our aerogel-based gradient generator allows control of the light intensity on any area of a well plate. This technique allows us to control light intensity over a well plate-sized area with a resolution limited only by the pixel density of the LCD.

*Nutrient conditions can inhibit CO\(_2\) fertilization in microalgae.* The data from the *Chlamydomonas*
*reinhardtii* experiment represents 768 unique treatments with 3-4 replicates, for a total of ~3000 experimental units (See **Fig. 2** and **Fig. S5**). Under control conditions (25 °C, nutrient replete, light replete), the influence from CO₂ on the growth of *Chlamydomonas reinhardtii* follows Monod kinetics, saturating at ~5,000 ppm CO₂ (**Fig. 2b**). This response is consistent with the literature\textsuperscript{162,242-244}. However, the extent to which CO₂ is beneficial for growth is typically reduced under nutrient stress. Under decreased nitrogen (**Fig. 2d**), the growth influence of CO₂ saturates at ~2000 ppm CO₂. Under decreased phosphorus (**Fig. 2j**), elevated CO₂ has only a small effect on growth rate. In the case of phosphorus, acidification could also play a role in reducing growth rates at high CO₂ (> 10,000 ppm) since the phosphates in HSM act as a buffer and reduced phosphate levels would lower buffering capacity.

**CO₂ fertilization in microalgae can vary depending on stressors.** The magnitude of inhibition is dependent on CO₂ levels. A current question is the dependence of CO₂ fertilization on nutrient availability\textsuperscript{164,245}. Here, an increase in CO₂ results in increased inhibition under decreased nutrient or light supply, likely because cells lack the resources to take advantage of the increased CO₂ availability (**Fig. 3a-c**). When different stressors are added in combination with warming, their inhibitory effect is typically additive (**Fig. 3e-g**). When all four (nitrogen limitation, phosphorus limitation, light limitation and warming) stressors are present, growth is nearly completely arrested, regardless of the CO₂ level (**Fig. 3h**). This increased inhibition implies that the perturbation of the primary productivity-driven negative feedback loop by nutrient limitation will become increasingly important as CO₂ levels increase.
Since rising temperatures occur concurrently with rising CO$_2$ levels, the combined effect of rising CO$_2$ and temperature is particularly important. Temperature influences how primary producers respond to rising CO$_2$ by decreasing gas solubility, increasing metabolism, increasing photorespiration$^{246}$, and modulating inorganic carbon uptake mechanisms$^{247}$. The combination of these mechanisms make the CO$_2$ response difficult to predict$^{162,163,222,246,247}$. Here, the inhibitory effect of elevated temperature is prominent at lower (< 10,000 ppm) CO$_2$ levels but decreases at higher CO$_2$ levels (Fig. 3d). A potential mechanism for the reduced inhibitory effect of temperature at higher CO$_2$ is that a higher CO$_2$ level offsets the increased photorespiration$^{246}$ and the lower solubility of CO$_2$ at higher temperatures (1.45 g/L at 25 °C vs. 1.33 g/L at 28 °C). This effect suggests that CO$_2$ supersaturation in lakes$^{224,225}$ and soils$^{226}$ may contribute to thermotolerance in microalgae.

**Multi-resource independent co-limitation occurs in Chlamydomonas reinhardtii.** Increasingly, the importance of multiple resource co-limitation is being recognized$^{164,248}$. Here, we observed growth

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**Figure 3:** Inhibition of growth rate from stressors at different CO$_2$ levels. (a) Light intensity 30 μmol·m$^{-2}$·s$^{-1}$, compared to 60 μmol·m$^{-2}$·s$^{-1}$ in the standard case (b) Phosphorus concentration, 0.675 mM starting concentration compared to 13.5 mM (c) Nitrogen concentration, 0.94 mM starting concentration compared to 9.4 mM (d) Warming, 28 °C compared to 25 °C (e) Decreased light in combination with warming (f) Decreased phosphorus in combination with warming (g) Decreased nitrogen in combination with warming (h) All four stressors in combination.
responses to four resources: CO$_2$, light, nitrogen, and phosphorus. While two-way resource co-limitation is relatively well studied\textsuperscript{164}, our platform allows us to observe up to four-way co-limitation. Here, cultures with a high level of any single resource, excluding nitrogen (two-tailed t-test $P = 0.19$), exhibited a significantly higher (two-tailed t-test, $P = 0.02$ for light, $P = 0.03$ for phosphorus, $P = 0.03$ for CO$_2$) growth rate compared to the low resource cultures (Fig. 4) – suggesting light, CO$_2$, and phosphorus are independently co-limiting with each other for \textit{Chlamydomonas reinhardtii}, in contrast to Liebig’s law of the minimum – which states that growth is constrained solely by the scarcest resource \textsuperscript{164,249}. We did not observe a significant increase in growth rate due to increased nitrogen compared to corresponding treatments with decreased nitrogen (two-tailed t-test, $P = 0.002$) unless other resources were increased first (Fig. 4i,h). This independent co-limitation supports the hypothesis that under co-limited conditions a microalgal cell must allocate energy and membrane porters between the active transport of multiple limiting resources\textsuperscript{250}. Under this hypothesis, the addition of one co-limiting resource alleviates the limitation by other resources\textsuperscript{250}.

Further, these results are consistent with the hypothesis that CO$_2$ is an aiding resource - facilitating uptake or use, due to its direct role in the photosynthetic energy supply\textsuperscript{164}. Here, CO$_2$ increases growth when other individual resources are both low and replete (Fig. 4m-o). CO$_2$ also increases growth when multiple other individual resources are both low (Fig. 4g-l) and replete (Fig. 4a-f). Similar behavior can be observed with light (Fig. 4a,b,j,k), the other direct input into photosynthesis. The ability of CO$_2$ to aid in resource uptake dampens relative fertilization penalty under resource limitation or use when resources are replete.
Figure 4: Co-limitation of *Chlamydomonas reinhardtii* growth rate by multiple resources. (a)-(f) Growth rates of cultures with combinations of resources, starting with low resources (LR) conditions, followed by different sequences of N, P and light addition, then lastly CO$_2$ addition for all cases. (g)-(l) Growth rates of cultures, starting with low resources (LR) conditions, followed by the addition of CO$_2$ for all cases, then addition of different sequences of N, P and Light. For points with elevated CO$_2$, maximum and minimum growth rates from all the levels of elevated CO$_2$ are shown in black along with the midpoint, shown in red. (m)-(o) Growth rates of the low resource cultures compared with cultures with either a single resource replete (CO$_2$ or another resource) or a combination of CO$_2$ enrichment and another replete resource. Error bars represent standard deviations in all cases with $n = 3-4$. 

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Pollution can Inhibit CO₂ Fertilization in Flora. We observed that the growth response of *Lemna gibba* to CO₂ concentrations follows Monod kinetics, with saturation occurring between 1200 ppm and 2000 ppm in standard media, in agreement with the literature. However, pollutants can alter this CO₂ response (Fig. 5). LAS (a commonly used surfactant) inhibits growth under all tested CO₂ conditions and results in shrunken and discolored fronds – indicating poor health (Fig. 5a). Moreover, under LAS stress, CO₂ fertilization is effectively suppressed (Fig. 5c). At 2,000 ppm CO₂ and LAS we observed approximately 40% (two-sided t-test, P = 0.001) fewer fronds when compared to the treatment without LAS. CO₂ stimulation of *Lemna gibba* growth in the presence of LAS saturated at a relatively low CO₂ concentration of ~1000 ppm compared to without LAS. Consequently, the percent inhibition by LAS tracks the Monod response to CO₂, with high CO₂ levels, in effect, enhancing the toxicity of LAS (Fig. 5d). We postulate that the increased LAS inhibition at high CO₂ levels is due to reduced chlorophyll concentration, apparent from the discolored fronds. With lower chlorophyll levels, light harvesting is less efficient causing growth to become more light limited than carbon limited. In short, the LAS stress may weaken the negative climate feedback effect of increasing primary production by inhibiting light harvesting.

In contrast to LAS, inhibition by titania nanoparticles (used as a pigment for industrial and consumer applications) was not observed at any individual CO₂ level, consistent with existing literature. Moreover, titania nanoparticles did not cause any obvious discoloration or deformation of the fronds. Low levels of titania nanoparticles are considered relatively harmless and can even have stimulatory effects on plant growth by enhancing chloroplast activity under current atmospheric CO₂ conditions. However, minor inhibition emerges at CO₂ levels 1000 ppm and above - indicating a possible interactive effect between titania nanoparticle toxicity and CO₂ levels.
Figure 5: Images of *Lemna gibba* cultured for 7 days (a) without and (b) with the surfactant LAS, a common pollutant. (c) Mean frond counts of *Lemna gibba* after 7 days with (grey squares) and without (black circles) LAS (n = 5 for each condition, error bars represent standard deviations) (d) mean inhibition by LAS at CO₂ concentrations from 400 to 4500 ppm. Images of *Lemna gibba* cultured for 7 days (a) without and (b) with titania nanoparticles (g) Mean frond counts of *Lemna gibba* after 7 days with (grey squares) and without (black circles) titania nanoparticles (n = 5, error bars represent standard deviations) (h) mean inhibition by titania nanoparticles at CO₂ concentrations from 400 to 4500 ppm.

*Communities can influence response to CO₂.* Elevated CO₂ levels are expected to have a detrimental effect on brine shrimp nauplii growth due to acidification. **Duniealla salina** is part of the typical diet of *Artemia salina* and therefore growth stimulation by elevated CO₂ in **Duniealla salina** could have a positive effect on the growth of *Artemia salina* nauplii despite acidification. Using our platform, we studied these direct and indirect effects for six different CO₂ concentrations as shown in **Fig. 6.** Our results show that a moderate positive effect (P = 0.04 calculated from Pearson’s correlation coefficient) of elevated CO₂ is present at the early stages of development where growth (measured by abdominal length) increased due to greater food availability (**Fig. 6b**). However, this effect loses significance when titania nanoparticles (**Fig. 6c**), which impede the growth of microalgae, are introduced. These results suggest that the impact of climate change drivers on heterotrophs, such as *Artemia salina*, must be considered in context with other stressors and with the effects of those same drivers on their food supply, which is typically tied to lower trophic levels.
Figure 6: (a) Representative image of an *Artemia salina* sub-adult grown from a nauplius (b) Growth response to CO₂ of *Artemia salina* cultured with live *Duniealla salina* in brine without titania nanoparticles. Black circles represent mean abdominal lengths; grey squares represent individual abdominal lengths; error bars represent standard deviations (c) Growth response to CO₂ of *Artemia salina* cultured with live *Duniealla salina* cultured in brine with titania nanoparticles.

**Discussion**

The real world is complex and multivariate, and thus it is difficult to understand how multiple stressors may impact biota with current technologies. In particular, responses to global change cannot be fully understood with experiments that do not account for local variables in nutrients and pollutants in combination with effects of climate change. To address this challenge, we developed a simple but powerful platform, leveraging the rapid and controlled gas diffusion through aerogels in combination with spatial light control, for parallel screening of growth responses to multiple parameters in microcosms. The ability to rapidly investigate the effect of multiple parameters is a step toward overcoming the throughput barrier to performing detailed full factorial studies that capture the complexity of multiple stressors.

Our technique enables the observation of phenomena that have implications for the role of primary productivity in the context of rising CO₂ levels with unprecedented throughput. Our method allows investigating a wide range of CO₂ conditions in combination with other environmental parameters. We found that the stimulation of growth by increased CO₂ is highly sensitive to other parameters, for both the microalgae and the multicellular plant studied here and is often constrained to favorable nutrient conditions. The multiplexing provided by our approach also enables the examination of multi-resource co-limitation of primary productivity. Lastly, the flexibility of our method makes it suitable for studying effects to communities in the context of climate change. We studied the direct effect on *Duniealla salina* and a subsequent indirect effect on *Artemia salina* and demonstrated that increased primary productivity has the potential to mitigate the stress of ocean
acidification. We expect that our approach will be applicable in furthering our understanding of the ecological impacts of climate change in concert with other environmental stressors. Collectively, these results demonstrate the potential of this method to screen multiple environmental parameters in the context of global climate change. This work highlights the opportunity for new methods to integrate several of the most complex environmental variables into ecological experiments that measure impacts to individual organisms and biological communities.

Methods

Aerogel-based gradient generator construction. Hydrophobic aerogel monoliths (Aerogel Technologies P-AT.A.X103, medium density, large panels) were purchased from Aerogel Technologies Inc. The aerogel monoliths were cut to size with a fine-toothed saw, sanded flat with 320 grit sandpaper, and thinned to a thickness of ~6 mm. Care was taken not to contact the aerogel with solvents that would collapse the pore structure and hinder gas diffusion. The experimental apparatus consisted of a standard well plate and aerogel sandwiched between a PMMA back plate and a PMMA layer with source and sink channels 6 mm tall and 6 mm wide spaced 6.8 cm apart, center to center (6.2 cm edge to edge) (Fig. S6b). All PMMA components were fabricated using a CO₂ laser cutter (Universal Laser Systems M-360). PMMA parts were bonded in a Carver hot press. The open areas of the aerogel are sealed with PET tape to ensure no-flux conditions where desired (ie. The source edge and the edges orthogonal to the channels). A silicon gasket was used to ensure a seal between the aerogel and the layer with the source and sink channels. While silicon is gas permeable, it does not permit advective mass transport. Clamping force was applied using ¼-20 machine screws and wing nuts. The highest and lowest concentrations in the CO₂ gradient were set by adjusting the concentration of CO₂ in the gas flowing in the source and sink channel respectively. All gas inlets were maintained at constant pressure using pressure regulators.

For the oxygen measurements, a custom PMMA manifold (Fig. S1) was used to position a needle-type optode (Presens Precision Sensing GmbH) at four sample points spaced at a pitch of 20 mm. The optode measurements were conducted on two devices using ~12 mm thick aerogels (Aerogel technologies P-AT.A.X103 medium density, tiles). The optodes were punctured through a custom silicon sheet that acted as a septum. For the pH indicator tests, a saturated bromothymol blue solution was adjusted to show blue at room CO₂. A 96-well clear plate was filled with 200 μL per well of the bromothymol blue solution and interfaced with the gradient generator. The source channel in the device used for experiments was provided with CO₂-enriched air and the sink
channel with plain air. This test was also conducted with a methyl red indicator solution (Fig. S11). We ensured that CO₂ consumption did not affect our gradient by conservatively modeling the gradient with CO₂ consumption in COMSOL (Fig. S3).

**Spatial Light Control.** An Epson EX3220 LCD projector was used as a light source which allows high-resolution spatial control of light intensity by adjusting pixel grey values. Light intensities were measured using a quantum meter (Li-COR), incident to the PMMA backplate. The projector was mounted at a tilt approximately 15 degrees from the vertical and manually aligned with the well plate to ensure that there was no spatial bias in light intensity. The absence of spatial bias was confirmed by measuring light intensities on both sides of a well plate. The light output from the projector was periodically monitored using a quantum meter to ensure that the light intensity did not decrease due to a worn lamp. If the light intensity was found to be low, the lamp was changed. Cultures were illuminated from the bottom of the well plate since the top of the well plate is occupied by the opaque gas gradient generator. However, the experiments were run with the well plate positioned upside down to allow illumination from above. Black-walled well plates were chosen to eliminate light cross-talk between wells (Fig. S12).

**Chlamydomonas reinhardtii experiments.** *Chlamydomonas reinhardtii* strain CC-124 (also known as 137c mt) was obtained from the Chlamydomonas Resource Centre (University of Minnesota, St. Paul, MN) and maintained on Sueoka's high salt medium²⁵⁷ (HSM) at 25 °C and approximately 30 μmol·m⁻²·s⁻¹ in several 250 mL culture flasks. KCl was used to ensure all experimental media had the same salinity¹⁶²,²⁵⁸. *Chlamydomonas reinhardtii* is particularly suited to our demonstration because it is a widely studied model alga and it is known to be amenable to small scale culture⁶⁴,¹⁶². Variations on HSM were used as experimental media. Prior to experiments, cultures were acclimated for at least 7 days in experimental media in 250 mL culture flasks¹⁶². All media was sterile filtered with 0.22 μm vacuum filtration units prior to use.

Experiments were conducted on black-walled 1,536-well plates for five-parameter experiments sealed with a breathable membrane²⁵⁹-²⁶¹ to reduce evaporation and prevent cross-contamination (Diversified Biotech Breathe-easy). The membrane also acts as a gasket to interface the well plate with the aerogel. Microalgal cultures were loaded into the well plates using a Perkin Elmer Flexdrop IV reagent dispenser set to 9 μL per well for 1536-well plates after resuspension in fresh experimental media. The volumes were chosen based on previously reported experience with *Chlamydomonas reinhardtii* experiments conducted in the same well plate formats²⁶⁰.
The well plates were interfaced with the aerogel gradient generator by sandwiching the well plate between a transparent PMMA backplate and the aerogel. The source channel was provided with a continuous gas flow of 30,000 ppm CO\textsubscript{2} and the sink channel was provided a continuous gas flow of 400 ppm CO\textsubscript{2} to provide a CO\textsubscript{2} concentration gradient from 400 to 30,000 ppm. The CO\textsubscript{2} concentrations were chosen to represent the range of concentrations in lakes and soils – which are typically supersaturated with CO\textsubscript{2}\textsuperscript{224,225}. The well plate was aligned with the first row of wells ~2 mm outside of the sink channel. The experiments were conducted over 4 days in batch. Due to the relatively small liquid volumes and high surface area to volume ratios in 1536-well plates the gas inlets were hydrated by bubbling through deionized water upstream of the gradient generator to further prevent evaporation. A resistive heater coupled to a temperature controller was used to set experimental temperatures. Fans were used to ensure an even temperature distribution throughout the experimental apparatus.

Optical density at 750 nm was measured using a BMG Pherastar FS plate reader. Prior to optical density measurements the well plates were vortex mixed and centrifuged to suspend the cells and eliminate bubbles. Wells containing bubbles after centrifugation were excluded from the analysis, these wells were relatively rare – consisting of fewer than 1% of the total wells used. During optical density measurement, the breathable sealing film was replaced with an optical-grade sealing film (Greiner VIEWseal). Growth rate was calculated using the following formula\textsuperscript{162}:

\[
\text{Growth Rate} = \frac{\ln(OD_2) - \ln(OD_1)}{t_2 - t_1}
\]

**Lemna gibba experiments.** *Lemna gibba* was obtained from the Canadian Phycological Culture Collection (University of Waterloo) and maintained on Hoagland’s media at 25 °C and approximately 30 μmol·m\textsuperscript{-2}s\textsuperscript{-1}. *Lemna gibba* experiments were conducted in custom bottomless 60-well plates fabricated from PMMA sealed with a breathable membrane on both sides (Diversified Biotech Breathe-easy). A custom well plate was used to obtain a suitable well size for culturing *Lemna gibba*. Each well of the custom well plate was initially loaded with a single plant with a pair of fronds (leaf-like appendages). An initial loading of 2 fronds and 500 μL of experimental medium was loaded in each well. The well plates were interfaced with the aerogel gradient generator by sandwiching the well plate between a PMMA backplate and the aerogel, as with the microalgae experiments.
The aerogel gradient generator was used to apply a CO\textsubscript{2} concentration gradient from 400 to 4500 ppm by providing gas streams of 400 ppm and 4500 ppm in the sink and source channel respectively. Continuous light at 60 \(\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\) was provided over the course of the experiment.

For cases with LAS stress 25 mg/L sodium dodecylbenzenesulfonate was added. For cases with titania nanoparticles 100 mg/L of 25 nm titania particles (Evonik Aeroxide) were added to the growth media. An endpoint frond count was taken after 7 days. Heavily discolored fronds were excluded from the count\textsuperscript{262}.

\textit{Atermia salina experiments.} \textit{Artemia salina} cysts were obtained from Brine Shrimp Direct. \textit{Artemia salina} cysts were incubated in f/2 nutrient enriched brine (a 6% sea salts solution)\textsuperscript{263} for 24 hours to hatch. \textit{Duniellia salina} (UTEX 1644) was obtained from the UTEX Culture Collection of Algae and maintained in f/2 nutrient enriched brine at 25 °C at ambient CO\textsubscript{2}, and approximately 30 \(\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\) in several 250 mL culture flasks. Freshly hatched nauplii were individually loaded into 48-well plates using a micropipette with wide-orifice tips along with a 1 mL suspension of live \textit{Duniellia salina} (OD\textsubscript{750} 0.03 measured in a 1 cm cuvette) in f/2 nutrient enriched brine as a food source. A temperature of 25 °C was maintained and continuous light with an intensity of 80 \(\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\) was provided. After 7 days the animals were euthanized by heating to 40 °C, allowed to settle on the bottom of the well plate, and imaged on a microscope. Abdominal length measurements were made manually in ImageJ from the microscope images (Supplementary Fig. 10). The occasional (less than 10% of the animals) animal that floated to the surface, hampering imaging, upon death was excluded from the analysis. For the experiments with titania nanoparticles, 100mg/L 25 nm titania nanoparticles were added to the brine.

\textbf{Statistics.} Graphs were created and statistics were calculated using Microsoft Excel. Significance was calculated using the two-sided Student’s t-test directly or from Pearson’s correlation coefficient.

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Elizabeth II Graduate Scholarships in Science & Technology, NSERC CGS Scholarships and the MEET, NSERC CREATE Program. Ongoing infrastructure support from the Canadian Foundation for Innovation and operational support through the NSERC Discovery Program is also gratefully acknowledged, as is imaging support from the Centre for Microfluidic Systems. Plate reading and preparation equipment used in this study was provided by The 3D (Diet, Digestive Tract and Disease) Centre funded by the Canadian Foundation for Innovation and Ontario Research Fund, project number 19442 and 30961.
High-throughput assessments of how future climate conditions may impact biological communities

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Supplementary Information

Supplementary Equation 1:

\begin{equation}
\frac{dC(x,t)}{dt} = D \frac{d^2C(x,t)}{dx^2}
\end{equation}

At equilibrium:

\begin{equation}
\frac{dC(x)}{dt} = 0
\end{equation}

With

\begin{align}
(3) \quad & C(0) = C_1 \quad & C(L) = C_2 \\
(4) \quad & C(x) = Kx + C_1
\end{align}
Figure S1: (a) Layout of oxygen sensing points. Oxygen measurements were taken from the four staggered points. The points were equally spaced in the horizontal direction. Distance was measured from the first point on the left (the low oxygen side). The total distance between the all points was 6 cm (b) The points were situated between two parallel channels on the opposite side of a 1.5 cm thick aerogel.
**Figure S2:** Assembled gas gradient generator with a 48-well plate. The edges of the aerogel are sealed with the yellow PET tape. The top plate, containing the channels, was fabricated out of 2 layers of 6 mm thick PMMA. PMMA ring spacers were used to align the well plate and the aerogel. The bottom plate was fabricated from 1 layer of 6 mm PMMA.

**Figure S3:** COMSOL simulation of the effects of CO₂ uptake by cultures (a) The effect of CO₂ uptake on the gradient profile (b) Visual representation of the gradient under typical CO₂ uptake with the aerogel gradient generator according to the simulation, units on the color scale are in mol/m³.
Supplementary Table 1: Parameters used for the COMSOL simulations. We assume that Chlorophyll is 5% of the dry weight\textsuperscript{186}.

<table>
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<td>50\textsuperscript{264}</td>
</tr>
</tbody>
</table>

Figure S4: (a) Projection set up. The LCD projector is used to generate a light pattern over a well plate to control the light intensity in the well. Light intensity measurements (through the bottom of a black-walled well plate) were taken under uniform illumination to ensure that there was no spatial bias in light intensity. (b) Assembled gas gradient generator with 1536-well plate and experimental light pattern projected. The bright lanes correspond to an intensity of 60 µmol·m\textsuperscript{-2}s\textsuperscript{-1} while the dark lanes correspond to an intensity of 30 µmol·m\textsuperscript{-2}s\textsuperscript{-1}. (c) Image used to project light pattern for 1536-well plate experiments. The bright lanes correspond to an intensity of 60 µmol·m\textsuperscript{-2}s\textsuperscript{-1} while the dark lanes correspond to an intensity of 30 µmol·m\textsuperscript{-2}s\textsuperscript{-1}.
Figure S5: CO$_2$ response curves at 0.675 mM phosphorus showing the interactive effect CO$_2$ (0-3000 ppm), Temperature (25 °C and 28 °C), Irradiance (30 μmol·m$^{-2}$·s$^{-1}$ and 50 μmol·m$^{-2}$·s$^{-1}$), and nitrogen (9.4)

Figure S6: (a) Design of custom 60-well plate used for *Lemna gibba* experiments. All dimensions are in mm. The plate was fabricated from 4 layers of 3 mm thick PMMA. (b) Channel layout. All dimensions are in mm.
Figure S7: Measurement of brine shrimp abdominal length. Red line indicates length measurement.

Figure S8: Photograph of an entire 60-well plate of *Lemna gibba* after 7 days of growth. The left half was subject to standard Hoagland’s solution while the right half was subject to Hoagland’s solution with LAS. A CO₂ gradient ranging from 400 ppm (bottom) to 4500 ppm (top) was applied.
Figure S9: Photograph of an entire well plate of *Lemna Gibba* after 7 days of growth. The right half was subject to standard Hoagland’s solution while the left half was subject to Hoagland’s solution with titania nanoparticles. A CO$_2$ gradient ranging from 400 ppm (bottom) to 4500 ppm (top) was applied.

Figure S10: A full well plate of *Artemia Salina* after 7 days of growth from freshly hatched nauplii. The right half was subject to brine with titania nanoparticles while the left half was subject to regular brine. A CO$_2$ gradient ranging from 400 ppm (top) to 4500 ppm (bottom) was applied.
**Figure S11:** Methyl red indicator test. Methyl red changes from yellow to red in response to lower pH (higher CO₂).

**Figure S12:** Illustration of crosstalk. This clear 96-well plate was subject to an image with 3 light levels (and 8 CO₂ levels) without changing other parameters. However, because of the clear walls cross talk between the wells created a more continuous light gradient and corresponding growth response.
8.2 Predominance of sperm motion in corners

This section contains the article Predominance of sperm motion corners, which has been published in Scientific Reports on May 13 2016. My contribution as second author includes designing the research, performing the experiments, analyzing the data, and writing the paper. Additional authors for the work include Reza Nosrati, Qiaozhi Liu, and David Sinton.

Abstract

Sperm migration through the female tract is crucial to fertilization, but the role of the complex and confined structure of the fallopian tube in sperm guidance remains unknown. Here, by confocal imaging microchannels head-on, we distinguish corner- vs. wall- vs. bulk-swimming bull sperm in confined geometries. Corner-swimming dominates with local areal concentrations as high as 200-fold that of the bulk. The relative degree of corner-swimming is strongest in small channels, decreases with increasing channel size, and plateaus for channels above 200 μm. Corner-swimming remains predominant across the physiologically-relevant range of viscosity and pH. Together, boundary-following sperm account for over 95% of the sperm distribution in small rectangular channels, which is similar to the percentage of wall swimmers in circular channels of similar size. We also demonstrate that wall-swimming sperm travel closer to walls in smaller channels (~ 100 μm), where the opposite wall is within the hydrodynamic interaction length-scale. The corner accumulation effect is more than the superposition of the influence of two walls, and over 5-fold stronger than that of a single wall. These findings suggest that folds and corners are dominant in sperm migration in the narrow (sub-mm) lumen of the fallopian tube and microchannel-based sperm selection devices.

Introduction

Sperm motion near surfaces plays a key role in natural fertilization, but the role of the complex and three-dimensional (3D) structure of the female tract on sperm migration near surfaces is largely unknown. During the journey to the egg, sperm encounter various rheological, biochemical, thermal and geometrical conditions. Specifically, viscosities range from 1 to over 100 mPa s\textsuperscript{266,267}, pH ranges from 6.5 to 8.5\textsuperscript{266,268}, temperatures range from 35 to 38 °C\textsuperscript{269} and confinement on the order of 10 to 100 μm is common\textsuperscript{270,271}. These variations enable sperm guidance and possible selection mechanisms, namely rheotaxis\textsuperscript{272-275},
chemotaxis, thermotaxis, and boundary-following navigation. With respect to the geometry, the fallopian tube becomes remarkably folded and confined with narrow lumen and corners as the sperm progresses along its journey. Emerging assisted reproduction methods also employ microconfined geometries to mimic the female tract for in vitro sperm selection and to coordinate fertilization. There is a lack, however, of quantitative understanding of how geometrical complexity and confinement influence sperm motion.

The study of sperm-surface interaction began in 1963, with Rothschild’s discovery of surface accumulation behaviour in bull sperm. This phenomenon has been studied for variety of microswimmers, by considering the effect of geometrical constraints, hydrodynamic effects, and flagellar beat pattern. These studies established that microswimmers, including sperm, accumulate near boundaries mainly due to a combination of hydrodynamic forces and steric repulsion. Surface confinement has also been shown to alter the swimming pattern and flagellar waveform of sperm. Recently we discovered an intermittent, fully two-dimensional slither swimming mode whereby the full sperm length oscillates within 1 μm of the surface. Denissenko et al. demonstrated that the migration ability of human sperm in a microchannel depends critically on the channel geometry, with corners contributing to boundary-following navigation. However, previous studies were limited to orthogonal imaging of the channel with a large depth of field (imaging a 1D distribution across the channel width), thus, could neither resolve nor quantify the distribution of sperm swimming close to a wall vs. those swimming close to a corner (requiring a 2D distribution across the channel).

Here, we resolve and quantify corner vs. wall vs. bulk swimmers using confocal imaging microchannels head-on. Our results demonstrate the strong preference of the bull sperm (~75 μm in length) to swim near corners (within 15 μm of the intersection of two walls) in rectangular microchannels, with local areal concentrations as high as 200-fold that of the bulk. The relative degree of corner-swimming preference is heavily dependent on channel size and shape, with channels above 200 μm resulting in similar corner, wall and bulk distributions. Furthermore, we characterize the effect of viscosity and pH on corner-swimming preference of sperm in square microchannels. Together, combined corner and wall swimmers account for over 95% of the sperm distribution in small rectangular channels, which is similar to the percentage of wall swimmers in similarly-sized circular microchannels. In the context of reproduction, this strong corner-swimming behavior highlights the role of geometrically complex and confined environment within the female
tract on sperm navigation. In the context of assisted reproduction, corner-directed sperm motion plays a dominant role in sperm migration within microchannel-based sperm selection devices.

Results and Discussion

*Corner-swimming in square microchannels.* The distinction and quantification of the swimming preference of sperm in the microchannel cross-sectional area was enabled by head-on confocal imaging, as shown in Fig. 1. The device comprises of a vertical and a horizontal layer (see Methods). The vertical layer, a 7.2 mm long microchannel, was aligned with the horizontal layer such that the microchannel cross-section was located at the center of a cylindrical chamber in the horizontal layer (Fig. 1a). The device was pre-filled with a biologically relevant buffer, mimicking the natural environment *in vivo*. Flow was inhibited by the dead-end structure of the device as well as relatively high fluid viscosities. The vertical channel exits into the cylindrical chamber which leads to eight horizontal trap reservoirs. The trap reservoirs use ratchet shape geometries to prevent sperm from re-entering the chamber. To characterize the effects of geometry and confinement, both rectangular and circular cross-section vertical channels, of varying dimensions, were tested. This characterization is relevant to both (*i*) sperm migration through the folded epithelium of the fallopian tube and the effect of confinement on sperm guidance *in vivo*, as well as (*ii*) sperm migration through microfluidic devices that generally have rectangular or square cross-sections.
Confocal microscopy was used to image migrating sperm through the vertical channel, at a cross-section 20 μm above the channel exit to the cylindrical chamber (Fig. 1b). The thickness of the focal plane was confined to 12.8-14.0 μm along the axial channel direction, to image a representative vertical channel cross-section, well before the channel exit. Sequences of bright-field and green fluorescence images with 500 ms interval were recorded for 30 min (see Movie S1 and S2). Bright-field (Fig. 1c,d top) and fluorescence (Fig. 1c,d bottom) imaging were used to locate channel walls and sperm, respectively. In contrast to previous imaging layouts that only give a 1D distribution by using the side-view of the microchannel, the method presented here images the channel head-on, providing the full 2D distribution of sperm. As a result we can accurately distinguish (i) a bulk swimmer at the center of the channel from a wall swimmer at the central part of the wall and (ii) a wall-swimming sperm at the center of the wall from a corner-swimming sperm at the channel corner.

Fig. 2 shows the cross-sectional distribution of sperm in square microchannels with side-lengths of 50 μm, 100 μm, and 400 μm. Sperm within 15 μm of only one wall were considered as wall swimmers and sperm within 15 μm of two walls were considered as corner swimmers, all other sperm were considered as bulk swimmers. The 15-μm threshold for wall proximity is based on previous works, indicating that microswimmers, including sperm, are most densely accumulated within 15-20 μm of the surface with their concentration decaying exponentially with distance from the surface\textsuperscript{303–306}. The 2D scatter distributions of sperm across the microchannels are shown in Fig. 2a-c with the relative density of sperm indicated via the color bar (for each plot). The plots of Fig. 2a,b are shown inset in Fig. 2c to give clarify the geometries. The results indicate the predominance
of corner-swimming as points with red and green colors in the plots indicate that many sperm swim near the corners during the imaging period.

Fig. 2d quantifies the percentage of bulk-swimming (PBS), percentage of wall-swimming (PWS), and percentage of corner-swimming (PCS) sperm as a function of the microchannel size. The corner-swimming tendency was predominant in both the 50- and 100-μm channels, accounting for 82% and 76% of sperm, respectively. In the significantly larger 400-μm channel corner-swimming accounting for 27% of sperm. In contrast to corner-swimming, wall-swimming increased with channel size, specifically, with 50, 100 and 400 μm microchannels having 16%, 22% and 54% wall-swimming sperm, respectively. Similarly, bulk-swimming also increased from less than 2% for 50 μm and 100 μm channels to 19% in 400 μm channels. Thus as the channel size increases, the concentration of corner swimming sperm decreases, while the concentration of wall and - to a lesser extent - bulk swimming sperm increases. This shift is mainly due to larger channel perimeter.

Figure 2. Corner-swimming preference of sperm in square microchannels. Cross-sectional distribution of 1176, 1457, and 1188 bull sperm in square microchannels with side-lengths of (a) 50 μm, (b) 100 μm, and (c) 400 μm, respectively. For comparison, the 50 μm and 100 μm channels are shown to scale inset in the bottom left corner of the 400 μm channel plot. The color bar represents the relative density of sperm in each graph. (d) Percentage of bulk swimmer (PBS), percentage of wall swimmer (PWS), and percentage of corner swimmer (PCS) sperm as a function of microchannel size. Wall-swimming sperm values do not include corner swimmers. Each point represents experiments with at least three samples with a minimum of 874 sperm imaged in each experiment. Values are reported as mean±s.d. (e) Histograms of sperm distance from the closest corner for each case. A total number of 800 sperm is
and higher area-to-perimeter ratio.

In terms of areal concentration, the relative area corresponding to corner-swimming regions decreases as channel size increases (36%, 9% and < 1% for 50, 100, and 400 μm channels respectively). Although only 27% of sperm swim in the corners of a 400 μm channel, the corner regions correspond to less than 1% of the channel cross-sectional area. Local areal concentrations in the corners reach over 200-fold that of the bulk for both 100 and 400 μm channels (227- and 213-fold, respectively).

Fig. 2e shows histograms of sperm distance from the closest corner, indicating a sharp right-skewed distribution for all channel sizes. For channels with 50 μm and 100 μm size, the maximum number of sperm was captured within 10-15 μm of the closest corner. For channels of 400 μm in size, the distribution had a long tail and a broad peak shifted to 20-25 μm from the closest corner. Furthermore, Fig. 2e shows that wall-swimmers are not uniformly distributed along the walls. Rather, the wall swimmers are more densely concentrated near the corner regions, with frequency decreasing along the wall.

Both wall- and corner-swimming preferences originate from hydrodynamic interaction of sperm with surfaces\textsuperscript{306,307}. It is clear from the experiments that the corner effect is stronger than simply the superposition of the influence of two walls. A simple superposition would predict a two-fold increase in relative density, whereas results here demonstrate over 5-fold increase in corners (maximum relative density of 0.51) relative to walls (maximum relative density of 0.12), as seen in Fig. 2a. Also from the perspective of flow theory, near-wall swimming hydrodynamics are well approximated by a single image of a dipole\textsuperscript{304,308} and subsequently an attractive force toward the surface\textsuperscript{303,306,307}. The presence of a corner, however, requires a second reflection of both the sperm flow field and the image\textsuperscript{309,310}. The resulting hydrodynamic effect is nonlinear, and greater than a direct combination of two walls. Recent computational modelling indicated that a simple bacterium near two orthogonal walls oscillates along one of the walls while remaining in close proximity to the corner, demonstrating that a corner has an effect distinct from the superposition of two walls\textsuperscript{311}. These findings suggest that multiple physical boundaries confine the 3D swimming trajectories of sperm to 1D trajectories along the corners, amplifying the progressive motion.

**Corner-swimming in rectangular microchannels.** To further analyze the corner-swimming preference, 100-μm high rectangular microchannels with widths of 50, 100, 200, and 400 μm were tested. The 2D scatter distributions of sperm, with red points concentrated at the corners, indicate the strong corner-swimming preference (Fig. 3a). Similar to square microchannels, the corner-
swimming preference decreased with microchannel size (Fig. 3b). Both 100×50 μm and 100×100 μm devices demonstrated a strong corner-swimming preference, with 76% corner-swimming and 22% wall-swimming sperm. By increasing only the channel width to 200 and 400 μm, corner-swimming decreased to 60% and 42%, respectively. In contrast to corner-swimming, wall-swimming increased with channel width to 34% in 200-μm wide channels and even higher to 52% (24% higher than the corresponding corner-swimming) in 400-μm wide channels. Thus as the channel width increases with height fixed, the concentration of wall swimmers increases at the expense of corner swimmers. A shift into the bulk is detectable, but not as significant as in square channels since rectangular channel cross-sections have relatively more boundary (lower area-to-perimeter ratio).

In terms of areal concentration, the relative area corresponding to corner-swimming regions decreases as channel width increases (18%, 9%, 4.5%, and 2.25% for 100-μm high microchannels with widths of 50, 100, 200, and 400 μm, respectively). Similar to square channels, the results indicate a strong corner-swimming preference for larger channels. Specifically, local areal concentrations in the corners reach over 150-fold that of the bulk for 100, 200 and 400 μm channels (227-, 169-, and 255-fold, respectively).

Histograms of sperm distance from the closest corner (Fig. 3c) show a similar trend to that of square channels (Fig. 2c), with wall swimmers most densely concentrated near the corner regions.
For 50 and 100 μm wide channels, the maximum number of sperm was captured within 5 and 10 μm of the closest corner, respectively. The peak was broadened and shifted up to 15 μm for both 200 and 400 μm widths. For rectangular channels, the higher concentration of wall swimmers near the corners results in differing densities of sperm on short and long channel walls. Fig. 3d plots the average linear density of wall-swimming sperm (percentage of wall-swimming per unit length of the microchannel wall) for both fixed height and variable width walls. Average linear density decreased by 42% and 75% along the fixed height and variable width walls, respectively, as the channel width increases from 50 to 100 μm then plateaus for larger sizes. The decreasing trends of average linear density with channel size indicates that the increase in wall-swimming preference for larger channels is mainly due to larger channel perimeter, and not due to capturing higher number of sperm per unit length of the wall. Higher density of wall-swimming sperm on short walls is attributed to the relative proximity to the corners, where sperm are densely concentrated (Fig. 3c). Additionally, the results indicate that the wall-swimming preference for sperm more than 100 μm from the wall (about one body length) is negligible as the linear density plateaus for channels wider than 200 μm.

The corner- and wall-swimming preferences reported here are not attributed to either hyperactivation or changes in swimming velocity of boundary-following sperm. First, hyperactivation could not have induced corner or wall swimming, as only progressively motile sperm will be able to swim a relatively long distance (7.2 mm, 100 body lengths) to reach the imaging section. Second, the imaging period (30 min) is long enough to allow slow swimmers to reach the imaging section.

Swimming preference in circular microchannels. Fig. 4 shows the swimming preference of sperm in circular microchannels with 100-, 250- and 510-μm diameter. Similar to rectangular microchannels, sperm exhibited a strong wall-swimming preference in circular microchannels (Fig. 4a). The wall-swimming preference decreased as the diameter of the microchannel increased, as shown in Fig. 4b. Specifically, wall-swimming linearly decreased from 98% to 91% and 85% by increasing the channel diameter from 100 to 250 and 510 μm, respectively. In terms of wall-swimming tendency and channel diameter, this is a strong linear trend ($R^2=0.99$), reflecting a straightforward dependence on area-to-perimeter ratio in the absence of corners. Importantly, the percentage of wall-swimmers for circular microchannels is comparable with combined percentage of wall and corner swimmers in square and rectangular microchannels with similar hydraulic diameters.
The proximity of imaged sperm to the circular microchannel wall is plotted in Fig. 4c. Similar to the distribution obtained for square and rectangular channels, the maximum number of sperm was captured within 5 \( \mu m \) of the wall for the smallest channels, with the peak shifting to 10-15 \( \mu m \) for larger channels. The preferred range of 10-20 \( \mu m \) is well established as the equilibrium distance for accumulation at planar surfaces\(^{303-306}\). Why sperm accumulate more closely to the surface of small channels, as observed here, is not fully clear. We expect the tighter surface accumulation is due to the proximity of the opposite wall, specifically, when geometrical confinement is comparable to the hydrodynamic interaction length-scale - both on the order of 100 \( \mu m \) (about one body length). Thus sperm travel closer to the walls in smaller channels, where the confinement matches that of the hydrodynamic length-scale.

**Influence of media on corner-swimming.** To study the influence of media properties on corner-swimming preference, we tested 100\( \times \)100 \( \mu m \) devices filled with buffers with viscosities of 20 and 100 mPa s (with pH of 7.5) and buffers with pH of 6.8, 7.5, and 8.2 (with a viscosity of 20 mPa s). Corner-swimming increased with both viscosity and pH, as shown in Fig. 5. Specifically, corner-swimming slightly increased from 76% to 82% by increasing the viscosity from 20 to 100 mPa s, with slight decrease in wall-swimming from 21% to 16%, respectively. Similarly, corner-swimming slightly increased from 68% to 76% and 79% by increasing the pH from 6.8 to 7.5 and 8.2, respectively (with corresponding wall-swimming of 29%, 21%, and 18%). The increase in corner-swimming preference by increasing viscosity and pH of the swimming medium is attributed to the change in flagellar waveform and beating pattern which alters the hydrodynamics of sperm motion\(^{267,297,312}\). Specifically, increased viscosity increases the drag forces that act on the sperm and

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**Figure 4.** Swimming preference of sperm in circular microchannels. (a) A representative cross-sectional distribution of 596 bull sperm in circular microchannels of 250 \( \mu m \) in diameter. Color bar represents the relative density of sperm in each graph. (b) PBS and PWS sperm as a function of microchannel diameter. Each points represents experiments with at least three samples with a minimum of 494 sperm imaged in each of the experiments. Values are reported as mean±s.d. (c) Histograms of sperm distance from the wall for all circular microchannels. A total number of 494 sperm is included in each case.
as a result suppresses the torsion and yaw in the swimming trajectory, while increased pH alters the flagellar waveform and increases sperm motility. Both viscosity and pH increase surface accumulation and potentially corner-swimming. The results indicate the corner-swimming remains predominant across the physiologically relevant range of the viscosity and pH in vivo.

Conclusion

We resolved and quantified corner- vs. wall- vs. bulk-swimming bull sperm in both rectangular and circular channels by confocal imaging microchannels head-on. Our results demonstrate the strong preference of sperm (~75 \( \mu \)m in length) to swim near corners (within 15 \( \mu \)m of the intersection of two walls) in rectangular microchannels. The remarkably folded and confined lumen of the fallopian tube narrows towards the egg and presents a swimming environment far more intricate than a single flat surface. The predominance of corner-swimming highlights the role of this increasing complexity of the fallopian tube in sperm guidance.
The corner-swimming preference originates from hydrodynamic interactions between sperm and surfaces. We demonstrate that the corner accumulation is more than the superposition of the influence of two walls, and over 5-fold stronger than that of a single wall. As channel size increases, the concentration of corner swimming sperm decreases, while the concentrations of wall swimming and - to a lesser extent - bulk swimming sperm increase. This shift in corner-swimming is mainly due to larger channel perimeter and higher area-to-perimeter ratio. In terms of area, local areal concentrations in the corners reach over 200-fold that of the bulk for both 100 and 400 μm square channels. The distribution of the wall-swimming sperm along the wall is non-uniform with wall-swimmers most densely concentrated near corner regions. Testing with different media properties indicated that corner-swimming remains predominant across the physiologically relevant range of the viscosity and pH in vivo. These findings suggest that multiple physical boundaries confine the 3D swimming trajectories of sperm to 1D trajectories along corners and folds, amplifying the progressive motion.

The results demonstrate that boundaries play a significant role in sperm guidance, as over 95% of the sperm traverse the channel near the walls and corners in small channels. The percentage of wall-swimmers for circular microchannels is comparable with combined percentage of wall and corner swimmers in square and rectangular microchannels with similar hydraulic diameters. For all three channel shapes studied here, we observed a tighter surface accumulation behavior when geometrical confinement is comparable to the hydrodynamic interaction length-scale - both on the order of 100 μm (about one body length). Thus sperm travel closer to the walls in smaller channels, where the confinement matches that of the hydrodynamic length-scale.

In the context of natural reproduction, these findings highlight the role of the geometrical complexity and confinement, typical of the fallopian tube in the isthmus and the ampulla, on sperm navigation. The highly folded epithelium of the tract results in narrow lumen and corners which are likely to confine the 3D swimming trajectories of sperm to 1D trajectories along the corners, amplifying the progressive motion – potentially providing a route to the egg. In addition, the changes in chemical and rheological properties of the fallopian tube can also serve as a mechanism to influence the swimming preference of sperm with respect to the boundaries and direct the sperm towards the oocyte. In the context of assisted reproduction, corners are common in emerging microchannel-based sperm selection methods. The predominance of corner swimming potentially inspires improved selection strategies for in vitro fertilization. In the context of assisted
reproduction, these findings highlight the dominant role of corner-directed sperm migration within microchannel-based sperm selection devices. The corner-swimming preference is of profound importance in developing new microfluidic technologies for motility-based sperm selection, since rectangular microchannels are the most established geometries to fabricate a microfluidic device.

Methods

Device fabrication. A microfluidic device was designed and fabricated to quantify the distribution of sperm in the cross-sectional area of a microchannel, as shown in Fig. 1. The device consisted of a vertical and a horizontal layer. The vertical layer contained an inlet and a microchannel. The semi-circular inlet served as a guide for sperm to swim into the microchannel. The vertical layer was aligned with the horizontal layer such that the microchannel cross-section was located at the center of the observation. The vertical channel exits into a cylindrical chamber (1.5 mm in diameter) in the horizontal layer. This chamber leads to eight trap reservoirs which use ratchet shape geometries\textsuperscript{280,301,302} to prevent sperm from re-entering the chamber. The ratchets are arrowhead-shaped with a concave sections around their entrance to redirect sperm back in to the trap, ensuring a unidirectional flux of the cells into the traps. The microfluidic device was designed in AutoCAD and printed on a photomask (CAD/Art Services, Inc., OR, USA). For devices with rectangular microchannels, masters with 50 µm, 100 µm, 200 µm, and 400 µm heights were fabricated from negative SU-8 photoresists (MicroChem, Newton, MA, USA) using standard soft lithography technique\textsuperscript{313}. The master for the bottom layer was always fabricated using SU-8 2075 with features 100 µm in height. Both layers were fabricated using Poly-dimethylsiloxane (PDMS) (Silgards 184: Dow Corning, MI, USA) substrate with 1:10 mixing ratio. A 1.5 mm Miltex Dermal Biopsy punch was used to punch a hole at the center of the horizontal layer. The geometry of the respective top and bottom layers were closed by bonding a plain layer of PDMS and a Micro Cover Glass No. 1 (Rectangular, 22×50 mm, VWR, PA, USA) using a hand-held corona treater (BD-20AC, Electro-Technic Products Inc., IL, USA). The microchannel in the vertical layer was cut to be 7.2 mm in length (including the inlet part), manually aligned at the center of the hole in the horizontal layer, and bonded using uncured PDMS.

For devices with circular microchannels, the inlet was designed as a disk 3 mm in diameter and 0.8 mm thick. The master for the inlet layer was fabricated by cutting a plastic disk from a 0.8 mm thick sheet of Polymethyl methacrylate (PMMA) (Plastic Word, Toronto, Canada) using a M-360 CO\textsubscript{2} laser, the disk was then bonded to a petri dish using Chloroform (Sigma-Aldrich Corp, MO, USA). Sufficient PDMS was poured to form an 8 mm thick layer, then a Miltex Dermal Biopsy punch
was used to punch a 1.5 mm diameter hole at the center of the inlet. Tubes 7.2 mm in length and inside diameter of 100 µm, 250 µm, and 510 µm (1.58 mm in outside diameter, biocompatible, DuPont FEP Tubing, Fisher Scientific, Canada) were pushed through the punched hole to form circular microchannels. The bottom layer was fabricated and the layers were bonded similarly as described for devices with rectangular microchannels.

**Buffer preparation.** HEPES-buffered saline (HBS) (135 mM NaCl, 5 mM KCl, 12 mM D-Glucose, 25 mM HEPES, 0.75 mM Na₂HPO₄·2H₂O) supplemented with 1mg/mL Poly(vinyl Alcohol) (PVA) with 0.5% and 0.875% Methyl cellulose (MC) (M0512; Sigma-Aldrich Corp, MO) was used to prepare non-Newtonian viscoelastic buffer with nominal viscosity of 20 and 100 mPa s at 20 °C according to manufacturer's manual, respectively. The viscosity of the buffer with 0.5% and 0.875% MC at 37 °C were measured using a Brookfield LVDV-E digital viscometer (Brookfield Engineering Laboratories, Inc., MA, USA) with spindle LV2 at 100 r.p.m. to be 18.95±0.15 and 88.53±1.40 mPa s, respectively. All viscosities values stated in the text are nominal values at 20 °C unless otherwise mentioned. Finally, a 1 M solution of NaOH (VWR, PA, USA) was used to adjust the buffer pH to 6.8, 7.5, and 8.2. The buffer was stored at 4 °C and used within two weeks of preparation. Buffer with 0.5% MC and pH of 7.5 was used for all of the experiments unless otherwise stated.

**Semen sample preparation.** Bull semen with approximate concentration of 50 million sperm per milliliter and 50% motility were purchased in 500 µL straws (ABS Global Inc., Canada) and stored in liquid nitrogen. Before the experiments, bull semen was thawed in a 37°C water bath for 5 min and extracted using an artificial insemination syringe. To stain live sperm with green fluorescence, 10 µL of 50-fold diluted solution of SYBR14 (Component A, LIVE/DEAD sperm viability kit, L-7011; Invitrogen, NY, USA) was added to 500 µL of semen and incubated at 37 °C for 10 min. This staining step was required to ensure that we can leverage the relatively thin focal plane in confocal microscopy to capture sperm while they swim normal to the focal plane. The bull semen was kept at 37 °C at all times, and experiments were conducted within 10 min of staining.

**Experimental procedure.** The device was filled by submerging it in buffer and applying vacuum pressure (-30 psi) for at least 1 hour and stored for about 1 hour inside a 37 °C incubator until use. All experiments were then performed at room temperature. Previous works have demonstrated that sperm motility characteristics at room temperature remain comparable to sperm motility at 37°C, for up to 3 hours. The chip was mounted to a Nikon A1 confocal microscope stage. A 40× magnification microscope objective (NA=0.6, WD=3.6 mm) was used for all of the confocal microscopy experiments except for the ones conducted with devices with the
microchannel dimension of 400 μm or larger where a 10× magnification microscope objective (NA=0.5, WD=4.0 mm) was used. A 1.2 AU pinhole was used during the confocal microscopy experiments, resulting in 12.8 μm and 14.0 μm depth of the focal plane for 40× and 10× magnification objectives, respectively. The focal plane was positioned along the vertical microchannel, 20 μm above the channel exit to the cylindrical chamber, ensuring only sperm inside the vertical channel cross-section were being imaged (section A-A’ in Fig. 1b).

Following this step, 30 μL of fluorescently labelled semen sample was pipetted into the inlet of the device. Since the semen sample was introduced at the entry of a prefilled dead-end microchannel, no flow was maintained within the microchannel during the experiments and sperm swam along the channel based on their own preference (tested using a prefilled device with buffer containing fluorescent particles). Progressively motile sperm must swim 7.2 mm along the vertical microchannel to reach the imaging section. After observing the first sperm in the microchannel cross-section in live mode of the microscope software, Confocal imaging system was used to capture sequences of bright-field and green fluorescence images with 500 ms intervals for 30 min. The bright-field images was used to recognize the channel walls in the fluorescence images. The freely available image processing software ImageJ was used to manually locate sperm across the microchannel and a custom written script in Matlab was used to process the data. It is noteworthy that the sperm swim aligned with the wall and the center of the sperm head was tracked for each point. Sperm with distance smaller than 15 μm from a wall were considered as wall swimmer sperm and sperm with distance smaller than 15 μm from two walls were considered as corner swimmer sperm, all other sperm were considered as bulk swimmer sperm. The ratio of corner swimmer, wall swimmer, and bulk swimmer sperm to total number of imaged sperm was stated as percentage of corner swimmers (PCS), percentage of wall swimmers (PWS), and percentage of bulk swimmers (PBS), respectively.

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Author contributions

R.N. and D.S. designed research; R.N., P.J.G., and Q.L. performed research; R.N., P.J.G., Q.L., and D.S. analyzed data; and R.N., P.J.G., Q.L., and D.S. wrote the paper.

Additional information

Supplementary Information accompanying this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.
8.3 Nanomorphology-enhanced gas-evolution intensifies CO₂ reduction electrochemistry

This section includes the manuscript “Nanomorphology-enhanced gas-evolution intensifies CO₂ reduction electrochemistry”, in *ACS Sustainable Chemistry and Engineering*, where I am second author. My contribution includes both manuscript editing and advice regarding formulating the mathematical model describing multispecies transport such that it can be solved using MATLAB. The skillset to contribute to this manuscript was developed during the process of the manuscript of the penalty of photosynthetic growth. Additional authors for this work include Thomas Burdyny, Yuanjie Pang, Cao-Thang Dinh, Min Liu, Edward H. Sargent, and David Sinton. Adapted with permission from: Nanomorphology-Enhanced Gas-Evolution Intensifies CO₂ Reduction Electrochemistry, Thomas Burdyny, Percival J. Graham, Yuanjie Pang, Cao-Thang Dinh, Min Liu, Edward H. Sargent, and David Sinton, ACS Sustainable Chemistry & Engineering 2017 5 (5), 4031-4040, DOI: 10.1021/acssuschemeng.7b00023. Copyright 2017 American Chemical Society.

Abstract

Nanostructured CO₂ reduction catalysts now achieve near-unity reaction selectivity at increasingly improved Tafel slopes and low overpotentials. With excellent surface reaction kinetics, these catalysts encounter CO₂ mass transport limitations at current densities circa 20 mA cm⁻². We show here that - in addition to influencing reaction rates and local reactant concentration - the morphology of nanostructured electrodes enhances long-range CO₂ transport via their influence on gas-evolution. Sharper needle morphologies can nucleate and release bubbles as small as 20 µm, leading to a four-fold increase in the limiting current density compared to a nanoparticle-based catalyst alone. By extending this observation into a diffusion model that accounts for bubble-induced mass transport near the electrode’s surface, diffusive transport can be directly linked to current densities and operating conditions, identifying efficient routes to > 100 mA cm⁻² production. We further extend this model to study the influence of mass transport on achieving
simultaneously high selectivity and current density of C2 reduction products, identifying precise
control of the local fluid environment as a crucial step necessary for producing C2 over C1 products.

SYNOPSIS: Catalyst surface morphology is used to manipulate gas-evolution from electrochemical
CO₂ reduction thereby increasing maximum reaction intensity and efficiency.

KEYWORDS: Nanomorphology, CO₂ Reduction, Gas-evolution, Limiting Current, Electrocatalysis,
Mass Transport

Introduction

The electrochemical conversion of CO₂ into fuels, both gaseous and liquid, is an emerging
approach to mitigate fossil fuel based carbon emissions. Competing reactions, poor surface binding
energies, low reactant solubility and electrode stability all limit system performance and are major
obstacles preventing electrochemical CO₂ reduction from widespread adoption. To advance
the field two distinct approaches have been adopted in literature: 1) improving electrode kinetics,
for instance through nanostructured electrodes with low activation potentials, high selectivity and
high Tafel slopes; and 2) improving mass transport, such as increasing long-range CO₂ flux using
pressurization, flow or gas diffusion electrodes. The field has advanced greatly in improving
reaction kinetics while further gains remain available in understanding and improving CO₂
transport to electrodes. Additionally, as electrocatalytic kinetics improve, transport limitations
become more significant and ultimately limit performance.

Recent electrocatalysts for the CO₂ reduction reaction (CO₂RR) report high Tafel slopes and lower
activation overpotentials when converting CO₂ into CO, HCOOH and multi-carbon products. In each
case performance advancements are attributed to engineered surface mechanisms. These strategies
range from high energy surface structures that improve CO₂ surface binding energies; oxide-
derived materials that preferentially bind CO₂/CO; and sharp morphologies resulting in
field-induced reagent concentration.\textsuperscript{329} As current is increased, however, the overpotential needed to further boost current density steadily increases due to pH gradients formed at the electrode and a decrease in the local concentration of CO\textsubscript{2}.\textsuperscript{330,331} At even higher currents, the CO\textsubscript{2} concentration at the electrode becomes depleted due to both consumption of CO\textsubscript{2} in the electrochemical reaction and unfavourable local pH conditions, limiting the maximum current density of CO\textsubscript{2} conversion. This limit cannot be overcome via CO\textsubscript{2} electrokinetics at the electrode surface. Increasing system pressure\textsuperscript{322,332} and fluid flow\textsuperscript{333} via devices are often quoted as a means to improve current density and selectivity in state-of-the-art electrodes. Understanding the mass transport dynamics at play in experimental systems is essential, however, due to the direct influence of mass transport on observed catalyst performance. As an example the limiting current density of state-of-the-art electrodes has been experimentally shown to be enhanced by different surface morphologies (e.g. \textasciitilde{} 5 mA cm\textsuperscript{-2} for a flat Ag metallic surface\textsuperscript{334}, \textasciitilde{} 15 mA cm\textsuperscript{-2} for Au-oxide nanoparticles\textsuperscript{326}, \textasciitilde{} 55 mA cm\textsuperscript{-2} for Au nanoneedles\textsuperscript{329}). Additionally, Rosen et al. reported a shift in the limiting current from \textasciitilde{} 10 mA cm\textsuperscript{-2} to \textasciitilde{} 35 mA cm\textsuperscript{-2} when switching from Ag nanoparticles to a nanoporous Ag structure under the same testing conditions.\textsuperscript{335} These differences imply a significant impact of the electrode surface itself on the flux of CO\textsubscript{2} from the bulk to the electrode, although the exact mechanisms are unclear. There is a need to better understand the complex interactions between the catalyst and the surrounding fluid such that experimental results can be better interpreted and, more importantly, future CO\textsubscript{2} reduction systems can be designed to reach higher currents.

To gain a more in-depth understanding of catalyst/electrolyte interactions several studies have investigated the dynamics of CO\textsubscript{2} and ion concentrations within a prescribed fluid diffusion layer at an electrode surface (\textasciitilde{} 10 – 1000 \textmu m).\textsuperscript{336–340} These studies allowed for the performance impacts of pH and CO\textsubscript{2} gradients, electrolyte concentration and temperature to be explored, identifying that significant overpotential losses and CO\textsubscript{2} depletion occurs at relatively low current densities. The diffusion thickness which controls the limiting current density, however, is assumed to be a fixed
value independent of operating conditions and electrode morphology. In parallel, other efforts have shown that mass transport due to gas-evolution is a primary driver of the diffusion thickness with transport increasing with current density and bubble release frequency.\textsuperscript{341-344} Hydrogen evolution applications have subsequently utilized gas-evolution and electrode morphology to increase the physical stability of electrodes by reducing the residence time of formed hydrogen bubbles\textsuperscript{345} while also increasing efficiencies by increasing active area and reducing ohmic drops and hysteresis at larger currents.\textsuperscript{346-349} Applying these lessons to CO\textsubscript{2} reduction applications, the morphology of a CO\textsubscript{2} reduction catalyst can be similarly manipulated to enhance gas-evolution mass transport and increase the maximum intensity of CO\textsubscript{2} reduction electrochemistry. By combining existing CO\textsubscript{2} electrochemical models with gas-evolution theory and experimental observations a more complete model can be developed which accounts for the interconnected factors governing CO\textsubscript{2} reduction, illuminating existing and future experimental results.

In short, rapid recent advancements in catalyst performance coupled with low CO\textsubscript{2} solubility demand that transport limitations within CO\textsubscript{2} reduction systems be better understood and addressed. Herein we combine experimental observations with a mathematical model to quantify the role that catalyst nanomorphology plays in significantly enhancing mass transport for CO\textsubscript{2} reduction to CO and increasing the intensity at which the reaction can occur. Experimentally we visualize gas-evolution on established electrocatalyst electrode morphologies - nanoneedles, nanorods and nanoparticles - and use the resulting observations to obtain a volume-averaged gas-evolution diameter for the surface. Combined with an electrochemical model accounting for mass transport from gas-evolution we compare our predicted findings with experimental results from Au nanoneedles and a similarly selective nanoparticle catalyst, emphasizing the influence of morphology-enhanced gas-evolution on the limiting current of experimental systems. Using this new perspective we discuss the impacts of electrode kinetics and CO\textsubscript{2} availability on product selectivity and partial currents as operating currents are varied. Finally, we extend our new
interpretation of CO\textsubscript{2} reduction modelling to multi-carbon (C2) products to identify existing mass transport limited performance and suggest an alternative experimental approach for achieving both high ethylene selectivity and low current densities on a copper catalyst.

**Modelling Section**

To model the performance of an electrochemical CO\textsubscript{2} reduction reaction it is necessary to take into account the combined effects of the electrode kinetics of the catalyst and the catalyst's interaction with the electrolyte. The electrode kinetics are modelled using the Butler-Volmer equation and experimentally obtained parameters for CO\textsubscript{2} reduction and hydrogen evolution (see Supplementary Information). The electrolyte/catalyst interactions are modelled as a 1-D diffusion system as discussed in the following sections.

**Calculation of the diffusion thickness**

As reactants (both CO\textsubscript{2} and H\textsuperscript{+}) are consumed at a catalyst's surface during an electrochemical reaction the concentration of molecules in the electrolyte will vary from that of the bulk. The concentration gradient is characterized by a diffusion layer adjacent to the electrode whose thickness directly correlates to the reaction's limiting current density and polarization losses. In previous electrochemical models for CO\textsubscript{2} reduction the diffusion layer thickness is prescribed as a specific value independent of current density or electrode morphology. Here we approximate the thickness of the diffusion layer as a function of operating parameters, allowing it vary with current density.

In a 1-D planar system the thickness of the diffusion layer is equal to the diffusivity of the species of interest, $D_i$, divided by the mass transfer coefficient, $k_m$, present in the system:

$$
\delta_i = \frac{D_i}{k_m}
$$

(1)

The mass transfer coefficient can be found by accounting for the combined effects of the two primary mass transfer mechanisms present in the system: convective bulk flow and bubble-induced momentum from gas-evolution.
\[ k_m = k_{\text{conv}} + k_{\text{bubble}} \]  \hspace{1cm} (2)

Mass transfer effects for convective flow can be found using the well-known correlation for flow over a flat plate where the average mass transfer coefficient calculated from the Sherwood number is:

\[ k_{\text{conv}} = \frac{\bar{Sh}D_i}{L} = \frac{0.664D_i}{L} \left( Re_L \right)^{0.5} \left( Sc \right)^{0.333} \]  \hspace{1cm} (3)

where the characteristic length, \( L \), is the length of the electrode in direction of flow. The Reynold’s number can be approximated from the stirring or flow conditions of the electrolyte.

For gas-evolution, several mechanisms contribute to the replenishment of reactant at the electrode surface including bubble growth, break-off and wake flow. A number of numerical correlations have been created to describe these mass transfer processes using a combined mathematical and empirical approach. Collectively, however, these correlations are in relative agreement as described by Vogt et al.\textsuperscript{341,342} To describe the effects of bubble break off where fluid immediately replaces the departing bubble, \( Sh_1 \), we use the Roušar correlation\textsuperscript{350,351} and to describe the combined effects of bubble growth and wake flow, \( Sh_2 \), we use Vogt’s correlation for low electrode bubble coverage:\textsuperscript{342}

\[ Sh_1 = \sqrt{\frac{12}{\pi}} Re_G^{0.5} Sc^{0.5} \Theta^{0.5} \]  \hspace{1cm} (4)

where \( \Theta \) represents the fraction of the electrode area shielded by bubbles during a bubble’s residence time and thus not available for reactions. The ratio \( R_a/R \) in Eq. 5 represents the ratio of inactive electrode area below a nucleated bubble as it grows. The Reynold’s and Schmidt numbers for gas-evolution are described as:

\[ Re_G = \frac{\nu_{\text{gas}} d_b}{A} \]  \hspace{1cm} (6)

\[ Sc = \frac{\nu}{D} \]  \hspace{1cm} (7)
For gas-evolution the bubble departure diameter, $d_b$, acts as the characteristic length scale while $\nu$ is the kinematic viscosity of the electrolyte. The volume flux from gas-evolution ($\dot{V}_{\text{gas}}/A$) represents the effective gas velocity and is defined as:

$$\frac{\dot{V}_{\text{gas}}}{A} = \frac{j_{\text{evolved}} RT}{n_e F P}$$

(8)

where pressure, $P$, and temperature, $T$, are generally known during experiments and the gas-evolving current, $j_{\text{evolved}}$, is the sum of all currents which form products that depart in the gaseous phase.

As described by Vogt the mass transport effects from bubble break-off and growth/wake flow occur simultaneously and compete with one another. These can thus be combined into a single Sherwood number from which the overall mass transfer coefficient from gas-evolution can be extracted:

$$S_{h_{\text{bubble}}} = (S_{h_1}^2 + S_{h_2}^2)^{0.5}$$

(9)

For our analysis we assume that the bubble coverage, $\theta$, is a constant 25% while $R_a/R$ is assumed to be 0.75.$^{342}$ It is worth noting that the mathematically derived Sherwood numbers in Eq. 4-5 assumed a flat electrode and a bubble contact angle of 90°. As nanostructured electrodes have a much higher electrochemically active surface area and are generally hydrophilic, the mass transfer coefficient calculated here is conservative, under-predicting most CO$_2$ reduction electrodes due to the reduced fraction of inactive surface where bubbles contact the electrode surface.

From Eqs. 1-10 the thickness of the diffusion layer can be calculated as a function of the gas-evolving current density, bubble departure diameter, pressure and fluid flow velocity. The bubble departure diameter is an experimentally determined quantity which depends upon the morphology and wettability of a catalyst’s surface.

**Numerical modelling of the diffusion layer**
With the diffusion layer thickness calculated as a function of operating conditions, the concentrations of reactants within the diffusion layer can be approximated using the Nernst-Planck equation.\textsuperscript{336,340,338} Here we utilize a similar approach to Gupta et al. assuming that the bulk electrolyte remains saturated with CO\textsubscript{2} during the reaction and the effects of migration are negligible.

We first account for the chemical equilibrium formed between CO\textsubscript{2}, HCO\textsubscript{3}, CO\textsubscript{3}\textsuperscript{2}, OH\textsuperscript{−} and H\textsuperscript{+} in an aqueous solution governed by the following equations:

\begin{align*}
CO_2(aq) & \rightleftharpoons CO_2(g) \quad (11) \\
CO_2(aq) + OH^- & \rightleftharpoons HCO_3^- \quad (12) \\
HCO_3^- + OH^- & \rightleftharpoons CO_3^{2-} + H_2O \quad (13) \\
H_2O & \rightleftharpoons H^+ + OH^- \quad (14)
\end{align*}

whose respective equilibrium constants $K_0$, $K_1$, $K_2$ and $K_w$ are determined as functions of temperature and salinity as described in the Supplemental Information. From these equations the bulk concentrations of each component can be found for different electrolyte concentrations, temperatures and operating pressures. The “salting out” effect of CO\textsubscript{2} due to the addition of NaHCO\textsubscript{3} and KHCO\textsubscript{3} is also accounted for as described by Eqs. (S5)-(S7).

Conservation of mass can then be applied within the diffusion layer bounded by the catalyst surface ($x = 0$) and the bulk electrolyte ($x = L$) resulting in the following governing equations:\textsuperscript{340}

\begin{align*}
\frac{\partial [CO_2]}{\partial t} &= D_CO_2 \frac{\partial^2 [CO_2]}{\partial x^2} - [CO_2][OH^-]k_{1f} + [HCO_3^-]k_{1r} \quad (15) \\
\frac{\partial [HCO_3^-]}{\partial t} &= D_{HCO_3^-} \frac{\partial^2 [HCO_3^-]}{\partial x^2} + [CO_2][OH^-]k_{1f} - [HCO_3^-]k_{1r} - [HCO_3^-][OH^-]k_{2f} + [CO_3^{2-}]k_{2r} \quad (16) \\
\frac{\partial [CO_3^{2-}]}{\partial t} &= D_{CO_3^{2-}} \frac{\partial^2 [CO_3^{2-}]}{\partial x^2} + [HCO_3^-][OH^-]k_{2f} - [CO_3^{2-}]k_{2r} \quad (17) \\
\frac{\partial [OH^-]}{\partial t} &= D_{OH^-} \frac{\partial^2 [OH^-]}{\partial x^2} + [CO_2][OH^-]k_{1f} + [HCO_3^-]k_{1r} - [HCO_3^-][OH^-]k_{2f} + [CO_3^{2-}]k_{2r} \quad (18)
\end{align*}

with the diffusion coefficients, $D_i$ and equilibrium rate constants ($k_{1f}$, $k_{1r}$, $k_{2f}$, $k_{2r}$) found in Supplementary Information. At the catalyst surface the flux of CO\textsubscript{2} and OH\textsuperscript{−} are proportional to the
CO₂ reduction current density and total current density, respectively. At the edge of the Nernst diffusion layer the concentrations of each species equal the bulk concentrations as determined from the equilibrium expressions in Eqs. 11-14. The concentration of each species in diffusion layer can then be solved by prescribing a current density and Faradaic efficiency.

Modelling electrochemical CO₂ reduction

From the steady-state concentrations of CO₂ and OH⁻ at the catalyst surface found using Eqs. 11-18 the polarization overpotentials resulting from pH gradients and the depletion of CO₂ reactant are post-calculated as follows:

\[ \phi_{pH} = \frac{2.303RT}{F} \left( \log ([OH^-]_{x=\delta}) - \log ([OH^-]_{x=0}) \right) \]  \hspace{1cm} (19)

\[ \phi_{CO_2} = \frac{RT}{nF} \log \left( \frac{[CO_2]_{x=\delta}}{[CO_2]_{x=0}} \right) \]  \hspace{1cm} (20)

where \( R \) is the universal gas constant, \( F \) is Faraday’s constant and \( n \) is the number of electrons required for the CO₂ reduction reaction. On the gold catalyst used in this paper only CO₂ reduction to CO and hydrogen evolution are assumed to take place which are both two electron processes:

\[ 2H_2O + 2e^- \rightarrow H_2 + 2OH^- \]  \hspace{1cm} (21)

\[ CO_{2(aq)} + H_2O + 2e^- \rightarrow CO + 2OH^- \]  \hspace{1cm} (22)

The overpotential losses can then be added to the previously obtained Butler-Volmer curves for CO₂ reduction to CO and hydrogen evolution to predict the voltage vs. current curve for the cathodic reaction.

During the reaction both CO and H₂ evolution are present with both contributing to the evolved gas in Eq. 8 and subsequently the diffusion thickness and polarization losses. Modelling the electrochemical reaction from the Tafel curves without prescribing a Faradaic efficiency then requires an iterative process as the polarization losses are post-calculated and the effects of CO₂ depletion only affect the CO evolution reaction. To that end an initial Faradaic efficiency is assumed and the simulation run to gain an initial estimation of the polarization losses. From the resulting overpotentials a new Faradic efficiency is calculated and the simulation is re-run until the iterated

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Fig. 1: Impact of operating conditions on the maximum performance of gas-evolving CO$_2$ reduction systems. a) Effect of various monodisperse bubble departure diameters on diffusion layer thickness at P = 1 atm, b) effect of diffusion thickness on the CO limiting current density at 100% CO selectivity for various total and partial pressures (0.5 M KHCO$_3$ electrolyte), c) effect of Faradaic Efficiency and hydrogen evolution on the limiting current density in CO production on Au catalysts.

value converges. At convergence the applied electrode potentials for CO$_2$ reduction and H$_2$ evolution are equal and the partial current densities sum to the prescribed total. Finally, the limiting current density for CO$_2$RR is determined to be the effective current density where the concentration of CO$_2$ at the electrode surface is equal to the rate at which it is generated by bicarbonate equilibrium.

Results and Discussion

Influence of gas-evolution on mass transport and CO$_2$ reduction intensity

Mass transport in electrochemical CO$_2$ reduction systems is imperative given the low solubility of CO$_2$ in aqueous systems and the large increase in currents needed to reach industrially relevant currents. Within aqueous electrochemical systems gas-evolution is known to be a primary driver of transport but depends on factors such as current density, bubble dynamics and wettability among others. Here we utilize mass transfer relations for gas-evolution and convective flow to estimate an effective diffusion layer thickness (Nernst diffusion layer) which can be used in a broader electrochemical CO$_2$ reduction model for predicting CO$_2$ reduction performance.

Using Eqs. 1-10 we calculate the expected diffusion thickness for various monodisperse bubble departure diameters and currents from gas products (gas-evolving currents) as seen in Figure 1a.
Since most aqueous systems are stirred, the approximated diffusion layer for a stirred beaker at ~500 rpm is also shown\textsuperscript{352} assuming a local flow velocity of 1 cm s\textsuperscript{-1} and electrode width of 0.5 cm. Figure 1a implies that the effective mass transport at the electrode due to bubble growth and departure is much greater than a moderately stirred beaker, particularly at current densities > 10 mA cm\textsuperscript{-2} where the diffusion thickness for even large bubbles is half that of a stirred beaker alone. As departure diameter is decreased the number and frequency of bubbles on a catalyst's surface increases resulting in a boost in mass transport. An increase in the gas-evolving current further decreases the diffusion thickness as observed experimentally elsewhere.\textsuperscript{353,354} Previous CO\textsubscript{2} reduction models\textsuperscript{331,340,338,337,339} have utilized a fixed diffusion layer rather than accounting for the large variability that occurs as current density and bubble departure diameters are altered.

The effects of altering the bulk CO\textsubscript{2} concentration are observed in Figure 1b by varying the total and partial pressure of CO\textsubscript{2} assuming an electrode selectivity of 100% towards CO. While all three curves are proportional the absolute current densities are very different owing to the increased dissolved CO\textsubscript{2} at higher pressures. Observing the 1 atm curve we can see that overcoming the commonly seen limiting currents of ~20 mA cm\textsuperscript{-2} requires diffusion thicknesses of less than 50 µm. Reaching these thicknesses would then require either a significant increase in forced convection or enhanced product release from the catalyst surface. Figure 1b also shows that pressure and mass transport have an additive effect on CO\textsubscript{2} availability and hence the maximum CO\textsubscript{2} reduction intensity while highlighting the need for pure CO\textsubscript{2} inputs even in high mass transport cases.

Given the importance of selectivity on performance, the impacts of Faradaic efficiency are also analyzed for a catalyst capable of producing CO and H\textsubscript{2}. As shown in Figure 1c as H\textsubscript{2} evolution is increased the maximum intensity for CO production subsequently increases following from the increase in evolved gases. The relative impact of bubble diameters also indicates that only small bubbles diameters are expected to greatly increase the absolute CO\textsubscript{2} reaction intensity.
Although mass transport from gas-evolution has been studied both experimentally and numerically, several limitations exist when applying the derived relations to experimental catalysts. For instance the Sherwood numbers in Eqs. 4-5 generally assume a horizontal, flat electrode in a stagnant reactor. Directionality and convective flow both effect gas-evolution dynamics but variations versus stagnant conditions are generally considered minimal in low flow conditions (< 0.1 m s⁻¹) versus the overall transport mechanism.³⁵⁴,³⁵⁵ Electrode bubble coverage is another assumption which factors into both the derivation and use of Eqs. 4-5 and varies between 0.1 and 0.3 for the current densities of interest.³⁵⁶ While mass transport from gas-evolution is greatly reduced for lower values of bubble coverage Vogt found the approximation to under-predict experimental findings as bubble coverage tends towards zero. These combined factors reflect some uncertainty but the overall calculated magnitude of the mass transfer coefficient remains similar to that predicted in Figure 1 for various operating conditions.

**Nanomorphology-enhanced mass transport**
Nanomorphology has been effective in improving electrode kinetics and reducing the overpotentials needed to convert CO\textsubscript{2} at low currents via a number of surface based approaches. To maintain record efficiency and selectivity at higher currents, however, the long-range flux of CO\textsubscript{2} to the electrode must be increased. As the reduction of CO\textsubscript{2} into CO necessarily results in gas-evolution – which in turn promotes dissolved CO\textsubscript{2} flux – enhancement of this phenomenon by manipulating nanomorphology provides a secondary means for the catalyst surface to influence reaction rates.

To assess the influence of nanomorphology on mass transport we first visualized CO gas-evolution using a dark field microscope for three different electrode morphologies shown in Figure 2a-c: Au nanoneedles, nanorods and nanoparticles. The nanoneedles had a noticeably smaller bubble departure diameter with a mean diameter of 23 \( \mu \text{m} \) based upon the volume-averaged bubble diameter (Figure 2d and Supplementary Video). Under otherwise similar conditions, bubbles on the nanorod and nanoparticle surfaces had average release diameters of 31 \( \mu \text{m} \) and 97 \( \mu \text{m} \), respectively (Supplementary Video). In the case of nanoneedles and nanorods a large
number of small bubble trains emanated from the surface with some larger bubbles seen in the nanorod case which increased the overall diameter. For the nanoparticles, however, bubbles resided on the surface for much longer times allowing for growth via diffusion. Exposed carbon paper in areas where the catalyst was removed showed the greatest bubble release diameters as expected from the reduced wettability versus gold. Through their structure, nanoneedles reduce the bubble contact diameter decreasing the forces adhering it to the surface, and the increased agitation promotes bubble detachment. The effervescent generation of small bubbles from the nanoneedles (Figure 2) provides improved transport as quantified by the small diffusion thickness (Figure 1) and intensified CO$_2$ reduction. While the volume-averaged bubble diameter can be used in Eqs. 4-10 to calculate an effective diffusion layer thickness it is accurate only for monodisperse or narrow bubble distribution. For bimodal or polydisperse distributions a collective mass transfer coefficient should instead be calculated using a weighted average of the mass transfer coefficients of bubble sub-populations. As detailed in the Supporting Information, including measured bubble size distributions resulted in variations of only 5% in terms of diffusion thickness for nanoneedles and particles (details in the Supporting Information).
Using our varying-diffusion-layer electrochemical model (Eqs. 1-22), we predict the performance of Au nanoneedles and a similarly selective nanoparticle CO$_2$RR catalyst with a flatter morphology. We chose Au-oxide nanoparticles$^{326}$ as an appropriate fair point of comparison due to the similar product, high selectivity, good Tafel slope and defined microstructure. The electrode kinetics of each morphology are separated from transport related effects by using the experimentally determined Tafel slopes and exchange current densities (see Supporting Information online). Inputting the system operating conditions into our 1D model, we can then simulate the expected overpotentials and polarization losses for a range of current densities and average bubble size in the system (see Modelling Section for details). The Faradaic efficiency of CO and H$_2$ evolution are

Fig. 3: Comparison between predicted and experimental steady-state current versus voltage curves for assumed bubble departure diameters of 20, 50 and 100 µm highlighting the impact of morphology on limiting current density and polarization losses. a) Performance of Au nanoneedles$^{12}$ in 0.5 M KHCO$_3$, b) Performance of Au-oxide nanoparticles$^9$ in 0.5 M NaHCO$_3$. 
calculated in the model by accounting for the combined effects of the electrode kinetics, electrolyte conditions and polarization losses. Figure 3a and b show the predicted CO partial current density versus overpotential for three different bubble departure diameters with the experimental results overlaid. Also included is the CO evolution Tafel curve (m in our simulation best matched the nanoparticles an inputted mean bubble diameter of 100 experimental limiting current density of 15 mA cm$^{-2}$, comparing well with our experimentally observed bubble diameters on Au nanoparticles (Figure 2d) and similar to that reported elsewhere on a Pt catalyst. For nanoneedles, the curve simulated using a 20 µm bubble diameter was needed to boost the limiting current to > 50 mA cm$^{-2}$, giving a similar result to the observed bubble diameter from Figure 2d. In both cases the partial CO current slowly increases after temporarily plateauing as hydrogen evolution provides the further mass transport needed to supply additional CO$_2$ (see Figure 2d). Together, these results show the importance of morphology-dependent gas-evolution dynamics on experimental CO$_2$ reduction performance and their importance in modelling electrochemical systems.
Fig. 4: a) Electrochemical cell for CO\textsubscript{2} reduction showing either a flatter or a sharper nanostructured electrode (scale bar is 20 \textmu m). b) Schematic depicting the influence of electrode morphology on bubble departure diameter and the resulting thickness of the diffusion boundary layer. Fresh electrolyte fills the region previously occupied by nucleated bubbles on the electrode’s surface.

In Figure 4 we illustrate how electrode nanomorphology can increase long-range transport by influencing gas-evolution, resulting in a four-fold increase in limiting current density of CO production for nanoneedles over nanoparticles. Similar to what has been found in heat transfer analyses along a surface\textsuperscript{357}, the enhanced mass transport stems from a reduction in the departure diameter of bubbles from the electrode surface. Designing electrodes that provide this level of mixing can then be more efficient and potentially forego high-velocity forced fluid flow that would otherwise be necessary to provide sufficient CO\textsubscript{2} to a surface. Further pairing the above passive approach for increasing current density with a pressurized system a high current density and product purity system may also be possible as an alternative to gas diffusion electrodes which limit product purity in the effluent stream and have poorer catalyst flexibility.

From both Figure 3a and b it is also possible to see the effect that mass transport has on polarization losses in the system, particularly as the current approaches the limiting value. These losses become inevitable as CO\textsubscript{2} concentration decreases and supports the findings of Chen et al.,\textsuperscript{337} regarding the importance of operating electrochemical CO\textsubscript{2} reduction cells at 80 – 90% of their maximum current density. It is important to note that differences in electrode morphology in turn result in large differences in the overall electrochemically active surface area which impacts both electrode kinetics and the current density normalized by the active surface area.\textsuperscript{329} In addressing the limiting CO2RR current density of the electrode, however, the geometric current density is of
primary importance as the flux of CO$_2$ to the catalyst is dependent on the planar area of the electrode rather than the specific surface area of catalyst.

Further optimization of electrode morphology for mass transport purposes is also possible as exhibited by bubble trains as small as 10 µm in portions of the nanoneedle and nanorods electrodes (Figure 2d). Facilitating bubble departure could be accomplished, for instance, using a more organized needle structure or even including non-participating structures which promote bubble nucleation and detachment. Increasing electrode wettability would achieve a similar result but changes to the surface would simultaneously impact electrode kinetics. Finally, adding a non-ionic surfactant to the liquid at close to the critical micelle concentration can lower the surface tension of water from ~72 mN m$^{-1}$ closer to ~33 mN m$^{-1}$, enough to aid in the release of bubbles from the surface at a smaller diameters.$^{358-360}$ As confirmation, by adding 0.8 times the CMC of Triton X-100 to the electrolyte we were able to reduce the residence time of bubbles by over half with an average departure diameter from the catalyst surface of 40 µm (Figure S1 and Supplementary Video online), indicating another route to higher CO$_2$ mass transport via gas evolution.

**Effect of CO$_2$ availability on CO$_2$ reduction selectivity**

Selectivity towards specific CO$_2$ reduction products (CO, HCOOH, C$_2$H$_4$, etc.) over H$_2$ evolution has been the central subject of numerous theoretical$^{361-363}$ and experimental$^{364,365}$ works. These efforts explore direct conversion kinetics but leave out CO$_2$ availability as a driver of CO$_2$RR selectivity, and the corresponding impact of mass transport as current increases.

Using nanoneedles, we identify the contributing factors of CO$_2$ availability towards selectivity. From the reported Tafel slope the ideal CO$_2$RR curve for the FIRC needles is shown in Figure 5a. Also using the experimental results, we extract the HER (Hydrogen Evolution Reaction) Tafel curve and plot within the same figure (see Modelling Section)-depletion (denoted by $\mathcal{F}$) assuming a predicted polarization losses from pH gradients at m. As current density increases these losses volume averaged bubble departure diameter of 20
steadily increase proportional to the OH− and CO2 concentration at the electrode. It is important to note that the overpotential due to a pH gradient at the electrode surface is a function of the total current density and affects both reaction products. The total and partial current densities for CO and H2 are then plotted in Figure 5b next to experimental data. Finally the selectivity towards CO as well as the electrode CO2 concentration for a range of overpotentials are plotted in Figure 5c, agreeing well with the Au needle experimental data.329

Three interesting conclusions can be made from the plots in Figure 5. Firstly, as long as the Tafel slope of CO2RR is greater than that of HER and the activation overpotential is lower, the high Faradaic efficiency of CO production is maintained provided ample CO2 is supplied to the electrode. Concentration polarization losses are then kept small until ~45 mA cm−2 where selectivity slowly begins to decrease. Secondly, as H2 evolution increases at higher overpotentials the thickness of the diffusion layer continues to decrease due to additional gas generation and mass transport, as seen in Figure 1a and Figure S2. The partial current density of CO then sees a small rise at higher voltages instead of plateauing, albeit at lower FE. Without transport increasing with gas-evolution the absolute CO partial current would remain constant after the limiting current. Finally, the poor CO Faradaic efficiency below 0.2 V is the result of more favourable H2 evolution even though H2 evolution kinetics are worse at higher overpotentials (highlighted inset in Figure 5a). This widely seen trend is due to the 0.10 V lower activation potential for H2 evolution (E°H2 = 0 V versus E°CO2 = -0.10 V RHE).

The analysis here can be applied to any mixture of gas and liquid CO2RR products and can be used to determine losses and the limiting current density even when a large number of products are present (eg. on a copper electrode). The greatest CO2RR current densities will be seen at higher volumes and ratios of gas products as described by Figure 1a and b. Systems with purely liquid CO2RR products have shown lower current densities than gaseous products (eg 5 – 10 mA cm−2 for formate), implying that performance suffers in the absence of bubble-induced transport.
Fig. 5: a) Butler-Volmer curves for CO$_2$RR and HER for gold nanoneedles with CO$_2$ saturated 0.5 KHCO$_3$. Inset shows more 20 $\mu$m bubble departure diameter. b) Partial and total simulated current densities. c) Selectivity and CO$_2$ availability of the catalyst for CO2RR using the loss adjusted CO$_2$RR and HER curves in a). Red experimental data is from Au nanoneedles in 0.5 M KHCO$_3$. Favorable HER kinetics at low overpotentials (B) for a
**High intensity production of C2 products**

Here we extend the experiment-validated idea of a variable diffusion layer and current-dependent mass transport to C2 reduction products in an effort guide future experiments towards both high product selectivity and current density. To date both experimental and modelling efforts have shown the importance of a high local electrode pH on ethylene (C₂H₄) selectivity due to the pH-dependent pathway for methane production allowing for high Faradaic efficiencies to be achieved on copper electrodes using low buffering capacities (0.1 M) and increased system pressures. Operating at too high a pH, however, shifts carbonate equilibrium away from CO₂. As a consequence, while lower electrolyte buffering capacities results in high ethylene selectivities the ethylene partial current is constrained at higher currents due to a shortage of CO₂. Rather than operating at a high local pH the local environment of the catalyst should be regulated to achieve both the desired ethylene selectivity and current density. In Figure 6 we briefly show the sensitivity of common operating conditions (buffering, pressure, mass transport) on the local pH and CO₂ availability versus a standard operating condition (0.1 M KHCO₃, 1 atm) using our model accounting for current dependent mass transport.

For simplicity we assume a Faradaic Efficiency distribution of 40% ethylene, 40% hydrogen and 20% liquid HCOOH in all cases though the exact ratio of methane/ethylene would change with current and pH. A bubble departure diameter of 100 µm is assumed to represent a typical electrode surface while a baseline convective flow from stirring is also assumed as before (500 rpm, Figure 1a). The effects of buffering capacity and pressure on CO₂ solubility and carbonate equilibrium are again taken into account in the Supporting Information. Termination of a line indicates the point where no CO₂ is present at the catalyst surface which in all cases happened close to a pH of 12.
Observing the curves in Figure 6 the relative impacts of changing each operating condition on the maximum current density and pH can be seen. First, systems with low buffering capacity will reach high pH values at overall lower current densities allowing for higher ethylene/methane ratios to be produced at low currents. The penalty with this approach, however, is the CO$_2$ at the electrode being depleted at modest currents of ~ 40 mA cm$^{-2}$. The maximum ethylene partial current is then limited to less than 16 mA cm$^{-2}$ as has been obtained experimentally. As the buffer is increased to 0.2 M the current needed to reach higher local pH increases, implying that more methane will be produced at lower currents. Higher total currents, however, are possible as compared to the more weakly buffered case. When the electrolyte concentration is brought back to 0.1 M and the pressure increased to 3 atm a similar initial trend exists as in the 1 atm case. Due to the increased CO$_2$ concentration at higher pressures, however, the system remains buffered and is allowed to reach total currents of over 65 mA cm$^{-2}$ before depleting all available CO$_2$. It is worth noting that the increase in current over the 1 atm case is only 25 mA cm$^{-2}$ showing the necessity of either further buffering or mass transport to ensure that the 3-fold larger initial CO$_2$ concentration is fully-utilized for ethylene production. Finally we see that in the case of enhanced mass transport from a smaller

![Fig. 6](image-url) Fig. 6: Local electrode pH as a function of current density for various electrolyte KHCO$_3$ concentrations, pressures and bubble departure diameters. Selectivity fixed as 40 % C$_2$H$_4$, 40 % H$_2$ and 20 % HCOOH. The termination of a line indicates that CO$_2$ has been fully depleted.
bubble diameter of 20 µm, maximum currents of over 100 mA cm\(^{-2}\) are possible even at a low electrolyte concentrations. Each of the proposed approaches is a demonstration of how higher ethylene partial currents can be reached by preventing the local electrode pH from getting so high that CO\(_2\) is lost to a shift in the carbonate equilibrium. Controlling pH with sufficient combinations of electrolyte, pressure and mass transport then provides an avenue to both high ethylene selectivity and current density. These results highlight the importance of combining modelling and experimental efforts to ensure that the best operating and testing conditions are identified to maximize performance.

**Conclusions**

Widespread application of CO\(_2\) reduction will necessitate high current densities and progress in both electrode kinetics and mass transport. These are linked by the low solubility of CO\(_2\) in aqueous media and the importance of process conditions on measured performance. In addition to influencing reaction rates and local reactant concentration, we show that the morphology of nanostructured electrodes can also enhance long-range CO\(_2\) transport via their influence on gas-evolution, effectively extending the reach of the nanostructure into solution. This is supported by an electrochemical model which deviates from previous CO\(_2\)RR efforts with the inclusion of bubble-induced mass transport via a variable diffusion layer thickness. This relatively straightforward approach replicated trends observed experimentally, most notably differences in limiting currents achieved with different electrode morphologies. A more complete understanding of the transport mechanisms affecting the selectivity and current densities of experimental electrodes and their results is then enabled as compared to previous works. Application to gas, liquid or combinations of products can help to guide experiments towards more promising operating conditions as well as interpreting existing results. The complexity of the model would need further enhancement for scenarios where factors such as surface poisoning affect product composition but could be incorporated into the underlying electrode kinetics. One instance of this involves CO surface
poisoning which can either promote or delay hydrogen evolution at lower voltages. Collectively, this work illustrates how electrode morphologies influence not only short-range reaction and concentration effects but long-range CO$_2$ transport, and provides a framework for quantifying the impacts of operating parameters and electrode kinetics on product selectivity and efficiency.
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