**Increasing algal photosynthetic productivity by integrating ecophysiology
with systems biology.**

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Abstract

Oxygenic photosynthesis is the process by which plants, algae and cyanobacteria convert sunlight and CO₂ into chemical energy and biomass. Previously published estimates suggest algal photosynthesis is, at best, able to convert ~ 5-7% of incident light energy to biomass and there is opportunity for improvement. Recent analyses of *in situ* photo-physiology in mass cultures of algae and cyanobacteria show that cultivation methods can have detrimental effects on a cell's photo-physiology – reinforcing the need to understand the complex responses of cell biology to a highly variable environment. A systems-based approach to understanding the stresses and efficiencies associated with light energy harvesting, CO₂ fixation and carbon partitioning will be necessary to make major headway towards improving photosynthetic yields.

Keywords:

Biofuels, photosynthesis, algae, cyanobacteria, systems biology

Oxygenic photosynthesis is inefficient

Algae and cyanobacteria could represent the next generation of biofuels and provide a major source of sustainable transportation fuel in order to reduce society's dependence on fossil fuels [1, 2]. There are several technological hurdles to overcome before the process can become commercially viable including the essential improvement of the photosynthetic process [3]. Previously published exercises have suggested that algal photosynthesis is, at its best in controlled culture conditions, able to convert $\sim 5\text{-}7\%$ of incident light energy to biomass [4]. Pushing photosynthetic efficiencies in mass culture towards this level will drastically reduce the land area and associated infrastructure required to produce biofuel [5].

Our understanding of algal photosynthesis has reached a critical knowledge base. We know the major players of light capture and carbon fixation, as well as how loss terms associated with them can be physiologically quantified. What is unclear is how algae and cyanobacteria integrate rapid, irregular changes in their light and CO_2 environment into the regular day/night rhythms associated with growth in any reasonable production environment. Understanding the systems biology associated with the stresses experienced in a photobioreactor (including light, CO_2 , O_2 , heat and nutrient stress) will be essential for pushing algal photosynthetic yields up to their theoretical maxima of 12% [4].

Photosynthetic rates saturate at a fraction of full sunlight ($\sim 10\text{-}20\%$, depending on species and environmental conditions). In full sunlight, processes such as non-photochemical quenching of light energy (NPQ) and alternate electron transport (AET) reduce the probability of damage from photo-oxidative stress (Box 1), but they also reduce the overall efficiency of algal photosynthesis. Inevitably, there is damage to the

photosynthetic apparatus despite the aforementioned protective mechanisms. The reduction in overall rates of carbon fixation, electron transport or the ability to convert light energy to chemical energy associated with these processes is termed photoinhibition. Reducing the impact of photoinhibition represents a major target for increasing overall efficiency. However, we know very little about the role of these processes, or any other cellular processes for that matter, in mass culture.

In the first part of this opinion, I give an overview of what is known about the physiology of photosynthesis in mass cultures of algae and show that photoinhibition is a significant issue in these systems. This is followed by a summary of the advances in systems biology as applied to photosynthesis in algae and cyanobacteria. I propose that the integration of these two approaches will allow the field to better identify and quantify the inefficiencies associated with the large-scale autotrophic cultivation of algae and cyanobacteria.

The ecophysiology of dense algal growth

Dense culturing is required to reach high aerial yields of algal biomass, which effectively reduces light penetration into the culture to only a few centimeters [6]. The cells perceive this as a net light-limited scenario and photo-acclimate. Photoacclimation involves an increase in the cellular concentrations of pigment-protein antenna complexes, photosystems or both to capture more light and vice versa. This further reduces light penetration into the cell suspension. It also increases the probability of overwhelming the light harvesting reactions in full sunlight, increasing the loss of energy as NPQ, AET (Box 1) or photoinhibition. Reducing the impact of this phenomenon has been an active area of

research. Strains engineered to have constitutively small antenna have higher photosynthetic efficiency at high light intensities and, in some cases, higher biomass productivity [7-9].

Dense culturing also creates a complex light environment for the photoautotrophic organism: cells are rapidly moved from an excess light environment into near complete darkness. The cellular physiology in these conditions has not been well studied and the dynamics of mixing, depth of the culture and cell density will all vary in different industrial-scale scenarios. For instance, the conditions experienced in a well sparged, thin (5cm) tube photobioreactor will be quite different from a 1 acre or larger sized raceway pond mixed by paddlewheels [10-12]. A successful engineering strategy to increase photosynthetic yields must anticipate the cellular response to these conditions and for that we need more observations of the photosynthetic physiology in industrially relevant conditions.

***In situ* physiology**

There have been relatively few published studies investigating the physiology of photosynthesis in algal mass culture. This is probably due to the financial constraints of constructing and operating large scale algal aquaculture facilities. But the data reported thus far are beginning to provide insights into the physiology associated with industrial scenarios.

Torzillo et al. [13] found that reducing the biomass of the diatom *Phaeodactylum* to 0.3 from 0.6 g l⁻¹ in a 4.85 cm tubular photobioreactor reduced the photochemical efficiency of photosystem II (PSII) and electron transport rates before solar peak irradiance, presumably reducing overall carbon fixation rates. Cells grown at the lower density in

small ponds (10cm depth) had little inhibition of photosynthesis and the highest aerial productivity. These results suggest more exposure to full sunlight reduced the overall efficiency of the process. All cultures had some degree of NPQ capacity although less than has been observed in algae grown in constant light in the lab. In a separate study, *Chlorella* and *Scenedesmus* grown in 50-liter tubular photobioreactors also showed NPQ capacity along with inhibition of PSII [14].

Cultures of the cyanobacteria *Anthrospira* (*Spirulina*) also show reduced inhibition of PSII activity when grown at higher densities (0.5 vs. 0.3 OD) when incident sunlight was shaded by 25% or with more rapid mixing [15]. Photosynthetic rate tracked insolation in the shaded culture compared to the uncovered culture, which clearly had 4 hours of photoinhibition around noon.

Nannochloropsis grown in flat panel bioreactors behaved as though acclimated to high light, while those grown in highly mixed, small ponds appeared acclimated to low light. Both showed photoinhibition just after peak irradiance and rapidly relaxing NPQ [16]. Interestingly, the cells grown in flat panels experienced higher temperatures and this led to a severe inhibition of photosystem II activity and slowed recovery of the maximal rates of photosynthesis [17]. This suggests that we should be considering the effects of other abiotic stresses on photosynthesis as well.

The examples from above show us, perhaps unsurprisingly, that there is clear variation between species and that there is variation between culture conditions. The observations that photochemical efficiency (or productivity) is increased by reducing incident light or increasing culture density are also intriguing considering this goes against the common view that more light penetration into the culture will increase productivity.

The choice of cultivation method affects the delivery and removal of gases within the culture as well. Dense cultures of algae or cyanobacteria will quickly deplete CO₂ from the media due to their rapid photosynthetic rates and increase ambient O₂, increasing the probability of photorespiration (Box 2). This likely has some effect on the observations reviewed above. However, it is currently unclear how cells respond to the oscillations in CO₂ supply that could occur in different industrial scenarios, although we know that even under “standard” lab or environmental conditions, many algae require a carbon concentrating mechanism to avoid CO₂ limitation of photosynthesis [18]. The high concentrations of oxygen that build up in small algal culture ponds were shown to severely inhibit photosynthetic rates of *Pleurochrysis carteriae* and this inhibition was further exacerbated by high temperatures [19]. Similarly, *Spirulina* cultures had a 20% reduction in photosystem II efficiency and overall biomass productivity when grown in 53 vs 20% mg/l O₂ [20].

We need to improve our understanding of what these observations mean for the overall efficiency of energy transduction from absorbed light to biofuel molecules of interest. Stresses associated with growth in variable environments change the metabolic demand for NADPH and ATP and compete with desired biomolecules for these substrates. This increased demand can be for resource acquisition (i.e. active pumping of bicarbonate or C4 style photosynthesis), to repair damaged enzymes or to detoxify harmful side products such as reactive oxygen species or the byproducts of photorespiration. Additionally, induction of energy dissipation pathways and damage to the photosynthetic systems will also reduce the total amount of energy available for fuel precursors or cell

maintenance [21]. A systems approach for understanding photosynthetic metabolism will allow us to identify, quantify and predict the flow of energy and carbon.

Systems biology of algae and cyanobacteria – state of the art

Systems approaches to predicting and manipulating metabolism have resulted in increases of heterotrophic fermentation yields [22] and also can be used to predict increases in plant biomass functions [23]. The development of these tools for algal and cyanobacterial systems over the last few years has now poised us to tackle the inefficiencies mentioned above. Model organisms such as *Chlamydomonas*, *Synechocystis*, *Synechococcus*, *Phaeodactylum*, *Thalassiosira* and most recently *Nannochloropsis* all have fully sequenced genomes and extensive molecular biology toolboxes. The field of algal systems biology is in its infancy and it is already being used to predict targets for increasing carbon partitioning towards biofuel precursor metabolites [24] and to observe changes in cell physiology and biochemistry in response to a changing environment.

A recent systems analyses of photosynthesis in *Chlamydomonas* in response to a doubling of light fluxes from low, limiting light to about 10% of full sunlight [25] points the way forward. A combination of metabolomics, proteomics, transcriptomics and modeling showed that the reactions of the Calvin-Benson-Bassham cycle were substrate limited in low light. Photosynthetic rate increased within 20 seconds and calculations of metabolite turnover rates suggested that increases in CBB metabolites within this timeframe were needed to fully activate the carbon fixation cycle resulting in the increased consumption of NADPH and ATP. Fixed carbon was funneled into starch synthesis to accommodate the increase photosynthesis before growth rates increased. The abundance of proteins

associated with photosynthesis was not well correlated with transcript levels during the transition and it appears that there was considerable post-translational control of carbon flux through photosynthesis. Transcriptomics revealed a rapid induction of LHCSR1 transcripts, encoding a protein known to be involved in NPQ [26], suggesting that even a mild shift in light can lead to the dissipation of light energy – although this was not measured. These conclusions would not have been possible without an integrated systems approach.

A second example comes from a systems level observation of N-starvation in *Chlamydomonas* [27]. N-starvation is a common way to induce lipid accumulation in some eukaryotic algae with the lipid being used as precursor for biofuels. N-starvation caused a decrease in photosynthesis capacity and photosystem II efficiencies over the course of 6-48 hours. Transcripts encoding the tetrapyrrole biosynthesis pathway for the production of chlorophyll and heme are quickly reduced in response to low N before any measureable changes in photosynthesis capacity. The different components of light harvesting and carbon fixation show a complex pattern of expression and accumulation of transcripts and peptides. They observed considerably variable dynamics even within a single photosystem (also seen in [25]). I will note that the N-starvation study was performed on algae growing in the presence of a reduced carbon source, and the authors clearly show an increased reliance on heterotrophic pathways to maintain growth. However, the complexity of the response resulting in the reduction in photosynthetic efficiency of the algae clearly shows that coaxing higher conversion efficiencies from light to lipid during N-limitation will involve complex engineering of the photosynthetic apparatus. The results of this study, and the photoacclimation study mentioned above, clearly point to the importance of post-

translational regulation of energy flux in changing environments and understanding the diversity and extent of this regulation will be a challenge for the field in the near future.

A third example employs systems biology information that has been assembled into genome-wide metabolic models of algae and cyanobacteria [28, 29]. These techniques, along with observations of ^{13}C flux in *Synechocystis* PCC6803 have suggested considerable and unexpected co-ordination between photosynthetic and catabolic metabolisms during growth in the light [30, 31]. Photorespiration was negligible during a transition from dark to light, but there appears to be a significant increase in ATP demand by Malic Enzyme and the Oxidative Pentose Pathway [31]. Models and measurements such as these will be incredibly valuable for not only identifying pinch points associated with photosynthetic metabolism but also for testing *in silico* how changes in biomass composition and photophysiology interact to influence cellular demand for ATP/NADPH [32] and the overall regulation of photosynthesis.

There have been other efforts into understanding the comprehensive response of algae to changes in light. These studies have spanned techniques and algal diversity such as the transcriptome of diatoms [33] and the proteome of coccolithophorids [34]. Each indicates the importance of metabolic remodeling in response to the changes in cellular energy states. Two recent transcriptomic studies of two different diatoms have revealed the extensive changes in cell biology associated with growth in a day/night cycle, which will be an important consideration when designing any manipulations associated with primary metabolism [35, 36]. Nunn et al. [37] found that light harvesting proteins were 4 of the 5 most abundant proteins in the diatom *Thalassiosira pseudonana*. This suggest that reductions in antenna size could increase the nitrogen use efficiency and the overall

sustainability of an algal mass culture [38] by reducing the N-demand per cell that would normally be associated with excess light harvesting.

So far all these observations are all based on small-scale, single variable experiments in the lab. However, there is now some movement towards the implementation of realistic scale-down technology [39], which should greatly facilitate our steps towards understanding the complexity of metabolism in mass culture. However, I will caution that investing too much effort in these technologies without validating them against large-scale systems will slow our progress towards meaningful strain improvements. Our goal is to increase yields in open ponds and large photobioreactors, not in bench top shake flasks.

The interdisciplinary collaborations that would be required among the fields of systems biology, photosynthesis and algal physiology could push the way forward to answering key questions in the field (Box 3). These questions are by no means exhaustive and represent a spectrum of topics that will reveal global regulatory elements, individual rate-limiting enzymes and interacting pathways that are involved in overall photosynthetic efficiency. A complete picture of photosynthetic efficiency in algae and cyanobacteria growing in a complex environment will require the co-ordinated employment of systems techniques with measurements of photosynthetic rate. This is crucial to being able to interpret changes in terms of photosynthetic efficiency. Also required will be an extensive time series across a day-night cycle that observes changes in protein accumulation, transcript abundance and “steady-state” metabolite abundances. This will reveal the complex physiological responses associated with stresses, such as photoinhibition, and the dynamics of recovery. Short-term studies that investigate the flux of carbon over the time

scales of mixing (seconds to minutes) in a photobioreactor, coupled to the measurement of energy dissipation pathways will be necessary. This will reveal the changing fate of absorbed light energy during rapid oscillations from near-dark to full sunlight. It is my opinion that this is the best way to dissect the impacts of NPQ, AET and photorespiration on carbon fixation and partitioning as all these factors can respond in seconds to changes in light absorption. The integration of all the experiments described above into dynamic models can then be used to develop testable hypotheses and engineering targets for increasing photosynthesis *in situ*. This could include identifying important post-translational controls of metabolic flux in real-world production scenarios. Using this integrated approach we will be able to ask questions along the following lines: How much energy is lost to NPQ or AET in a dense photobioreactor in the morning vs. the afternoon? Can we dispense of these processes if cells are only exposed to saturating sunlight for 1 second out of every 20 seconds during a mixing cycle? Is more carbon funneled to storage products if NPQ is removed and CO₂ is in excess? Understanding the complex systems biology associated with photosynthesis *in situ* will be our best approach to refining these questions with meaningful insight.

Finally, I think it is important to investigate the diversity of biochemistry found in the broad range of algae and cyanobacteria that has resulted from a complex history of endosymbiotic events and horizontal gene transfers [40]. Major reductions in the costs associated with DNA sequencing and bioinformatics analyses have facilitated the discovery of unexpected biology of algae and cyanobacteria. Recent examples of this include the discovery of an Entner-Doudoroff glycolytic pathway in diatoms [41] and the surprise of a novel enzyme that completes the TCA cycle in cyanobacteria [42]. It will be important to

ascertain the impact of novel biochemistries on overall metabolism. There is no clear winner for the “best” biofuel strain yet and novel biological solutions to metabolic problems may be found anywhere.

Concluding Remarks and Future Perspectives

Our understanding of algal biology has reached a major milestone. Sensitive physiological methods to measure photosynthesis and physiology can now be coupled to systems biology approaches. Improving photosynthesis will require close collaboration between algal physiologists with experts in metabolic modeling. Together, they can take the tools that have been outlined above and apply them to discovering and manipulating new targets that are relevant to increasing the productivity of photosynthetic microbes.

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Box 1 – Light energy harvesting and associated loss terms

Only a portion of the light spectrum is able to drive the photosynthetic reactions (400-700 nm, photosynthetically active radiation -PAR), and any unused portion of it is immediately lost. The majority of light is absorbed by pigment/protein antennae complexes and this energy is transferred to the photosystems. In photosystem II (PSII) the energy of a red photon (regardless of the photon energy absorbed) drives charge separation and liberated electrons are subsequently donated to quinones. The electron that is removed from PSII is replaced by the sequential stepwise oxidation of a water molecule, yielding molecular oxygen. The electron passes through an electron transport chain, eventually replacing an electron in photosystem I (PSI), which has become oxidized from an independent photon absorption event. The electron lost from PSI is ultimately used to reduce NADP⁺ to NADPH. Protons are liberated from water during water oxidation and are also pumped across the thylakoid membrane during electron transport. This creates a proton motive force driving the formation of ATP. Light energy that is absorbed in excess of downstream demand for ATP and NADPH is dissipated by a number of different pathways. Non-photochemical quenching (NPQ) is a process that toggles the light harvesting antenna from a state of light absorption and transfer to the reaction centers, to a state of absorption and the dissipation of light energy as heat. Up to 80% of absorbed sunlight is thought to be lost through this process [9]. This process is under physiological and genetic control and the proteins involved vary between algae and cyanobacteria [43]. There is also considerable diversity in the pigments and proteins that make up the antennae complexes in algae and cyanobacteria. Alternate Electron Transport (AET) is a process where electrons are

removed from the electron transport chain and used to reduce oxygen to create water. This process helps oxidize an over-reduced electron transport chain and can consume up to 49% of total electron flow in excess light conditions in some species [44].

Box 2 –Carbon dioxide fixation and associated loss terms.

The NADPH and ATP generated from light energy harvesting (often referred to as the “light reactions”) are used to drive the Calvin-Benson-Bassham cycle. Ribulose-Bisphosphate Carboxylase/Oxygenase (RuBisCO) catalyzes the essential step of CO₂ fixation with the remainder of the cycle consuming NADPH and ATP to regenerate the substrate for RuBisCO. RuBisCO has several inefficiencies. It is a torpid enzyme, capable of only a few turnovers per second and it also can use oxygen as a substrate, creating 2-phosphoglycerate (photorespiration). Photorespiration is an energetically expensive process, involving metabolite transport between organelles (in the case of algae) and the loss of fixed CO₂ and NH₃. Algae and cyanobacteria have evolved many different mechanisms to reduce the impact of photorespiration. Carbon concentrating mechanisms (CCM) favorably increase the ratio of CO₂:O₂ near RuBisCO. CCMs can range from active pumping of inorganic carbon species into the cells (bicarbonate transporters) to a C₄-type mechanism that captures CO₂ into an organic acid which is then transported to, and decarboxylated at, the location of RuBisCO [18, 45]. There are energetic costs associated with the CCM. Despite the presence of these mechanisms, photorespiration may account for a significant fraction of RuBisCO activity in laboratory culture (up to 28%) [46].

BOX 3 - Outstanding Questions

- What are the actual losses associated with the complex process of photoinhibition *in situ*?
- How do we integrate the rapidly changing environment of algal mass culture into genome scale metabolic models?
- How are heterotrophic and photosynthetic metabolisms coordinated to supply energy to the cell throughout a day and night cycle?