High-throughput optimisation of light-driven microalgae biotechnologies

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Microalgae biotechnologies are rapidly developing into new commercial settings. Several high value products already exist on the market, and systems development is focused on cost reduction to open up future economic opportunities for food, fuel and freshwater production. Light is a key environmental driver for photosynthesis and optimising light capture is therefore critical for low cost, high efficiency systems. Here a novel high-throughput screen that simulates fluctuating light regimes in mass cultures is presented. The data was used to model photosynthetic efficiency ($\text{PE}_{\mu}$, mol photon$^{-1}$m$^{-2}$) and chlorophyll fluorescence of two green algae, Chlamydomonas reinhardtii and Chlorella sp. Response surface methodology defined the effect of three key variables: density factor ($D_f$, ‘culture density’), cycle time ($t_c$, ‘mixing rate’), and maximum incident irradiance ($I_{\text{max}}$). Both species exhibited a large rise in $\text{PE}_{\mu}$ with decreasing $I_{\text{max}}$ and a minimal effect of $t_c$ (between 3–20 s). However, the optimal $D_f$ of 0.4 for Chlamydomonas and 0.8 for Chlorella suggested strong preferences for dilute and dense cultures respectively. Chlorella had a two-fold higher optimised $\text{PE}_{\mu}$ than Chlamydomonas, despite its higher light sensitivity. These results demonstrate species-specific light preferences within the green algae clade. Our high-throughput screen enables rapid strain selection and process optimisation.

Green algae are oxygenic photosynthetic organisms which, like higher plants and cyanobacteria, have evolved over 3 billion years to tap into the huge energy resource of the sun. This energy is used to fix CO$_2$, releasing O$_2$ as a by-product and producing biomass rich in proteins, lipids, starch, bioactive compounds and phytoneutrients. Consequently, single celled green algae (microalgae) are increasingly being integrated into industrial production systems to realise solar driven biotechnologies. Microalgae technologies are already being exploited commercially to produce high value commodities (e.g. functional foods, feeds, protein therapeutics and chemicals) and the knowledge gained is driving down production costs toward the levels required to expand low value market opportunities including fuels and fertilisers as well as ecosystem services (e.g. water treatment and CO$_2$ sequestration). The first step of all solar driven microalgae processes is light capture and conversion to chemical energy (ATP, NADPH), and the optimisation of this step is therefore essential to develop high-efficiency economic solutions. In outdoor mass cultures, the light reaching the surface of the pond or bioreactor is highly variable over the day, ranging from light limiting during early/late hours of the day or periods of high cloud cover, to photo-inhibiting conditions (up to 2,000 $\mu$mol m$^{-2}$ s$^{-1}$) during mid-day in locations receiving high solar radiation. Within the culture itself, cells are exposed to high light gradients as they cycle from the illuminated surface (often inhibitory light levels) to deep within the culture (light limiting or dark conditions). This fluctuating light regime within the mass culture is governed by the optical properties of the culture (based on cell size, cell number and pigment content) while the frequency with which cells cycle between the light and dark zones is regulated by mixing rate as well as the photobioreactor geometry which influences the light distribution through the optical pathlength and the surface to volume ratio. The relatively rapid light fluctuations within the culture affect the photo-regulatory response, while the relatively slow environmental light fluxes affect photoacclimation, both leading to changes in the overall productivity of the culture. Defining and optimising the effects and interactions of the variables that govern a given light regime is a challenge that requires comparatively large experimental datasets which can be laborious and expensive to obtain using traditional pilot- or even laboratory-scale bioreactors. The high-throughput light screen method presented here has been designed to simulate light regimes encountered in mass cultured photobioreactors under ‘typical’ conditions.

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outdoor production conditions to enable process optimisation, model guided system design, species selection and a better extrapolation of laboratory results to field trials.

The light screen collected data from LED illuminated microwells, and Response Surface Methodology was employed to predictively model photosynthetic efficiency (PE$_p$), to define both main effects and the pair-wise interactions between the light factors that govern it and to identify the conditions that yield optimum productivity. As fluctuating light can effect photoregulation and photoacclimation, we also investigated some of these underlying mechanisms to assess the extent of their effect on PE$_p$

A full factorial experimental design was employed, with quadratic models fitted to the data to measure the PE$_p$ in response to variations of three key factors that govern the light regime to which cells in mass culture are exposed: density factor (Df, -), defined as the proportion of the time that cells are in the dark zone (I$_{max}$, s) compared with the total time in both light (I$_{light}$, s) and dark zones; cycle time (t$_c$, s), which is defined by the mixing rate, or the total time of a cell’s fluctuation between light and dark zones for one cycle along the culture depth; and maximum irradiance (I$_{max}$, μmol photons m$^{-2}$ s$^{-1}$) defined as the irradiance entering the photobioreactor at the illuminated surface (Fig. 1A). Dark was defined as <5μmol PAR at which respiration typically exceeds photosynthesis (the compensation point)$^{13,14}$. The three factors (Df, t$_c$, I$_{max}$) affect the average irradiance (I$_{avg}$), which is the integration of light experienced by the cells over the entire light cycle (Fig. 1B). Our miniaturised and automated screen enables the analysis of the interactions between the three light-dependent factors and generates a strain-specific model that can be used to optimise production conditions or predict productivities for different production scenarios.

This empirical model is an alternative approach to traditional models based on photosynthetic irradiance (P-E) curves. It only requires knowledge of the density factor, incident irradiance and mixing rate. The Df for a given species and reactor geometry can be easily found (indoor experiments) or given incident irradiance by measuring the depth of culture at the point where light is reduced to <5 μmol m$^{-2}$ s$^{-1}$ (i.e. start of the “dark zone”) and calculating the ratio of this depth to the total culture depth (usually fixed). This can be correlated to a range of optical densities (or biomass dry weights) to provide a simple method to establish what Df a reactor will have at a known culture density, pathlength and incident irradiance. Since Df has been determined as a critical factor in this and other studies, we believe that this is another useful modelling tool for process design.

Two biotechnologically relevant microalgal strains were analysed in this study: *Chlamydomonas reinhardtii* (*Chlamydomonas*), the model alga most used in photosynthetic studies$^{15,16}$ and for heterologous protein expression$^{17,18}$, and a strain of *Chlorella* sp. 11_H5 (*Chlorella*) isolated in Australia which was found to have high biomass productivity at laboratory and pilot scale$^{19,20}$. *Chlamydomonas* (originally isolated from soil)$^{21}$ has successfully transitioned from land to water in laboratory conditions, arguably owing to its robust and evolved photosynthetic machinery that protects it from oxidative stress and changing environmental conditions$^{22}$. Hence, understanding the interplay between photosynthetic regulation, photoacclimation and its effect on growth and biomass productivity would determine the feasibility of delivering functional microalgae biotechnologies. This paper presents a high-throughput miniaturised light optimisation screen (allowing up to 18 different combinations of light regime and up to 1,728 conditions), designed to identify species-specific illumination conditions that maximise photosynthetic efficiency and productivity to fast track systems optimisation.

### Results

#### High-throughput screen (HTS) of simulated light regimes in mass cultures.

To analyse the effects of varying levels of I$_{max}$, Df and t$_c$ (Fig. 1B) on the PE$_p$ of microalgae, light simulations were performed on dilute 150 μl microwell cultures (5 mm pathlength)$^{23}$, each illuminated using individual LEDs (Fig. 1C). The intensity of photosynthetically active radiation (400–700 nm, PAR) emitted by the LEDs was programmed (Arduino® integrated circuit and controller) to mimic a sinusoidal trajectory of a cell cycling in a one-dimensionally illuminated culture (i.e. an open pond) between the illuminated surface and the dark zone (Fig. 1B)$^{10}$. In this way, the light regime encountered by the incubated cells in each well was a function of the LED’s illumination profile, thereby allowing tight control of the levels of each factor (I$_{max}$, Df and t$_c$). The robotic arm was programmed to take the plates to a reader at determined time intervals where rapid measurements of optical density and fluorescence can be taken. Here, two strains were analysed for the initial HTS light simulations, however, this method can rapidly be used to model up to 32 strains run in triplicate in one experiment.

Figure 1A depicts the three levels of each factor (I$_{max}$, Df, t$_c$) and the real-world phenomena they represent based on information from literature$^{24–26}$ and on experimental data$^{27–29}$. A low (0.2) or high (0.8) Df represents a low or high cell/biomass density respectively (e.g. dilute cultures at the beginning of cultivation versus dense cultures at harvest in a batch production regime). The system is able to analyse any range between 10 ms fluctuations to constant light. The cycle time of 3–20 s represents typical ‘mixing’ cell cycle rates through the optical pathlength of photobioreactors, where a t$_c$ of 3, 10, and 20 s represents rapid, moderate or slow mixing, as might occur in a tubular PBR, thick flat panel PBR and open pond respectively. The t$_c$ is influenced by mixing and/or sparging rates, reactor pathlength, or a combination of the two, which can vary for individual reactors depending on cultivation regime. The I$_{max}$ values represent the incident solar radiation in the early morning and late afternoon (375 μmol m$^{-2}$ s$^{-1}$), mid-morning and -afternoon (750 μmol m$^{-2}$ s$^{-1}$) and noon (1500 μmol m$^{-2}$ s$^{-1}$) respectively. I$_{max}$ values are based on the average annual solar radiation levels for Brisbane, Australia$^{30,31}$, and are representative of other high solar regions that are suitable for outdoor microalgal production. The simulation of these three factors at three levels each via programmed changes in LED light flux over time are depicted in Fig. 1B. This approach provided a complete factorial design (3$^3$) of 27 combinations for model fitting of the main response variable, PE$_p$ (Table 1) and underlying responses at the level of PSII (Table 2).

A further dataset with a Df of 0.6 (at each level of I$_{max}$ and t$_c$) provided 9 independent data points used for model validation and goodness of fit (Table 1, validation data are indicated by *'). See section (Model validation shows that the light factors Df, I$_{max}$ and t$_c$ can be used to predict PE$_p$ accurately in *Chlorella* and moderately in
Chlamydomonas) for results). For all treatments, the combination of each Df and I_max also resulted in 12 unique integrated average irradiance levels, I_avg (mol photons m⁻² s⁻¹). Additional experiments compared the PE between cells exposed to fluctuating regimes with cells exposed to constant illumination (control) with the same I_avg to compare the effect of light regime and light dose (Fig. 2C, Supplementary Table S1, Supplementary Fig. S4).

Figure 1. Experimental design for high-throughput light simulations of cells cycling in outdoor microalgae mass cultures. (A) Depicts the 3 factors that affect the light regime experienced by cells cycling in mass cultures: I_max, D_f, and t_c, and the levels used for the full factorial experimental design which are based on ‘typical’ outdoor conditions. (B) Each combination of light factors was programmed by changing the light intensity of the LEDs over the cycle time, assuming cell cycling occurs in a sinusoidal trajectory. Here, I_max is the amplitude of the sine, simulating the maximum irradiance that a cell would receive when at the ‘surface’ of a mass culture, D_f is the proportion of time that PAR is below 5 µmol m⁻² s⁻¹ in one period; this simulates the fraction of time that a cell spends in the dark, depending on the culture density, and t_c is the period of one sine wave, that simulates the time required for a cell to cycle through the reactor. I_avg is the integration of light received, simulating the average irradiance or light dose received by the cell. Here t_light and t_dark are the time cells receive PAR (>5 µmol m⁻² s⁻¹) and no PAR (<5 µmol m⁻² s⁻¹) respectively. (C) The programmed LEDs form part of an 18-plate microwell robotic system. Chlamydomonas and Chlorella were incubated in 96-well plates placed on LED arrays with one LED per microwell and one unique light regime per plate. All light regimes occurred over a photoperiod of 16 h day⁻¹ and a dark period of 8 h day⁻¹.
μ during the exponential phase (after ~38 hours of light regime exposure) and normalised to the light received to Chlamydomonas (Chlorella but approximately the same for Chlorella). Factors (Fig. 2C), and over all but one factor (Fig. 2D–F). Overall, Figure 2C also shows the mean PE for Chlorella (~7.5%) than that obtained under fluctuating light of the same Iavg. For Chlamydomonas, this result concurs with other studies showing a negative impact of fluctuating light on

### Table 1. PE of Chlamydomonas and Chlorella under the experimental matrix of light regimes. All data are the mean of 3 replicates ± standard deviation. *Indicates data used for model validation. ‘Coded’ refers to the normalised values used for the quadratic model (Equation 2).

| \( J_{\text{max}} \) (Actual (\( \mu mol \text{ m}^{-2} \text{s}^{-1} \)) Coded) | \( D_1 \) Actual (−) Coded | \( t_c \) Actual (s) Coded | \( I_{\text{avg}} \) (mol m\(^{-2}\text{h}^{-1}\)) Coded | \( \text{PE}_\mu \) (mol photon\(^{-1}\text{m}^{-2}\)) Chlamydomonas Chlorella |
|---|---|---|---|---|---|
| 375 | 0.2 | −1 | 3 | −1.73 | 0.619 | 0.118 ± 0.0030 0.136 ± 0.017 |
| | | | 10 | 0 | 0.142 ± 0.018 |
| | | | 20 | 1 | 0.107 ± 0.0031 0.151 ± 0.026 |
| | 0.4 | 0 | 3 | −1.73 | 0.490 | 0.174 ± 0.0070 0.183 ± 0.012 |
| | | | 10 | 0 | 0.133 ± 0.0079 0.149 ± 0.001 |
| | | | 20 | 1 | 0.094 ± 0.0070 0.132 ± 0.018 |
| | 0.6* | − | 3 | −1.73 | 0.367 | 0.088 ± 0.0066 0.176 ± 0.007 |
| | | | 10 | 0 | 0.099 ± 0.0010 0.167 ± 0.011 |
| | | | 20 | 1 | 0.084 ± 0.0100 0.149 ± 0.007 |
| | 0.8 | 1 | 3 | −1.73 | 0.18 | 0.040 ± 0.0028 0.277 ± 0.022 |
| | | | 10 | 0 | 0.048 ± 0.0000 0.197 ± 0.014 |
| | | | 20 | 1 | 0.047 ± 0.0107 0.159 ± 0.006 |
| 750 | 0.2 | −1 | 3 | −1.73 | 1.242 | 0.078 ± 0.0037 0.039 ± 0.003 |
| | | | 10 | 0 | 0.063 ± 0.0013 0.054 ± 0.002 |
| | | | 20 | 1 | 0.053 ± 0.0022 0.076 ± 0.001 |
| | 0.4 | 0 | 3 | −1.73 | 0.979 | 0.060 ± 0.0121 0.087 ± 0.004 |
| | | | 10 | 0 | 0.061 ± 0.0040 0.087 ± 0.006 |
| | | | 20 | 1 | 0.049 ± 0.0020 0.095 ± 0.008 |
| | 0.6* | − | 3 | −1.73 | 0.738 | 0.079 ± 0.0030 0.099 ± 0.005 |
| | | | 10 | 0 | 0.065 ± 0.0016 0.082 ± 0.006 |
| | | | 20 | 1 | 0.049 ± 0.0030 0.182 ± 0.003 |
| | 0.8 | 1 | 3 | −1.73 | 0.360 | 0.063 ± 0.0073 0.134 ± 0.012 |
| | | | 10 | 0 | 0.046 ± 0.0023 0.072 ± 0.022 |
| | | | 20 | 1 | 0.020 ± 0.0027 0.097 ± 0.008 |
| 1500 | 1 | 0.2 | −1 | 3 | −1.73 | 2.480 | 0.051 ± 0.0027 0.021 ± 0.0004 |
| | | | 10 | 0 | 0.067 ± 0.0109 0.025 ± 0.002 |
| | | | 20 | 1 | 0.049 ± 0.0021 0.047 ± 0.006 |
| | 0.4 | 0 | 3 | −1.73 | 1.958 | 0.053 ± 0.0021 0.037 ± 0.004 |
| | | | 10 | 0 | 0.052 ± 0.0035 0.055 ± 0.001 |
| | | | 20 | 1 | 0.045 ± 0.0026 0.072 ± 0.011 |
| | 0.6* | − | 3 | −1.73 | 1.472 | 0.050 ± 0.0138 0.067 ± 0.001 |
| | | | 10 | 0 | 0.041 ± 0.0074 0.057 ± 0.006 |
| | | | 20 | 1 | 0.030 ± 0.0080 0.092 ± 0.003 |
| | 0.8 | 1 | 3 | −1.73 | 0.713 | 0.051 ± 0.0053 0.072 ± 0.001 |
| | | | 10 | 0 | 0.031 ± 0.0088 0.043 ± 0.006 |
| | | | 20 | 1 | 0.030 ± 0.0170 0.043 ± 0.007 |

**Photosynthetic efficiency under different light regimes.** The PE of Chlamydomonas and Chlorella under all 27 fluctuating light regimes are shown in Fig. 2A and B. Some similarities in the general trends of Chlamydomonas and Chlorella are evident, such as the effect of \( J_{\text{max}} \), where a large increase in PE occurred with decreasing \( J_{\text{max}} \). To better depict PE trends, individual treatments were averaged for each species over all factors (Fig. 2C), and over all but one factor (Fig. 2D–F). Overall, Chlorella exhibited a ~50% higher PE than Chlamydomonas (average PE of 0.099 ± 0.060 mol photon\(^{-1}\text{m}^{-2}\) and 0.066 ± 0.034 mol photon\(^{-1}\text{m}^{-2}\) respectively, Fig. 2C), in line with previous reports. Figure 2C also shows the mean PE obtained under constant light was ~80% higher in Chlamydomonas but approximately the same for Chlorella (~7.5%) than that obtained under fluctuating light of the same Iavg. For Chlamydomonas, this result concurs with other studies showing a negative impact of fluctuating light on
time-integrated photosynthesis and growth rates\textsuperscript{30,12,33,34}. Interestingly, for this strain of Chlorella, fluctuating light had little effect compared to constant light conditions.

For main effects of each factor, Fig. 2D shows at the lowest I\textsubscript{max} value, the mean PE\textsubscript{µ} increased up to two-fold for Chlamydomonas and 3.67-fold for Chlorella, respectively, indicating that photosynthetic light utilisation is compromised under high incident light (i.e. at noon under outdoor conditions)\textsuperscript{35–37}, especially for Chlorella.

The trends of D\textsubscript{f} (Fig. 2E) resulted in diametrically opposing responses: PE\textsubscript{µ} in Chlamydomonas performed best at a low D\textsubscript{f} (increasing up to 83\% from D\textsubscript{f} = 0.8 to D\textsubscript{f} = 0.2) while Chlorella at a high D\textsubscript{f} (PE\textsubscript{µ} increased up to 58\% from D\textsubscript{f} = 0.2 to D\textsubscript{f} = 0.8). Since mass cultures operating under high cell densities is advantageous to reduce downstream processing costs, these results suggest that Chlorella is more suited to mass cultivation than Chlamydomonas.

For both species, the effect of t\textsubscript{c} seemed minor (Fig. 2F). Cell cycling in the range analysed (t\textsubscript{c} = 20 s) while had little effect compared to constant light conditions.

### Table 2. Comparison of the factor coefficients of the quadratic model obtained from analysis of variance (ANOVA) for A) PE\textsubscript{µ}, B) \( \Phi \text{PSII} \) and C) F\textsubscript{v}/F\textsubscript{m} parameters for Chlamydomonas and Chlorella. *Represents significant effects at p-value < 0.05. n = 3 (PE\textsubscript{µ}), n = 2 (\( \Phi \text{PSII} \) & F\textsubscript{v}/F\textsubscript{m}).

| Factor | Chlamydomonas | Chlorella | Chlamydomonas | Chlorella | Chlamydomonas | Chlorella |
|--------|---------------|-----------|---------------|-----------|---------------|-----------|
| Intercept | 67.6          | 71.1      | 236.5         | 194.3     | 655.6         | 647.1     |
| R\textsuperscript{2} | 0.67          | 0.93      | 0.44          | 0.74      | 0.91          |           |
| D\textsubscript{f} | -20.0\*        | 20.5       | -35.7\*       | -8.1\*    | 16.4          | 16.6      |
| I\textsubscript{max} | -21.0\*        | 20.5       | -35.7\*       | -8.1\*    | 16.4          | 16.6      |
| t\textsubscript{c} | -6.6\*         | -6.6       | -5.5\*        | -3.3      | -2.0          | -0.9      |
| D\textsubscript{f} & I\textsubscript{max} | 16.0\*      | -10.3\*      | -29.6\*     | -6.8\*    | -6.1          | 9.7\*     |
| D\textsubscript{f} & t\textsubscript{c} | -1.1         | -14.7\*     | 0.8           | 0.9       | -3.6          | 3.9       |
| I\textsubscript{max} & t\textsubscript{c} | 3.2          | -9.5\*      | -5.0\*       | 3.9       | 3.0           | -6.5\*    |
| D\textsubscript{f} \^ 2 | -24.6\*        | 2.4        | -26.8\*      | -4.7      | 10.4          | 1.8       |
| I\textsubscript{max} \^ 2 | 14.2\*        | 28.0\*      | 19.1\*       | -4.9      | 31.8\*       | 1.7       |
| t\textsubscript{c} \^ 2 | 1.0           | 2.8        | 0.2           | 0.6       | 1.7           | -3.8      |

### Modelling light factor interactions using response surface methodology.

Response surface methodology of the complete factorial design\textsuperscript{46–48} was next employed to model and explore the interactions between the three input factors (I\textsubscript{max}, D\textsubscript{f} and t\textsubscript{c}) to PE\textsubscript{µ}. Furthermore, to determine the influence of photoregulation under fluctuating light on PE\textsubscript{µ}, supporting parameters at the level of PSII regulation for Chlamydomonas and Chlorella were also modelled from chlorophyll fluorescence data. These are: the operating efficiency of PSII (\( \phi \text{PSII} \)) – a measure of the proportion of absorbed light used for photochemistry; maximum quantum efficiency of PSII photochemistry (F\textsubscript{v}/F\textsubscript{m}) – an indicator of PSII inactivation via photoinhibition; and non-photochemical quenching (NPQ) – the apparent rate constant for heat loss from PSII\textsuperscript{44}. These parameters provide clues as to the underlying mechanisms of the observed PE\textsubscript{µ}.

The three levels of each factor (Table 1) were coded with the mid-point (coded as ‘0’) and this was halved in the experimental design such that the coded factors of the independent variables were calculated using the logarithmic equation,

\[ x_i = (1.4427 \ln(X_i) + A_i) \]

where, \( x \) is the coded factor level, \( X \) is the actual value of the factor, \( i = 1, 2, 3; A \) is the intercept value of the logarithmic function for each factor with \( A_1 = 1.3219, A_2 = -9.5507 \) and \( A_3 = -3.3219 \) for D\textsubscript{f}, I\textsubscript{max} and t\textsubscript{c} respectively.

Quadratic models (Equation 2) were fitted to the data:

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{i} x_i^2 \]

In Equation 2, \( Y \) is the predicted response variable (PE\textsubscript{µ}, \( \Phi \text{PSII} \), F\textsubscript{v}/F\textsubscript{m} or NPQ); \( \beta_0, \beta_i, \beta_{ij} \) and \( \beta_{i} \) are the coefficients for intercept, linear, interaction and quadratic effects respectively; \( x_1, x_2 \ldots x_k \) are the coded values of the input factors (\( i = j \)); and \( k = 3 \). Multiple regression of the data was used to obtain the regression coefficients.

Model validation shows that the light factors I\textsubscript{max}, D\textsubscript{f} and t\textsubscript{c} can be used to predict PE\textsubscript{µ} accurately in Chlorella and moderately in Chlamydomonas.

For the primary response, PE\textsubscript{µ}, the quadratic model demonstrated a moderate and high goodness of fit for Chlamydomonas (R\textsuperscript{2} = 0.67) and Chlorella (R\textsuperscript{2} = 0.93), respectively.
To assess whether the model fit was adequate to predict $P_{E_\mu}$ within the range analysed, the quadratic models were validated using an additional set of experimental data at $D_f = 0.6$ at each level of $I_{max}$ (9 experimental sets for each strain) (Table 1). Comparing the fitted models against the actual data gave a low $R^2$ of 0.456 for *Chlamydomonas* and a high $R^2$ of 0.882 for *Chlorella* (Supplementary Fig. S5). In general, the residuals showed a normal distribution and the Cook's distance plot showed only a small number of outliers for *Chlamydomonas* and *Chlorella* (Supplementary Fig. S5).

For *Chlorella*, these results indicated that the three light factors accounted for a high proportion of variation in $P_{E_\mu}$ observed and can be used to adequately predict their relationship to $P_{E_\mu}$. For *Chlamydomonas*, it seems there are more complex regulations of the photosynthetic machinery, which cannot be modelled with these factors alone.
The light factors of $I_{\text{max}}$ and $D_f$ significantly affect $PE_{\mu}$ under fluctuating light. The coefficient terms tabulated in Table 2 show the relative size and direction that each factor has on the response variables, while the three dimensional (3D) response surface plots and 2D contour plots graphically depict the interactions of two factors on the primary response of $PE_{\mu}$, where the third factor is set to the midpoint (Fig. 3).

For *Chlamydomonas*, the most significant factors affecting $PE_{\mu}$ were: $I_{\text{max}}$ ($p$-value $= 3.83 \times 10^{-8}$), $D_f$ ($p$-value $= 1.04 \times 10^{-8}$), and the interaction of $D_f-I_{\text{max}}$ ($p$-value $1.05 \times 10^{-4}$) (Table 2). Here, both high $D_f$ and high $I_{\text{max}}$ had similar negative impacts on $PE_{\mu}$, yet the interaction of $D_f-I_{\text{max}}$ had a positive effect, suggesting that dense cultures may offer some protection under high light whilst dilute cultures may improve $PE_{\mu}$ under low light. As expected, the 3D plots show the highest $PE_{\mu}$ values at a combination of low $D_f$ (i.e. not light limited) and low $I_{\text{max}}$ (i.e. not photo-inhibited) (Fig. 3A), however, the slight saddle shape of the interaction plot at high $I_{\text{max}}$ shows that the optimal $D_f$ is around 0.4 (at the mid-point) for *Chlamydomonas*.

The $PE_{\mu}$ of *Chlorella* was most significantly adversely affected by high $I_{\text{max}}$ ($p$-value $9.92 \times 10^{-37}$), and unlike *Chlamydomonas*, showed a significant positive response for increasing $D_f$ ($p$-value $4.67 \times 10^{-11}$). The $I_{\text{max}}-D_f$ interaction showed an exponential increase in $PE_{\mu}$ with a reduction of $I_{\text{max}}$ and an increase in $D_f$ (Fig. 3B). However, the significant negative interaction of $D_f-t_c$ (Table 2) suggests that long cycle times could adversely affect productivity.
in high density cultures (Fig. 3D). Overall, for Chlamydomonas a low Imax and low Df (Fig. 3A) and for Chlorella a low Imax and high Df (with moderate benefits of low tc) (Fig. 3 B and D) resulted in the highest PE_µ.

PSII regulation has a strong effect on PE_µ under fluctuating light. To assess some underlying mechanisms that may affect PE_µ, chlorophyll fluorescence measurements were taken to assess levels of stress and photo-inhibition (F_v/Fm), the operating efficiency of PSII (ΦPSII) and non-photochemical quenching (NPQ). The data was fitted to the quadratic model (Equation 2) to compare the magnitude of effect of the three light factors. Additionally, changes in the ratio of OD_680/OD_750 were used as a high-throughput proxy to determine photoacclimation via changes in chlorophyll content.

A high goodness of fit to the quadratic model was observed in Chlamydomonas for ΦPSII (R² = 0.89) and Fv/Fm (R² = 0.74) and, in Chlorella, for Fv/Fm (R² = 0.91), suggesting that PSII regulation is highly affected by the three light factors examined in this study and is a contributing factor to the observed PE_µ. Remarkably, all treatments for both species showed low NPQ (<0.3) relative to average values reported in literature (up to ~2 for Chlamydomonas and ~1.5 for Chlorella)15 and a poor goodness of fit to the quadratic model for both strains (see Supplementary Table S2). Other stressors, such as nutrient limitation, are also known to increase NPQ49. Since both strains were cultivated on previously optimised nutrients this may have contributed to the overall reduced NPQ in this study.

For Chlamydomonas, a significant (p-value = 1.79E-17) reduction in ΦPSII occurred at high Df (Table 2, Fig. 2E). This suggests that efficient electron transfer is compromised under high dark fractions for this alga and links ΦPSII to the reduced PE_µ, trends under high Df observed. Furthermore, increased OD_680/OD_750 measurement (a proxy for chlorophyll content per cell) was prominent with increasing Df (Fig. 4H), suggesting high dark fractions lead to increased cellular chlorophyll levels typical for low-light acclimation, which may further explain the lower efficiency of light utilisation (i.e. PE) at high Df (Fig. 2E). Remarkably, a high Imax actually improved both ΦPSII (Fig. 4A) and Fv/Fm (Fig. 4D) and lowered OD_680/OD_750 (Fig. 4G), despite a reduction in PE_µ (Fig. 2D). This suggests that while photosynthetic rates improved in Chlamydomonas under high light, the over-saturating irradiance could not be fully utilised by the Calvin-Benson cycle, suggesting other downstream mechanisms such as alternative electron sinks50 could become relevant under high light.

For Chlorella, the most significant factor corresponding directly to PE_µ was the effect of Imax on Fv/Fm, which gave a large negative coefficient in the model (Table 2) and showed a noticeable decline in Fv/Fm with increasing Imax (Fig. 4D). Like Chlamydomonas, increasing Df was found to have a positive effect on Fv/Fm (Fig. 4E),
also seen by the relative magnitudes of coefficients and their significance (p-value = 3.09E-07), and a significant positive interaction between D f and t c (p-value = 5.19E-03) similar to Chlamydomonas, Chlorella exhibited an up-regulation of OD_{600nm} (indicative of higher chlorophyll) at high D f (Fig. 4H, Supplementary Table S2).

In summary, these results suggest that Chlorella is sensitive to high light as seen by PSII inactivation but less sensitive to light/dark fluctuations. In contrast, Chlamydomonas is sensitive to strong light/dark fluctuations due to disrupted electron transport flows but seems to have better acclimisation strategies to cope with high light. These results suggest that maintaining Chlamydomonas at relatively dilute cultures is beneficial, whereas operating Chlorella at high densities is preferable, especially under high light.

**Optimisation predicts a two-fold higher maximum PE_p for Chlorella compared to Chlamydomonas.**

It is evident from the 3D surface plots (Fig. 3) showing PE_p response that the maxima occur at the extremes in most instances. The maximum PE_p values (at the mid-point, i.e. level 0) and their corresponding factor levels were used to obtain the maximum PE_p and optimum conditions. For both Chlamydomonas and Chlorella, the maximum PE_p values occurred at the minimum I_max (375 μE) and the minimum value of t c (Table 3). Using this combination of I_max and t c, the optimal D f values were found to be 0.24 and 0.8 for Chlamydomonas and Chlorella respectively. These combination of factor values results in a theoretical maximum PE_p for Chlorella than Chlamydomonas. As discussed in the section 3.3.1, the three light factors modelled only explains two thirds of the variation in PE_p for Chlamydomonas and these results are indicative only for this species.

**Concluding Remarks**

The HTS coupled with response surface methodology delivers a working statistical design for simultaneous light optimisation of several species of microalgae. This platform has been used to screen growth responses to nutrients and organic carbon sources20,23, and can be extended to screen other parameters such as CO_2 or growth contaminants (e.g. herbicides, antibiotics, bacteria or predating organisms), and could monitor other response variables such as lipid accumulation (e.g. Nile Red) and protein expression using fluorescence tags. Some limitations imposed by the microwell HTS can include high variation between replicates when trialled at conditions that are sensitive to light/dark fluctuations. In contrast, Chlamydomonas is sensitive to strong light/dark fluctuations due to minimal transmittance losses (e.g. by increasing surface to volume ratios or using specially designed light guides24) may be another strategy to improve PE_p rather than adjusting cycle time (by increasing mixing rates, gas sparging) particularly as the latter would require higher energy inputs with minimal gains in PE_p. Another important deduction of strain-specific characterisation for scale up was the detrimental effect of cycle time on PE_p for Chlamydomonas (~ 46%) versus a similar effect for Chlorella as compared to constant light. This signifies the application of our HTS outcomes toward strain selection as well as growth platform selection (i.e. open pond (slow mixing) versus tubular PBRs (faster mixing) or other designs) when going from laboratory (constant light)

| Species          | Condition | Predicted max PE_p | D_f | I_max | t_c |
|------------------|-----------|--------------------|-----|-------|-----|
|                  |           | (mol photon m^{-2} s^{-1}) | Coded | Coded | Coded |
| Chlamydomonas    | t_c midpoint | 0.116 | -0.75 | 0.24 | -1 | 375 | 0 | 10 |
|                  | I_max midpoint | 0.079 | -0.4  | 0.30 | 0  | 750 | -1 | 5  |
|                  | D_f midpoint | 0.113 | 0     | 0.40 | -1 | 375 | -1 | 5  |
|                  | Optima    | 0.126 | -0.73 | 0.24 | -1 | 375 | -1 | 5  |
| Chlorella        | t_c midpoint | 0.194 | 1     | 0.80 | -1 | 375 | 0  | 10 |
|                  | I_max midpoint | 0.117 | 1     | 0.80 | 0  | 750 | -1 | 5  |
|                  | D_f midpoint | 0.178 | 0     | 0.40 | -1 | 375 | -1 | 5  |
|                  | Optima    | 0.226 | 1     | 0.80 | -1 | 375 | -1 | 5  |

Table 3. Optimisation of PE_p and the respective factor levels around the mid-point of each factor, and around the optimised point for total predicted maximum PE_p within the ranges of the full factorial design.
to outdoor systems (fluctuating light). In both alga, as is typical of other species, high incident light has the most detrimental effect on PE\textsubscript{\textit{m}}. Therefore, efforts to diffuse light sources, such as done through the use of reflectors, or to use vertical flat panels or vertically stacked tubular photobioreactors to avoid direct sunlight at high light periods, may benefit from the ‘light dilution effect’.

Previous transcriptomic and proteomic studies in \textit{Chlamydomonas} have shown that acclimation to environmental stimuli is achieved by remodelling photosystem I and II antenna complexes, further highlighting the flexibility of their photosynthetic machinery\textsuperscript{52}. While \textit{Chlamydomonas} may possess the survival strategies required to acclimate to changing light conditions, typically for soil environments, they may not be tuned for high biomass productivity, unlike fast-growing strains like the \textit{Chlorella} strain used in this study, which despite seemingly lacking the level of regulatory sophistication, might be better suited for mass cultivation.

In conclusion, the HTS method developed here enables a rapid approach to optimise systems design, scale up operational conditions and species selection to advance feasible solar-driven biotechnologies.

Materials and Methods

Strains and pre-culture conditions. Liquid pre-cultures were prepared in triplicate (40 mL culture in 100 mL flasks) and inoculated with either \textit{C. reinhardtii} WT strain CC125\textsuperscript{53} or \textit{Chlorella} sp. 11.H5\textsuperscript{58} (Australian isolate) maintained on TAP\textsuperscript{54} agar (1.5\%). To ensure nutrients were non-limiting, photoautotrophic medium previously optimised for each species was used for \textit{C. reinhardtii} (PCM\textsuperscript{55}, N source NH4\textsuperscript{+}) and \textit{Chlorella} sp (OpM\textsuperscript{20}, N source urea). Flasks were maintained on shakers (200 rpm) in an enclosed incubation system at 23\textdegree{}C, 1\% CO\textsubscript{2} and a 16/8 hour light/dark cycle, illuminated with 100 \(\mu\)mol m\textsuperscript{-2} s\textsuperscript{-1} of overhead white fluorescent light for 5 days.

To ensure that the cultures were well synchronised to the light conditions being tested, flask pre-cultures first acclimated to a 16/8 h light/dark cycle were inoculated into microwell plates (150 \(\mu\)l), and gradually acclimated to the light intensity close to the mean \(I_{\text{avg}}\) before the first measurement. For the higher light intensity experiments \((I_{\text{max}} = 1500 \text{ \(\mu\)mol m}^{-2} \text{ s}^{-1})\), care was taken not to shock the low density cultures by subjecting them to a step-wise gradually increasing light regime (a detailed summary of the acclimation regimes is provided in Supplementary Table S3).

Automated HTS and lighting design. The design, structure and operation of the HTS system (Tecan Freedom Evo 150, Tecan Group Ltd., Männedorf, Switzerland) is as previously described\textsuperscript{20,23}. Briefly, the HTS system is an enclosed chamber fitted with three orbital shakers which hold six microwell plates each, a robotic manipulator arm that removes the plate lid and carries the plates to a reader (Infinite M200 PRO, Tecan Group Ltd., Männedorf, Switzerland, Fig. 1C) and atmospheric CO\textsubscript{2} control. Each of the 18 microwell plate positions is fitted with 96 ‘warm white’ LEDs positioned directly under each well of a 96-well plate. Each of the LED arrays is controlled by user defined scripts on an Adruino® integrated circuit controller and software, permitting 18 different light conditions to be tested in parallel. LEDs were fitted with a low pass LC filter to smooth the intensity signal from pulse width modulation to variable voltage, thereby eliminating ‘flashing light’ phenomena due to on/off signals. The spectrum of wavelengths of LEDs is compared against that of natural sunlight (see Supplementary Fig. S6). For simplicity, a sinusoidal mixing regime was assumed to allow tight control of the factors of \(I_{\text{max}}, D_{\text{f}}\) and \(t_{\text{c}}\), as has been used in previous studies\textsuperscript{56,57}. Pre-cultures were centrifuged (300g, 20 min, 18\textdegree{}C) and the pellet re-suspended in fresh medium. To minimise cell shading effects and ensure tight light control, a volume of 150 \(\mu\)l was chosen for a short pathlength of 5 mm and a semi-continuous cultivation regime was applied by daily culture re-suspended in fresh medium. To minimise cell shading effects and ensure tight light control, a volume of 150 \(\mu\)l was chosen for a short pathlength of 5 mm and a semi-continuous cultivation regime was applied by daily culture re-suspended in fresh medium.

Growth rate and photosynthetic efficiency (PE\textsubscript{\textit{m}}) measurements. Growth rates were calculated from 3-hourly OD\textsubscript{750} measurements. High-throughput automated measurements of OD\textsubscript{750} were used as a proxy for growth from which growth rates, \(\mu\) (h\textsuperscript{-1}), were calculated as the rate of change of OD\textsubscript{750},

\[
\mu = \frac{(\ln OD_{750}(t_2) - \ln OD_{750}(t_1))}{(t_2 - t_1)}
\]

where, \(t_1\) and \(t_2\) are the time points at which OD\textsubscript{750} was measured.

A 3-hour measuring frequency during the light period was used for the growth curve calculations. This frequency was chosen to limit evaporation and contamination issues. A detailed description of the growth curves, sampling points and lighting schedule can be found as Supplementary Figs S1 and S2.

The main response variable, PE\textsubscript{\textit{m}}, was assumed to be indicative of light utilisation efficiency of the microalgae, where the growth rate normalised to the average integrated PAR received,

\[
PE = \frac{\mu}{I_{\text{avg}}}
\]

And the \(I_{\text{avg}}\) is,

\[
I_{\text{avg}} = \int_0^t I(t)dt + 3.6 \times 10^{-9}
\]

In Equation 5, \(I_0\) is the cycle time, \(I(t)\) is the irradiance (\(\mu\)mol photons m\textsuperscript{-2} s\textsuperscript{-1}) at a given time of \(t_\text{c}\), and \(3.6 \times 10^{-9}\) is the conversion factor from \(\mu\)mol photons m\textsuperscript{-2} s\textsuperscript{-1} to \text{mol} photons m\textsuperscript{-2} h\textsuperscript{-1}. 
Chlorophyll fluorescence of photosystem II measurements. Photosystem II (PSII) kinetics were measured as a function of PSII chlorophyll fluorescence. Biological duplicates of each sample (dilution factor of 5) was added to a Fluorimeter cuvette (Sigma), dark adapted for 20 minutes and processed using the FluoroWin software (Photon Systems Instruments, Czech Republic). The quenching analysis protocol had the following settings: measuring light: 20% V; saturating pulse: 0.9 ± 8% V; actinic light: 51 s, 18.3 V (800 µmol m⁻² s⁻¹). Weak infrared pulses (730 nm) were applied for 5 s prior to measurement to quench Qₒ. The PSII parameters calculated from the quenching analysis were: Fm/Fm' (maximum quantum efficiency of PSII), ΦPSII (PSII operating efficiency), and NPQ (Non-photochemical Quenching) using respectively,

\[
Fm/Fm' = (Fm' - F0)/Fm
\]

\[
\varphi_{PSII} = (Fm' - F')/Fm'
\]

\[
NPQ = (Fm/Fm') - 1
\]

Photoacclimation via OD₆₈₀/₇₅₀. Chlorophyll a has a maximum absorbance at 680 nm. Therefore, OD₆₈₀ measurements were normalised to OD₇₅₀ (OD₆₈₀/₇₅₀) as a proxy of changes in chlorophyll absorbance between different light regimes.

Statistical Analysis. All data are expressed as Mean ± SD of three biological replicates (for automated readings) and two biological replicates (for the manual PSII measurements), each with multiple technical replicates as mentioned in section 5.2. MATLAB was used for the design and analysis of the response surface methodology. A p-value <0.05 was used for determining significant effects. Both contour and surface plots were developed for visualisation of the data and to predict the relationship and interaction effects on the light utilisation efficiency. Regression coefficient (R²) was used to resolve the goodness of fit. The fitted model using the regression coefficients was validated with an additional experimental dataset.

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Author Contributions
Jennifer Yarnold, Juliane Wolf and Ben Hankamer conceived and designed the experiments. Shwetha Sivakaminathan and Jennifer Yarnold performed the experiments. Shwetha Sivakaminathan, Jennifer Yarnold and Juliane Wolf analysed the data. Shwetha Sivakaminathan, Jennifer Yarnold and Juliane Wolf drafted the article. Ben Hankamer revised it critically and provided the funds and infrastructure for conducting the research at the Institute for Molecular Bioscience.

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