1. Introduction

The production of biofuels and other chemicals from photosynthetic microorganisms – primarily microalgae and cyanobacteria – has enormous potential because of the higher areal productivity of these organisms compared to plants, the relative ease of genetic manipulation, and the lack of requirement for arable land [1-5]. However, there are also considerable challenges at all stages of the production process, including water and nutrient use, cost-efficient cultivation and harvesting, and development of microorganisms that are robust and productive under industrial conditions and scales.

Cyanobacteria can grow more rapidly than eukaryotic algae and offer areal productivities estimated to be 350 barrel energy equivalents/acre/year, which is ~5x the estimated amount for algae [6, 7]. Interest in utilizing cyanobacteria as a microbial production platform for biofuel and biochemical production is increasing due to the ease of genetic manipulations of some strains like *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*).

Life cycle analysis (LCA) provides a system-level economic and environmental impact analysis. To identify the research targets that will have the most impact on the development of economical and environmentally sustainable photosynthetic biorefineries (PSBRs) based on cyanobacteria, we developed a preliminary LCA model. This model enables us to focus on four areas that limit the economic, environmental and resource scalability of PSBRs: (1) improving photosynthetic efficiency to improve productivity and biofuel net energy ratios; (2) developing accurate models of light effects on photophysiology to improve the robustness, energy consumption, and scalability of industrial-scale cultivation systems; (3) partitioning of cellular carbon towards biofuel molecules of interest to increase product yield and improve PBR economics; and (4) developing a high efficiency, low energy cell concentration system, which will lower production costs and improve biofuel economics.

The goals of this proposed project are to improve system level environmental, economic, and sustainability criteria through improvements in PSBR process design and organism design. Here, we assert that this effort will develop more economically and environmentally sustainable PSBRs through a set of aims to improve mixing/organism interactions, improve organism photosynthetic efficiency, produce novel products, and improve dewatering efficiency. All of these objectives are informed by system-level net-energy, environmental impacts, and scalability analyses of photosynthetic biofuels production processes to discover the areas of technological and biological development that will have large impact on PSBR sustainability metrics. For example, Fig. 1a shows the breakdown of energy consumption for a photobioreactor (PBR) microalgae-based biodiesel. The primary energy inputs to the process are sparge (99% of growth stage energy consumption, primarily functioning as a means for mixing), dewater (centrifugation), extraction, and conversion [11]. Fig 1 (b-d) illustrates the potential impact that our research targets may have on the LCA. Making a biofuel molecule that does not need transesterification (Fig 1b, Aim 3) reduces the conversion stage energy requirements. Improvements in photosynthetic productivities with less mixing, and the channeling of this increased production to the appropriate biofuel (Fig 1c, Aim 2 and Aim 3) will reduce the energy inputs into the growth stage. Improving dewater and separation technologies (Aim 4, Fig. 1d) reduces the Energy Ratio of the system to <0.1 (Energy Return on Investment>10). This is less than the energy of extraction for petroleum-based fuels. Aim 5 will continue to inform and validate the results of these biological and process engineering tasks. Based on a system-level understanding of the impacts of PSBRs, this research program provides an informed pathway for an economically and environmentally competitive biofuels process.

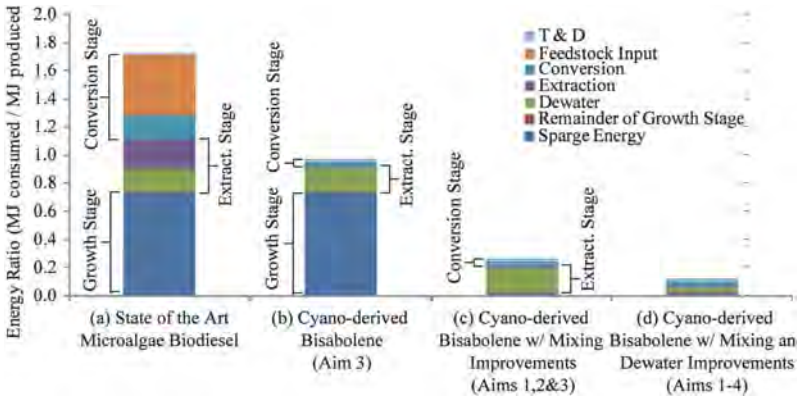

Figure 1. Allocation of biofuel production process energy to the process steps for the growth, extraction, conversion and transportation and delivery (T&D) process phases. This analysis is based on the cultivation of *Nannochloropsis* in a PBR using production-scale processes and growth rates, co-location of CO_2 sources, and no co-product credits.

The proposed research will use *Synechocystis* as a model photoautotrophic organism owing to its potential for high areal productivities, to the availability of a variety of molecular biology techniques needed for genetic manipulations [13, 14] and to the sequenced genome [15, 16]. The knowledge gained from the proposed work is expected to translate to other cyanobacterial strains of interest. Previous efforts have lead to intriguing but incremental improvements in either hydrocarbon feed stock production [8] or in laboratory photosynthetic efficiency [18].

2. Vision and Goals

Vision: A systems approach, guided by life-cycle analysis, that bridges photobioreactor analysis, photophysiology, and metabolic engineering, will enable the development of highly productive photosynthetic biorefineries.

The proposed research applies expertise from fluid dynamics, photosynthetic physiology, proteomics, metabolic engineering, and LCA to address the issue of increasing commodity yields. We intend to model the environment of different PBR strategies and use these models to design accurate scale-down systems for cultivating cyanobacteria in the lab. This will improve our ability to predict large-scale performance from bench-scale experiments. Observations of photophysiology within the lab will be used to quantify loss processes and provide targets of genetic improvements. This research will be coupled to the metabolic engineering of cyanobacteria to shuttle fixed carbon to molecules of commercial interest. These molecules include alkanes/alkenes, which are of broad interest as biofuels to many sectors including the Air Force. The same lab-scale PBRs will be used to ascertain realistic productivity potentials of these bioproducts at scale. To address a critical engineering need in the production of biofuels and biochemicals from photosynthetic microorganisms, a low-energy process will be developed for cell concentration during the initial harvest. Finally, LCA will integrate these results to assess the sustainability of different PBR, photosynthetic engineering, and cell concentration solutions, and suggest additional novel targets.

The goals of this project are to significantly advance the economic and environmental sustainability of cyanobacterial photosynthetic biorefineries. Our methodology will first be applied to increasing the sustainability and total productivity of a system that employs a model cyanobacterium to produce bisabolene. However, we strive to pioneer an integrated approach that can be applied to any combination of organism(s) and product(s). To accomplish these goals, we have developed a program of five specific aims:

1) Develop accurate models of the mixing dynamics and light environment experienced by cyanobacteria in production-scale PBRs, including closed bioreactors and open ponds, and translate these models to bench-scale bioreactors.
2) Quantify photosynthetic loss processes and efficiencies of *Synechocystis* in industrially relevant environments and tune photosynthesis for specific bioreactor conditions to achieve increased photosynthetic efficiency
3) Increase production of alkenes in cyanobacteria using an expanded genetic toolkit
4) Develop a low-energy cell concentration process based on inertial migration
5) Integrate and validate productivity efficiencies with life cycle assessments of photo-biorefineries.

3. Addressing the Required Photosynthetic Biorefineries Elements:

- PSBR 1: Process concepts based on foundational knowledge, and explore innovative ideas. Applying and gaining fundamental knowledge in engineering, life sciences, and environmental sciences is the foundation of all 5 Specific Aims. Aim 1 applies foundational principles in chemical engineering to understand the physical processes at play in large-scale PBRs. This will enable development of *accurate* bench-scale PBRs, which in turn will allow researchers to accurately predict behavior on a large scale from small-scale experiments. Aim 2 works to gain foundational knowledge about the photophysiology of cyanobacteria under realistic environmental conditions. Aim 3 applies foundational knowledge from molecular biology and biochemistry and gained in Aim 2 to strain design. Aim 4 applies foundational principles in chemical engineering to cell harvesting. Aim 5 applies foundational knowledge across disciplines to assess the process for sustainability.
- PSBR 2: Process concepts demonstrate innovative ideas for efficiently capturing CO_2 and sunlight. Aim 2 utilizes systems biology to gain a better understanding the complex interactions between the environment and the effect on capturing CO_2 and sunlight. This information will inform strain design and rational design of a terpenoid producing strain.

- PSBR 3: Process concepts demonstrate scalability and embrace a multi-scale systems approach.

Aims 1-3 address the issues of scalability. We are taking a unique approach in terms of working to accurately scale down commercial PBRs so that experiments performed on a lab-bench scale will more accurately predict the performance in commercial PBRs. Systems biology will be utilized to understand and link photophysiology to PBR design and will also be utilized to inform strain development in Aim 3.

- PSBR 4: Economic, ecological, and life cycle perspectives. Aim 5 directly addresses these issues.
- PSBR 5: Process concepts demonstrate sustainability of natural resource utilization. Aim 5 will provide an assessment of water and nutrient use in biofuel production processes based on photosynthetic microorganisms. As part of this work, process modifications (e.g., incorporation of hydrothermal gasification of waste biomass for nutrient recovery) to increase re-use of water and nutrients will be explored. Energy use is also a key consideration for these processes, and the novel inertial migration method to be developed in Aim 4 will result in energy savings. In addition, increased photosynthetic efficiency, with modifications to direct photosynthetically fixed carbon toward product formation, will result in more product formation per unit nutrient consumed.

4. Prior Research

4.A. Fluid dynamics: computational simulations and experiments

Co-PI Dandy and his research group have utilized well established computational fluid dynamics (CFD) techniques to rigorously simulate complicated three-dimensional incompressible flows, for a variety of applications that include microfluidic mixing, thermal and plasma chemical vapor deposition, *Chlorella* and *Spirulina* microalgae recovery, and dense medium plasma remediation. While not directly applicable to the Aim 1 goals of this project, the following example illustrates the geometric complexities that may be addressed, and demonstrates the potential of this approach as a design tool.

4.A.1. Dense medium plasma reactor (DMPR)

Plasma treatment is an appealing method for the disinfection of contaminated water. The process is characterized by the production of high oxidation potential species and a wide spectral emission. The UV radiation and oxidation species produced by the plasma discharge are effective in deactivating microbial species, as well as initiating oxidation reactions. The DMPR was developed for reacting liquid/vapor phase species in an induced plasma state using low temperature plasma chemistry. The reactor consists of a vessel containing a rotating array of 25 pin electrodes, stationary lower electrode, cooling system and gas introduction and discharge ports. CFD simulations are singularly suited to provide qualitative and quantitative information, which may not be readily accessible in the experiments, regarding the interaction of the liquid in the DMPR with the plasma and the characterization of the fluid in the vicinity of the plasma. The simulations were utilized to provide insight into the following questions that arose as a consequence of experimental results: (1) what is the volumetric flow rate of the liquid through the plasma as a function of pin array spin rate and reactor configuration? And (2) does spinning the pin array induce a pressure gradient along the surface of the pin electrodes, thus focusing the location of the discharge to a distinct region on the pin electrode surface away from the location where the radius of curvature is minimized? The results from the simulations successfully addressed these operational questions and led to significant reactor design modifications. [24] Although this study does not address the modeling of flow in PBRs, it illustrates the rigor that this approach can bring to develop a physical understanding of microscopic and macroscopic systems. And the approach allows incorporation of other complicating effects such as particle transport, nonisothermal behavior, and radiative transport.

4.A.2. PBR Mixing Characterization and Design

The commercially significant output of this study will be optimal configurations and levels of sparge mixing that maximize the economic, GHG and net energy performance of the growth stage of algae biofuels. Whereas the studies performed to date have concentrated on optimization of the reactor as an independent subsystem, this study will measure the degree of reactor optimization using system-level design metrics such as lifecycle energy use. Unlike the data that exists currently in literature, these results will be performed at commercially relevant scales. These data will explore the lower power region of the sparge mixed fluid environment to determine whether the tradeoff between algae growth rate and sparge power consumption leads to net costs or net benefits relative to the current state of the art. The economic and environmental viability of algae biofuels is highly dependent on the rate at which growth drops off with sparge energy in this region. If algal growth rates can be preserved at low mixing power, then the lifecycle energy of algae will be significantly improved at the lower power regime. Possible means for reducing this energy consumption include (1) separating the mass transfer and mixing functions of sparge into two

independently controlled and orthogonal systems, (2) coordinating sparge timing and flow to achieve chaotic, disperse energy dissipation within the reactor, or (3) changing reactor shape to allow for higher efficiency mixing and mass transfer. Preliminary data have been collected regarding the flow structure within sparged industrial-scale bioreactors (Fig. 2). These data will be used to validate and inform CFD and experimental investigations into reducing sparge and mixing energy consumption.

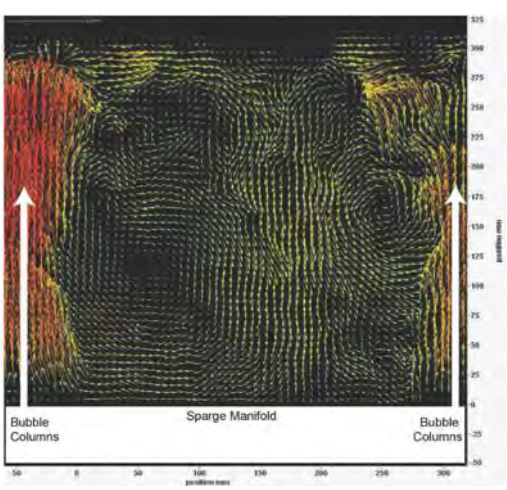

Figure 2. PIV data set showing preliminary test cases to determine flow structure within sparged industrial-scale bioreactors. Regions of high velocity correspond to the bubble columns. The net circulation established by the sparge system is evident in the central downward fluid motion.

4.B. Optimizing cell concentration based on inertial migration

When a dilute suspension flows in the laminar regime through an enclosed channel such as a tube, the suspended particles migrate radially to an equilibrium position; in a tube of circular cross section this equilibrium position is a very tight, well-defined annulus. With the advent and now ubiquitous use of soft lithography techniques, it is straightforward to construct multi-layer, massively parallel microfluidic networks (μFNs). The ability to use this approach to concentrate microparticle suspensions has been demonstrated with straight [25] and curved [26] microchannels. The Dandy group has experience designing and fabricating μFNs for use in biosensors, micromosaic assays, and biomarker sampling.[27-31] Capitalizing on the published literature and our microfabrication capabilities, we have conducted preliminary experiments to passively concentrate microparticles of size (2 μm) comparable to microalgae. Four identical microchannels were constructed in polydimethylsiloxane (PDMS). Two such channels are shown in Fig. 3(a). The channel dimensions (20 μm high, 50 μm wide) result in particle migration toward two equilibrium locations, and the serpentine structure causes secondary (Dean) flow that moves those locations next to the microchannel sidewall. The suspension subsequently enters an expansion to slow its velocity and aid in redirecting the particles into a smaller side channel. For subsequent passes to further concentrate the suspension a number of side channels are combined and sent through another serpentine channel. This approach was applied for 3 subsequent passes (Fig 3(b)), and it was determined that the suspension was concentrated from 0.01 vol% to 1.75 vol% (±0.08 vol%).

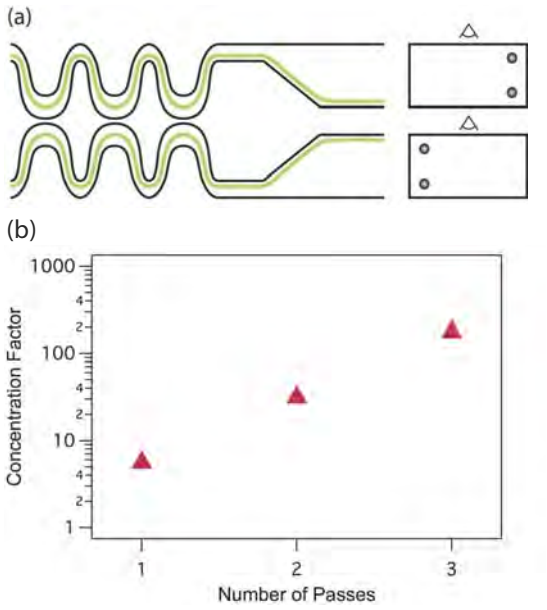

Figure 3. (a) Schematic of two adjacent serpentine microchannels demonstrating the combined effects of inertial migration and Dean flow on particle focusing. The suspension enters an expanded region to slow its velocity and facilitate splitting of a particle-enriched stream. (b) Laboratory results demonstrating the effect of sequential concentration of an initially 0.01 vol% suspension. The concentrated streams from adjacent microchannels were combined, and the process was repeated for two more passes. After three passes the concentration factor is 175.

4.C. Transcriptomic and proteomic profiling of photosynthetic microbes

Dr. Peers performed transcriptomic experiments aimed at discovering factors regulating productivity when he worked in the biofuel industry. Dr. Reardon has extensive experience with proteomic analysis [32], including method development and proteomic profiling of cyanobacteria and microalgae [33-36]. His laboratory operates an ABSciex state-of-the-art quadrupole/time-of-flight tandem mass spectrometer (see Facilities) for quantitative proteomics, and is developing methods for quantitative phosphoproteomics.

4.D. Physiological response of photosynthetic microbes to variable light exposure

Peers has broad experience investigating the response of the algal photosynthesis to abiotic stress. His work has resulted in the discovery of unexpected proteins involved in the light reactions of photosynthesis including electron transport [37], reactive oxygen detoxification [38] and in thermal dissipation of excess light energy [39].

Peers and Reardon performed preliminary experiments to explore the changes in cellular physiology and proteomes associated with growth in a variable light environment vs. constant light. Our first step was to compare the physiology and proteome of *Synechocystis* sp. PCC 6803 cultures grown in continuous (100µmol photons m^{-2} s^{-1}) and oscillating (200 µmol photons m^{-2} s^{-1}, 30s on/off intervals) light. Cultures were bubbled vigorously and their pH was monitored to guard against CO_2 depletion. Care was taken to keep harvested biomass low to ensure that the cells were nutrient replete. Proteins extracted from cell pellets were trypsin digested and collected peptides were analyzed with LC/MS/MS (ESI-TripleTOF, ABSciex, [40]). Acquired data were processed with PEAKS 6.0 (BSI) for protein identification and label-free quantitation. Any protein found to have 1-fold or greater change in intensity was screened for statistical significance using a paired t-test. Cultures in oscillating light had a maximum growth rate approximately half that of continuous light, despite the same total light exposure. Comparison of replicate analyses suggested significant shared coverage of photosynthetic proteins and the proteome in general. Eighteen proteins were identified as differentially accumulated, eight of which are directly involved in photosynthesis including proteins that play a component in the Calvin cycle (Fig. 4). The differential expression of the fructose 1,6-/sedoheptulose-1,7-bisphosphatase (glpX), which catalyzes two steps of the cycle in cyanobacteria, is intriguing as its activity been shown to be a key regulator in biomass productivity [41]. We also observed changes associated with light harvesting, oxidative stress and several proteins of unknown function. These results show that there are major changes in cellular physiology associated with growth in a variable light environment that are not intuitive.

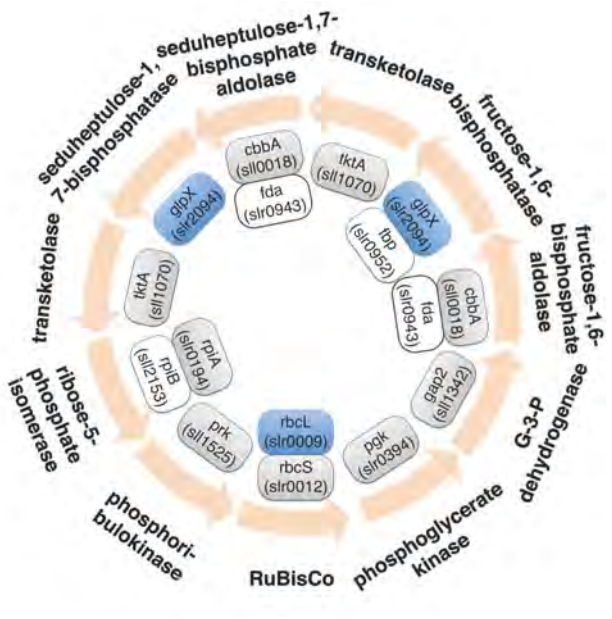

Figure 4. Schematic of Calvin Cycle. Blue boxes show gene IDs associated with proteins that show >2 fold accumulation in constant vs. fluctuating light. White boxes show genes with no peptide hits and grey boxes show genes with peptide changes <2 fold.

4.E. Metabolic engineering of photosynthetic microbes

4.E.1. Engineered photosynthesis: Dr. Peers engineered photosynthesis in photosynthetic microbes to increase productivity the results of which are contained in a patent application ("Enhancement of Biomass Production by Disruption of Light Energy Dissipation Pathways" US2012-0178134)

4.E.2. Tool Development in cyanobacteria: The Peebles lab has previously published her work on the development of a counter-selection marker for *Synechocystis* [42].

Characterization of promoters in *Synechocystis*: Engineering of promoters to balance gene expression of heterologous pathways have been extensively pursued in heterotrophic organisms such as *E. coli* and yeast but little work has been pursued in photoautotrophic organisms which undergoes diurnal cycles. As a result of these diurnal cycles, the cellular environment varies greatly over a 24 hr period. The Peebles lab has begun work to characterize promoters that function under different cellular conditions (constitutive and light induced) to allow for controlled gene expression.

For example, systems inducible via circadian rhythm changes allows for rational control during optimal cellular conditions [43, 44]. Examples of circadian control include the light induced *psbAII* and dark induced *lrtA* promoter systems in *Synechocystis.* The *psbAII* promoter region is responsible for generating the D1 protein in the photosystem II complex present in *Synechocystis* [45, 46]. During high light stress, the D1 protein degrades 15 times faster when compared to low light conditions. Due to this rapid

degradation, the *psbAII* promoter up-regulates gene expression during high light conditions [47, 48]. Because of this, the *psbAII* promoter is the most popular promoter used when engineering product production in photosynthetic cells [9, 10, 49, 50]. Several control regions on the *psbAII* promoter have been identified that modify polymerase binding during varying light conditions [48, 51, 52]. To better understand how the *psbAII* promoter regulates gene expression and to modulate gene expression, the Peebles lab has constructed and is testing variants of the *psbAII* promoter. To characterize the promoter manipulations, each promoter controls the expression of GFP_{mut3} and is transformed into the same location of the *Synechocystis* genome via homologous recombination. This GFP variant has been specifically designed for use in a photosynthetic organism [53]. We have observed that the native *psbAII* promoter is down-regulated by 1.5 to 5 fold as cell density increases. Our modified *psbAII* promoter shows higher expression and it is not as severely down-regulated at higher culture densities. These results indicate that the *psbAII* promoter is not an appropriate promoter to use to drive metabolism at high cell densities. It also highlights the need for molecular tool development to control cells in industrially relevant conditions. We will explore the development of novel promoters to be utilized for metabolic engineering in ***Aim 3***.

4.F. Life cycle assessment of photosynthetic microbial processes

Previous research has attempted to understand the lifecycle environmental impacts of microalgae growth and lipid production through modeling, experiment, and system-level analysis. Recent studies have been focused on representation of LCA informed by industrial-scale production data [11]. These results highlighted several weaknesses in the field that required experimental data to address. For example, many microalgae LCA studies to date have used the IPCC standard to represent the N_2O emissions due to N-fertilizer denitrification. This standard was developed (and is appropriate for) land-based crops, but its applicability to microalgae production was unknown. Bradley's group collaborated with denitrification experts in Environmental Engineering and air quality experts in Mechanical Engineering to perform experimental algae cultivation work that showed that the IPCC standards were overestimating the N_2O emissions of microalgae cultivation by a factor of 500 [54]. As N_2O emissions have a 100-year global warming potential 298 times that of CO_2, these results are of critical importance for microalgae-based biofuels net GHG emissions modeling. Recent work has concentrated on process-integrated microalgae LCA, air pollution production, microalgae growth kinetics, water footprint and US productivity potential [55-58].

5. Approach and Methodology

The research plan outlined below will enable us to achieve sustainable routes to biofuel and biochemical production in *Synechocystis* in large-scale PBRs. ***Work on each aim is intimately connected with others, as indicated in the text below.*** A synergistic approach will be achieved through regular communication among the PIs, postdoctoral scholar, and graduate students, and by having researchers associated primarily with one aim also sometimes participate directly in work on other aims.

5.A. Specific Aim 1: Develop accurate models for scale-down of PBRs (Dandy; Reardon)

Cyanobacteria in cultivation systems are rapidly mixed from near darkness into light fluxes that oversaturate the photosynthetic system, exacerbating inefficiencies of solar energy conversion. *We propose that tailoring the photosynthetic process to different cultivation system mixing dynamics will increase yields of commercially relevant products.* We will model the light environment experienced by cyanobacteria in a variety of possible reactor mixing regimes and will test the results in a scaled-down laboratory photobioreactor that mimics large-scale reactors. Multiple reactor types will be investigated from tubular photobioreactors (PBRs) to open ponds. Transcriptomics, proteomics, biomass production, and physiological assays of photosynthesis will be used to identify gene targets for monitoring energy status of the cell, to reveal novel genes involved in the photosynthetic process and to unveil key regulatory systems (***Aim 2***). Increasing the efficiency of light energy harvesting through photosynthesis is an important component for the success of cyanobacterial- and algal-based bioproducts. The larger the amount of absorbed light energy that can be used to fix carbon, the higher yields one can expect per unit area. Currently reported yields of PBRs are lower than theoretical calculations [59] and the inefficiencies of photosynthesis are believed to be a major loss term [60]. Understanding and manipulating the light environment within PBRs and the organisms within these systems is a critical need for increasing yields.

A key strategy in the proposed development of new cyanobacteria strains is the implementation of bench-scale PBRs that share common physical, chemical, and thermal characteristics with their large-scale counterparts. Accurately scaled-down reactors allow for experimentation that predicts performance on the industrial scale. These scaled-down reactors allow for scientist and engineers to test how engi-

neered microorganisms perform as biocatalysts under a variety of conditions at a reduced cost. These conditions can include different genetic manipulations, different media composition, culture conditions, etc. If the scaled-down reactor does not mimic the industrial scale reactors, the process will not perform as expected which ultimately results in a loss of resources. The problems associated with scale-up/scale-down in industry are well documented for heterotrophic microorganisms [61-65]. These problems are typically caused by poorly mixed regions and enhanced stress exposure within the large-scale reactors, which can cause metabolic shifts in the heterotrophic microorganisms [65]. Investigators have altered lab-scale conditions to mimic the stresses associated with industrial scale with success [66-69]. In addition, computational fluid dynamics has been utilized to describe the gradients and oscillations experienced by heterotrophic cell in the large-scale reactors [67].

Similar quality work in photoautotrophic microorganisms is lacking. The role of light in the growth of these photoautotrophic microorganisms adds a new dimension of complexity that must be considered during scale-up/scale-down of these cultures. To successfully scale up cyanobacterial cultures that produce biofuels at an industrially relevant level, we need scaled-down PBRs that accurately reflect the industrial scale photobioreactors so that we can test how modified phototrophic microorganisms will perform under different conditions. This information will drive the metabolic engineering strategies utilized to design a biofuel-producing strain of cyanobacteria. These accurately scaled-down PBRs do not exist. In work on Aim 1, we will develop scaled-down photobioreactors that mimic large-scale PBRs and will use these reactors to gain a better understanding of the photophysiology of the cyanobacteria (***Aim 2***) and to design metabolic engineering strategies for biofuel production (***Aim 3***). This information will also be utilized in the life cycle analysis to determine the sustainability of our process (***Aim 5***).

The transformative nature of this Aim is that it is one of the first attempts to intelligently scale down a commercial-sized PBR system to the bench scale so that advances made in developing new strains of cyanobacteria will directly scale upward to the full-sized, site-deployable PBR. Bench-scale PBRs will be an integral component in understanding photophysiology (***Aim 2***), evaluating performance of engineered strains (***Aim 3***), and for providing experimental parameters for LCA (***Aim 5***)

For this Aim, the team will employ three-dimensional computational fluid dynamics (CFD) simulations, coupled with dimensional analysis, to model liquid flow, energy and mass transport, cell distribution, and light intensity distribution **in several different, industrially relevant, PBR types**. It is recognized that proper design and analysis of PBRs is significantly enhanced through the use of CFD [70]. Even more important, it has been demonstrated that CFD and trajectory analysis are very useful for scale-down and scale-up.[71] *As an example of this approach,* we present the process for one type of PBR (note that we will investigate many different types in practice): Transparent tubes will be arranged in coils or U-bends [72, 73] to promote mixing through generation of Dean vortices [74, 75]. The CFD models will be applied to full-size tubular PBRs to ensure that they capture quantitatively the conditions and behavior of the large-scale systems. Once the transport, particle tracking, and light attenuation models have been validated, conditions will be selected for the proposed bench scale systems such that governing dimensionless quantities—including the Reynolds, Peclet, Dean, and Froude numbers—are equivalent between the large- and bench-scale systems. The CFD and light attenuation models will be applied to the reduced dimension geometries to quantify that local environmental conditions (fluid shear, vortex strength, CO_2 partial pressure gradients, and cell residence times) are representative of the larger systems.

While this effort does not represent the first time that CFD has been applied to study PBR characteristics, it is one of the first attempts to intelligently scale down a commercial-sized system to the bench scale so that advances made in developing new strains of cyanobacteria that will directly scale upward to the full sized, site-deployable PBRs.

Task 1: Validate against data from large-scale systems. The commercial package ANSYS will be used to carry out all CFD modeling. Specifically, the Fluent component will be run on co-PI Dandy's Linux cluster in parallel format. Data from published experimental and computational studies of PBRs will be compared against three-dimensional Fluent simulations that capture distributions of dissolved O_2 and CO_2, cell motion, and light transmission. The outcome of this task will be a fully validated numerical model for liquid flow, particle (cell) transport, chemical species transport, and light transmission.

Task 2: Perform dimensional analysis for scaling. The suite of results from Task 1 will be applied to develop appropriate dimensionless scaling factors to guide the design of the laboratory-sized PBR to be implemented in ***Aim 2***. It is anticipated that classic dimensionless analysis of rate data [76] will satisfactorily address the goal of this task. The dimensional analysis approach will be applied to both published data and the mathematical physics models underlying the Fluent code. The outcome of this task will be a

well-defined set of characteristic scales and parameters that will define the scaled-down PBR to be implemented in the next Aim.

Task 3: Validate against data from small scale PBR. In parallel with laboratory experiments using the laboratory scale reactor, CFD simulations will be performed to demonstrate the validity of the analysis in Task 2. The simulation results will be used to confirm reactor conditions are in line with the large-scale PBR counterparts. The outcome of this task will be the confirmation of a successful scale-down exercise. The designed bench-top PBR will accurately simulate, CO_2, light, O_2, shear and temperature environments found in a variety of closed photobioreactors and open ponds.

5.B. Specific Aim 2: Characterization and manipulation of *Synechocystis* photophysiology to increase productivity in industrially relevant operating conditions (Peers; Reardon)

Increasing the efficiency of light energy harvesting through photosynthesis is an important component for the success of cyanobacterial- and algal-based bioproducts. The larger the amount of absorbed light energy that can be used to fix carbon, the higher yields one can expect per unit area. Currently reported yields of photobioreactors (PBRs), which we define as both closed systems and open ponds, are lower than theoretical calculations [59] and the inefficiencies of photosynthesis are a major loss term [60]. Understanding and manipulating the light environment within PBRs and the organisms within these production systems is an important step towards increasing yields.

We aim to increase photosynthetic productivity of cyanobacteria in industrially suitable conditions. Cyanobacteria must be grown to high biomass to achieve reasonable aerial yields of biomass, however this leads to a situation where the light penetration into the PBR is severely reduced. Cyanobacteria are known to respond to low light by increasing light harvesting antenna size and the total amount of pigment in the cell in order collect more of the limiting resource [77]. This photoacclimation strategy further reduces the penetration of light into the PBR or pond. On the other hand, mixing of these cultivation systems moves cells into regions with light levels that supersaturate photosynthesis and create photooxidative stress [78]. In high light, cells induce photoprotective mechanisms such as nonphotochemical quenching (NPQ) [79, 80] and alternate electron transport that harmlessly dissipate excess energy, but significantly reduce energy flow to CO_2 fixation [81]. Photosynthesis in an industrially relevant PBR strives to balance these two contrasting behaviors that benefit algae in nature but end up being detrimental to industrial productivity. Correcting these wasteful processes has been recognized as one of the major biological engineering targets for photosynthetic microbe improvement [34].

NPQ is estimated to dissipate 80% of absorbed energy in full sunlight as a strategy to protect the photosynthetic apparatus from over-excitation, oxidative stress and photoinhibition. Alternate electron transport and photorespiration can further reduce energy conversion efficiencies. Twenty % of the electrons liberated from water can find their way into these pathways in *Synechocystis*, and this increases to 60% in low CO_2 conditions [82]. Genetic deletion of some proteins required for this activity results in damage to photosystem II [83]. However, the alternate electron transport pathway (which has many components) can also serve to increase ATP:NADPH ratios [84] so it can be recognized both as a path to adjust photosynthesis to metabolic demand as well as a way of protecting against oxidative stress. We do not yet know how these processes behave in an industrial setting and how they contribute to overall photosynthetic efficiency. The results shown in section 4D suggest our understanding of the photosynthetic system in fluctuating light is not clear. We anticipate that tuning our culturing to realistic conditions will reveal many uncharacterized proteins and pathways responsible for regulating photosynthetic efficiency.

Task 1: Measure photosynthetic efficiencies and loss processes in scale-down systems. Cells will be grown in lab scale PBRs (Phenometrcis ePBRs) in conditions designed and described in ***Aim 1***. Biomass productivity will be estimated by measuring Ash-Free Dry Weight of algal cells throughout a growth cycle. **Initial investigations will explore the differences in two contrasting environments: (a) a tubular bioreactor and (b) a one-acre pond, which represent two extremes of deployment technology for photosynthetic microbes.**

We will investigate the photosynthetic physiology and productivity of strains by using a combination of techniques: A) *Membrane Inlet Mass Spectrometry* – to measure oxygen evolution, dark respiration and the contribution of alternate electron transport and photorespiration (measured as $^{18}O_2$ consumption) to total electron flow. B) *Chlorophyll Fluorescence* - (Fluorescence Induction and Relaxation [85] and Pulse Amplitude Modulation [80] – to measure the maximal quantum yield of photosystem II (Fv/Fm), the functional absorption cross section of photosystem II, the oxidation rate of the plastoquinone pool and, to estimate the induction of NPQ. C) *Ash-Free Dry Weight* – to observe total biomass productivity changes.

Photosynthetic capacity (methods A and B) will be estimated at several constant light levels, but cells will also be exposed to modeled variations in light in separate experiments to observe the dynamic response of the photosynthetic apparatus. These assays will be performed throughout the day and night cycles of growth to thoroughly explore the behavior of photosynthetic light harvesting in realistic conditions. Initial experiments will inform future, detailed experiments aimed at understanding the molecular regulation of these processes (Task 2). We will further validate this technology by observing the behavior and efficiency of photosynthetic mutants, such as those with reduced antenna [18, 86], which are predicted to have increased productivity under in large scale cultivation.

Task 2: Proteomic and transcriptomic comparisons. Most 'omic data from photosynthetic microbes is collected from experiments aimed to observe changes associated with an acute stress imposed on benign growth conditions. While this has greatly increased our understanding of stress responses, it does not directly inform us of the molecular mechanisms at play in a realistic setting.

We propose to couple transcriptomic (RNA-seq, [87]) and proteomic profiling [32-35] to the experiments described above. We will contrast the transcriptome between light and dark in two different mixing environments that we feel span the spectrum of possible PBR conditions. RNA samples will be processed at the CSU core facility affiliated with the Infectious Disease Research Center; sequenced using the AB SOLiD 4 platform. Core facility staff routinely assist and train students and PIs on analysis software. These studies will identify novel genes involved in photoacclimation (comparing cells that are net light limited to those that spend more time in high light), expose pathways regulated by the day/night cycle and also suggest genetic control elements that can be used for tool development (***Aim 3***, Task 4). Proteomic studies will compliment this work and also reveal novel post-translational control of photosynthetic proteins via phosphorylation and thiol-disulfide redox modifications. Both processes are important for regulation of light energy harvesting in the laboratory ([88] and [89], respectively) but their response to a highly variable environment and light/dark cycling is unknown.

These studies will also expose unexpected regulation of many of the enzymes and pathways that will be manipulated in ***Aim 3***, further expanding out targets for increasing fuel molecule productivity.

Task 3: Manipulating photosynthetic productivity. Data collected in ***Aims 1-3*** will combine to direct us to novel targets that may increase photosynthetic efficiency. We will employ standard techniques of gene knockout by homologous recombination and also utilize the expression tools described in ***Aim 3***, Task 4 to control the expression of target genes. We have already uncovered several targets from our preliminary experiment (Section 4D above) including 4 genes of unknown function and components of the Calvin cycle that are differentially regulated between constant vs. variable light. Investigating cells in more realistic environments will likely reveal other strong candidates. We will also use site-directed mutagenesis to investigate the importance of certain post-translational modifications (identified in Task 2) to overall photosynthetic behavior. Cyanobacterial mutants with reduced antenna size have been shown to have increased productivity and reduced photoinhibition [18]. We will integrate the generation and study of such mutants (several phycobilisome-antenna mutants have already been generated in the Peers lab) into our work to discover the interplay between light energy capture and the systems biology of cyanobacterial bioreactors. Our work will disrupt the field's standard approach to predicting and engineering photosynthetic behavior and lead to an increase in areal photosynthetic productivity.

5.C. Specific Aim 3: Engineering of *Synechocystis* for the synthesis of biofuels (Peebles; Peers)

In this aim, we will incorporate the insights derived from the other aims (in an iterative fashion) into our rational design strategy to engineer increased productivity of value added molecules in *Synechocystis*. Our initial target is the sesquiterpene bisabolene, which can be utilized as a D2 diesel fuel replacement. We have chosen to focus on the production of only one molecule so that our efforts can be directed toward achieving the optimum productivity and yields. The insights we gain here will be directly transferrable to the production of other sesquiterpenes for both biofuel and biochemical applications. We will utilize *Synechocystis* sp. PCC 6803 as a proof-of-concept strain since a variety of molecular biology tools have been developed for this organism, and it is one of the more well-studied strains of cyanobacteria. The initial strategy for strain engineering will focus on local pathway manipulations to increase product yields and productivity. These manipulations will be combined with the insights gained from reactor modeling and from detailed studies of the response of cyanobacteria to the different reactor conditions (***Aim 2***). LCA (***Aim 5***) will identify the productivity targets that need to be reached for a sustainable process.

Past metabolic engineering efforts in cyanobacteria have garnered modest gains in product production (Table 1). These results are similar to the early years of work in *E. coli* to produce various products before more advanced tools and techniques were developed. These past cyanobacterial studies have been performed utilizing molecular tools not designed for optimal product production and have been performed under 24 hour light conditions which do not mimic the natural light:dark cycles these strains would experience in an industrial setting. This work will identify and characterize new molecular tools (such as promoters) to be utilized for *optimal product production in an industrial environment* based off hypothesis generated in **Aim 2**. Pathway optimization and genetic manipulations will build off an iterative approach of learning that is connected to all aims. The performance and sustainability of the strains will be evaluated in the bench scale photobioreactors (***Aim 1***) and through LCA (***Aim 5***). Our approach of manipulating carbon flux to biofuel molecules in industrially relevant conditions will revolutionize the way others approach the engineering of any photosynthetic microbial platform.

Table 1. Examples of cyanobacterial metabolic engineering efforts

Product	Organism	Production	Source
fatty acid	*Synechocystis*	197 mg/L	[8]
isoprene	*Synechocystis*	50 µg/g dcw	[9]
zeaxanthin	*Synechocystis*	2.49 mg/L	[10]
ethanol	*Synechococcus*	1.71 mM	[12]
acetone	*Synechocystis*	36 mg/L	[17]
fatty alcohols	*Synechocystis*	120 µg/L	[19]
1,2-propanediol	*Synechococcus*	150 mg/L	[20]
ethylene	*Synechocystis*	7.1 mg/L/h	[21]
isobutryaldehyde	*Synechococcus*	6.2 mg/L/h	[22]
2,3 butanediol	*Synechococcus*	9.8 mg/L/h	[23]

Task 1: Creation of a bisabolene production strain. Cyanobacteria naturally produce a variety of alkane and alkene molecules [90, 91] and thus serve as a suitable sustainable platform to engineer greater productivity of hydrocarbons. Terpenoids are a structurally diverse class of molecules that find use in a variety of biofuel and biochemical applications. In particular, the sequiterpenes have been identified as suitable replacements for diesel fuels [92, 93]. We have chosen the sesquiterpene bisabolene as a target molecule to demonstrate the feasibility of biofuel production from cyanobacteria for three key reasons. The first reason is that bisabolene once converted chemically to bisabolane has been identified as a suitable replacement for D2 diesel fuel. Second, this compound has been produced at titers of >900 mg/L in two different hosts systems (*E. coli* and *S. cerevisiae*), showing the potential for suitable production in a variety of organisms [94]. Finally, bisabolene showed low toxic effects in *Synechocystis*. We tested *Synechocystis* growth in 0 and 0.5 v/v bisabolene. There were no observable growth differences at these concentrations of bisabolene. At concentrations ≥1%, phase separation was observed. Similarly, no toxic effects were observed when *E. coli* or *S. cerevisiae* were grown in the presence of bisabolene [94]. In order to produce bisabolene in *Synechocystis,* we will need to express FPP synthase and (E)- α -bisabolene synthase Ag1 from *Abies grandis* [94]. Both genes will be will be optimized for codon usage and mRNA stability and bisabolene will be extracted and detected as previously described [94].

Task 2: Introduction of a heterologous mevalonate pathway. In nature, there are two major pathways that lead to the terpenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). One pathway, the mevalonate (MVA) pathway, is found in all eukaryotes, in the cytosol of plants, in archaea, and in a few eubacteria. The other pathway, the methyl-erythritol phosphate (MEP) pathway, is found in eubacteria, cyanobacteria, green algae, and the plastid of higher plants. Both of these pathways have been reviewed in detail elsewhere [95-98] and can be targets for pathway engineering for increased terpenoid production.

Why focus on the mevalonate pathway? There are several reasons that the mevalonate pathway was chosen for terpenoid production over the MEP pathway: (1) During autotrophic or heterotrophic growth the flux through acetyl-CoA (mevalonate precursor) is approximately 30-70 fold higher than the flux through the MEP pathway based on genome scale modeling [84]. (2) Endogenous regulation of the precursor pathways can be circumvented by heterologous expression of the mevalonate pathway. (3) Engineering of a heterologous mevalonate pathway in *E. coli* has led to high levels of terpenoid production of a variety of products including 900 mg/L bisabolene [94], 700 mg/L amorphadiene in shake flasks [99], and >27 g/L amorphadiene in bioreactors [100].

Approach. The seven mevalonate pathway genes will be introduced into *Synechocystis* engineered to produce bisabolene. This pathway has been well characterized in a number of organisms. Optimal production of mevalonate from acetyl-CoA has been shown in *E. coli* through the use of *Enterococcus faecalis* genes *mvaE* and *mvaS* [101, 102]. *mvaE* encodes a bifunctional protein that possesses ac-

tivities of both HMG-CoA reductase and acetyl-CoA acetyltransferase [103]. *mvaS* encodes HMG-CoA synthase. Genes from various other Gram positive sources (*S. pneumonia* and *S. aureus*) were tested in their ability to form mevalonate but performed poorly in comparison with *E. faecalis* [101]. *E. faecalis* genes also performed better than the optimized *S. cerevisiae* pathway [104]. Similarly, the pathway from mevalonate to IPP was tested in *E. coli* through expression of genes from various sources (*S. pneumoniae*, *S. pyogenes*, *S. aureus*, and *E. faecalis*, and from *S. cerevisiae*) [101]. It was found that genes from *S. pneumonia* and *S. cerevisiae* performed the best. *In Synechocystis, we will express mvaE and mvaS from E. faecalis; MK, PMK, and MPD from S. cerevisiae; and IDI from E. coli.* All genes introduced into *Synechocystis* will be optimized for codon usage and mRNA stability and will be inserted into the bisabolene producing strain. Bisabolene will be extracted and analyzed via GC as described [94].

Task 3: Redirect carbon flux from storage to product production during photosynthesis. In addition to directly utilizing pathway engineering to optimize bisabolene production, we will also need to consider ways to redirect global carbon flux away from glycogen synthesis and toward acetyl-CoA (and ultimately toward bisabolene) during photoautotrophic growth. It is important to note that glycogen biosynthesis cannot be completely knocked out since it is vital to the cell during the dark cycle [105]. Hypothesis that can redirect flux near the acetyl-CoA node can be derived from a detailed look at metabolic flux analysis results from genome scale analysis [84] and from 13C labeling experiments [106]. Other hypothesis will need a greater understanding of which genes are active during light and dark conditions before they are developed. These hypotheses will be formulated and tested as results from ***Aim 2*** are obtained.

Examples of hypotheses that can be generated follow. One hypothesis is that increasing the expression of phosphoglycerate mutase (PM) will drive flux toward pyruvate. The overexpression of this enzyme maybe difficult since it competes for 3-phosphoglycerate, an important metabolite in the Calvin-Benson cycle. Varying strength promoters developed by the Peebles lab will be used to fine tune expression of PM to achieve increased terpenoid flux with minimal effect on growth rates. A second hypothesis is that expressing the transcription factor Rre37 will increase expression of glycogen catabolism and glycolysis genes [107]. This should lead to increased availability of acetyl-CoA for bisabolene production.

Task 4: Develop novel control mechanisms for expression in realistic conditions. Aims 1 and 2 will generate an abundance of information from the transcriptome and proteome that will allow us to understand how cyanobacteria react to different environments. We suspect that this information will lead to novel hypotheses regarding gene control strategies for strain engineering. In photoautotrophic organisms, we lack a fundamental understanding of <u>when</u> genes should be expressed to achieve the highest possible titers, yields, and productivities. Beyond the traditional exponential vs. stationary phase and constitutive vs. inducible considerations you have in heterotrophic microorganisms, one also needs to consider the diurnal cycle and whether expression during one or both phase is optimal for production. Hypotheses will be investigated utilizing GFP_{mut3} [53] as a reporter (similar to preliminary work in Section 4.E.2).

We will also discover strong promoter elements by employing “promoter trap” techniques. This approach has been used in many different organisms including *Synechocystis* to identify novel genetic control elements [108]. We will swap out a *rbcL* promoter from a shuttle plasmid containing GFP as a reporter [53] with a random gDNA library of *Synechocystis*. Transgenic strains will then be grown in PBRs and cells with high GFP fluorescence will be selected out of the population using fluorescently activated cell sorting (FACS, CSU core facility). The phenotype will be verified and the vector sequenced for ID of the regulatory element and its native gene or operon. We anticipate this will be a powerful tool for discovering novel gene regulators in our system. Once we have several stable promoters, we will perform error-prone PCR on the isolated promoter region and use the techniques described above to re-isolate promoters with different strengths thus creating a toolbox that can be used to tailor protein or pathway expression for manipulation of photosynthesis or target molecule synthesis.

5.D. Specific Aim 4: Develop a cell concentration process based on inertial migration (Dandy)

A novel low-energy cell concentration process will be designed and tested. This system is based on the particle migration phenomenon that occurs when a dilute suspension of microparticles flow through microchannels [26]; velocity gradients push the particles to well defined positions near the channel walls, allowing them to be preferentially separated from the bulk flow. In the case of square microchannels there are four equilibrium positions, one at each wall midpoint; and in rectangular channels with sufficiently large aspect ratio, there are two equilibrium positions, at the midpoint of the shorter walls.[26] Computational fluid dynamics will be utilized to design the optimal geometry of the microchannels. This design will be fabricated using standard soft lithographic techniques with PDMS and then tested with cyanobacteria

suspensions. Once the design has been vetted, a multiplatform, multilayer configuration will be designed to process 10-50 L/h of the suspension as a proof-of-concept device. Based on our preliminary results, *in a single pass* this device will yield a 5- to 10-fold concentration enrichment of the cells which will have a significant impact on the sustainability of the process based on LCA analysis (***Aim 5)***. The plan is to take the serpentine channel configuration shown in Fig. 3(a) and apply this concept in a multi-pass, or multi-stage configuration, as illustrated by the two-pass system in Fig. 5 (the black lines represent microchannels). In this schematic, the dilute cyanobacteria suspension flows through 8 parallel serpentine microchannels. As shown in Section 3.B, the cells in each microchannel are focused to positions near a sidewall and subsequently removed via small side channels. In the concept drawing below, concentrated suspensions from 4 microchannels are combined and further concentrated in a second pass. Successive passes further concentrate the cellular suspension.

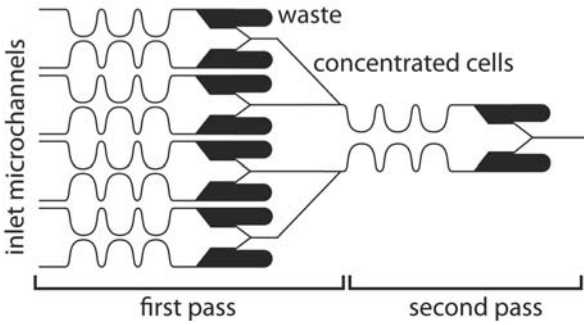

Figure 5. Soft lithographic techniques are used to create μFNs. The dilute cyanobacteria suspension is pumped through the parallel serpentine microchannels, where lateral forces and Dean flow migrate the particles to well defined positions near a shorter (vertical) sidewall. The particles exit through small side branches, thereby concentrating the suspension.

The reason that this microfluidic approach is well suited for the concentration of cyanobacteria at (near) commercial scale is its intrinsic scalability. That is, while the amount of suspension that can reasonably be pushed through a single microchannel is small (100 to 500 μL/min), the use of a massively parallel network of microchannels provides the necessary multiplication factor. To illustrate this concept, consider a group of 10 adjacent microchannels. The group consists of 10 inlet channels, 10 waste channels, and 10 recovery channels. Assuming that each inlet channel is 200 μm in width, the group of 10 channels has a footprint a little less than 4 mm across. The network configuration in Fig. 5 is multilayered: to avoid crossing waste and recovery channels at the end of the device, one type (waste) will be directed downward to a lower channel layer for collection. Using resistance factors it is possible to accurately predict the pressure drop (Δp) required to achieve a desired flow rate (Q): $Q = -\Delta p / K$, where K is the viscous resistance within a microchannel and may be calculated as[109]

$$K = \frac{12\mu L}{w^3 h}\left[1 - \frac{192w}{\pi^5 h}\sum_{n=1,3,5,\ldots}^{\infty}\frac{1}{n^5}\tanh\left(\frac{n\pi h}{2w}\right)\right]^{-1}.$$

Here, μ is dynamic viscosity, w and h are channel width and height, and L is channel length. Using this expression it may be determined that the total flow rate through the group of 10 inlet microchannels can readily be 1 to 5 mL/min, *at a cost of only several tenths of a psi per cm of channel*. Scaling this system up, 1000 groups of 10 can be expected to process 60 to 300 L/h of cyanobacteria culture.

Task 1: Design and optimize μFN. Three dimensional computational fluid dynamics simulations will be used to determine migration rates and equilibrium positions of cyanobacteria within the microchannels as functions of inlet channel geometry and flow rate. These results will directly inform the microchannel fabrication process. The outcome of this task is an optimal μFN design that balances achievable concentration factors with recovery efficiency, processing speed, and pumping costs.

Task 2: Fabricate and test model μFN. While the group of 8 channels was shown above for illustrative purposes, it is also an appropriate starting point for proving the applicability of the approach for concentrating cyanobacteria suspensions. Using the design parameters from Task 1, a series of experiments will be performed using neutrally buoyant microspheres (comparable in size to cyanobacteria) in water. The predictions from Task 1 regarding recovery efficiency, throughput, and pumping costs will be tested for inlet cyanobacteria concentrations ranging from 0.01 to 0.1%. The outcome of this task will be a functioning prototype capable of processing 0.06 to 0.3 L/h. In the event that the outcomes do not align with the predictions of Task 1 or the goals of the project, the system will be reevaluated and reanalyzed. Such actions may include the implementation of a multistage device where recovery streams are merged and passed through additional inertial migration concentrators.

Task 3: Scale up μFN. When Task 2 yields anticipated results, a multiplatform, multilayer configuration

will be designed to process 10 to 75 L/h of suspension. The goal of this task is to ensure that the concept is scalable in a linear manner, and in a way that minimizes system footprint and height, as well as minimizing pumping costs. The engineering challenge of this task is integrating feed streams with up to 3200 separate microchannels in such a way that each channel operates identically and without leaks. The outcome of this task will be a true proof of concept device and a design plan for further scale up.

5.E. Specific Aim 5: Sustainability, economic, and scalability analyses of process (Bradley)
Cyanobacteria-derived bioproducts have the potential to replace conventionally derived bioproducts, but the efficacy with which sustainability goals can be achieved is dependent on the lifecycle impacts of the cyanobacteria-to-bioproducts process including metrics of sustainability, economic performance and scalability. The design of sustainable biorefinery systems requires a means of interface between the micro-scale characteristics of the organism and process engineering, and the macro-scale system performance. As shown in Fig. 5, modeling and simulation can provide this interface both through the *attributional* evaluation of process characteristics in terms of system level performance metrics (through LCA, technoeconomic analysis and scalability analysis), but also through an iterative process of computational and experimental multidisciplinary optimization and *sustainable system design*. For this Aim, we will synthesize the toolset required to evaluate (task 1) and design (task 2) the photosynthetic biorefinery on the basis of system-level considerations including economics, scalability, and environmental sustainability.
Task 1: Attributional scalability and sustainability assessment of PSBR. Attributional analysis (including LCA, technoeconomic analysis, and scalability analysis) is the most common process for evaluating the environmental, economic, and scalability characteristics of a biorefinery, but performing these analyses with validity for industrial scales requires more detailed modeling of biology, process engineering, and resource availability than is usually performed [58]. We will develop a physical/empirical model of the microorganism-to-biofuels process for *descriptively* assessing its characteristics.

To enable a more valid model of the cyanobacteria growth, we will develop experimental characterizations of the culture under various light, temperature, and mixing environments. The productivity of the culture will be assessed on a batch-time scale to capture all aspects of the culture growth cycles. The productivity of large-scale reactors will then be modeled based on a finite volume characterization of the light, temperature and nutrient characteristics of the large-scale PBR. Previous work by the co-PI (Bradley) has developed experimentally-validated means of scaling the reactor-specific productivity [57], energy/materials consumption [11], and waste products of photobioreactors [54]. These methods have proven to be more valid for large-scale bioproduction systems modeling and consequential bioproducts LCA than the methods that existed previously in literature. Experimental validation can be performed using the full-scale commercial demonstration indoor/outdoor reactor system that is available at CSU [57].

Results from the growth-system characterization (Aims 1-3) and results from the low energy cell concentration experiment (Aim 4) will be integrated with lifecycle energetic and economic inventories to construct a lifecycle economic and environmental assessment of the PSBR system. The model for this cyanobacteria LCA is built upon previous work from Dr. Bradley's lab [11], one of the most comprehensive and comparable PBR LCA of microalgae-based biofuels performed to date. The scope of the LCA of the proposed biofuel system will be "well to wheels" comprehensive, and will include the stages of feedstock growth, transportation, chemical conversion, distribution, and end use for the proposed process. We will assign a functional unit to each of the proposed end uses of the bioproduct system, and will model both greenhouse gas and energetic lifecycle performance of each process.

Various methods will be used to allocate energy and emissions to the feedstocks and products depending on the details of how they are currently produced. For example, co-products and agricultural residues will in turn be considered to have energy content equivalent to their displacement value as cattle or aquacultural feed, to their economic value on the open market, and to their energetic value (LHV).

The massive scale of the demand for photosynthetic bioproducts also necessitates an understanding of the scalability and sustainability of the proposed processes. Scalability of the process must be assessed in terms of a variety of metrics; for this study, we will evaluate the process on the basis of resource consumption, land availability, water footprint (in terms of lifecycle, green, and blue water consumption metrics (as in [55])), and economics.
Task 2: Sustainability-informed design of PSBR organisms and processes. Based on the *descriptive* understanding of the lifecycle PSBR performance that has been developed in Task 1, Task 2 will develop a physical/empirical model of the microorganism-to-biofuels process for *predicatively* assessing the effect of biological, process, or economic changes on the system-level performance of the proposed sys-

tem. Task 2 represents the right side of the diagram shown in Fig. 6.
Under this task, system level assessments will inform the objectives of reactor-level and organism-level design. At the same time, and as demonstrated in preliminary work, expressing scientific advancement using system-level metrics can lead to an understanding of the transformative impacts of changes to the reactor-level and organism-level performance. This project proposes a spiraling development process where interactions between the system-level assessment and the other tasks will lead to transformational advancements in the state of the art.

In previous work, the PIs have used sustainability metrics to develop novel means of process engineering for microalgae biofuels. For example, in [54], we propose and experimentally validate the means to reduce the lifecycle GHG emissions of microalgae based biofuels by 43% through a process engineering change which reduces the direct N_2O emissions of algae cultivation. In industrial research work, the co-PI (Bradley) has developed industrial-scale PBR optimization studies resulting in 2x areal productivity improvements. These previous works followed the circulating system design process of Fig. 6, wherein the sustainability criteria (derived from LCA and other system-level analysis) informs the process design objectives, at the same time as the process characteristics and results are direct inputs to the calculation of sustainability criteria.

6. Expected Outcomes

The tasks associated with **Aim 1** will result in a sophisticated computational fluid dynamics model that quantitatively describes the physics within large- and bench-scale PBRs. The model will be useful for designing PBRs for optimal photosynthetic productivity of cyanobacteria in industrially relevant conditions. Moreover, the model will be broadly applicable to a wide class of PBR geometries, scales, and operating conditions.

Aim 2 will greatly expand our knowledge of microbial photosynthesis and its regulation. The approaches employed here will serve as a model for discovery in other photosynthetic microbes. Through systems biology approaches we will identify factors that impede photosynthetic efficiency and will deliver an engineered strain that overcomes these impedances with increased productivity.

Aim 3 will create a *Synechocystis* bisabolene biofuel producing strain that is engineered for increase yields and productivities in a PBR. The technologies and methodologies developed in this aim will be directly transferrable to other cyanobacteria strains. This strain will be ideally suited for increased production of other terpenoid-based biofuels and chemicals with minimal genetic manipulations.

Aim 4 focuses on the development of an inexpensive, low operating cost, efficient concentrator for cyanobacteria. While the approach is based on microscale flow—necessary because of cyanobacteria size—the approach is highly parallelizable and therefore arbitrarily scalable to meet industrial needs. Other than a pump, there are no moving parts involved in the concentration process. The platform will achieve 10 to 100x concentration factors with > 95% recovery.

The first outcome of **Aim 5** will be a comprehensive lifecycle analysis for these proposed bioproducts that can adhere to the same system boundaries and energy input/output assumptions as the published lifecycle analyses for conventional products, thereby enabling direct comparison and evaluation. The second outcome of this research will be the assessment of the environmental, economic and scalability impacts of the proposed PSBR. The final research outcome for this work is the development of the framework for the design of organisms and processes further advance system-level sustainability, scalability and environmental objectives.

In addition, the methods and approaches developed here can be applied to other photosynthetic microorganisms and other chemical products, either directly or indirectly depending on the system.

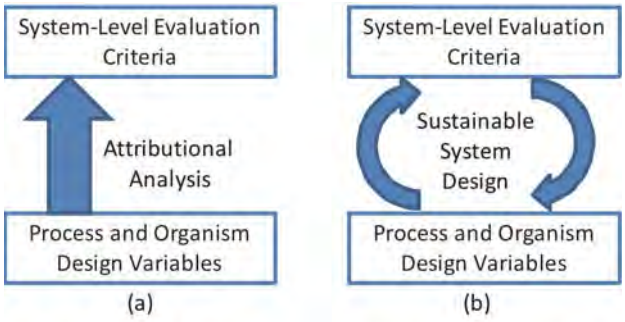

Figure 6. Two means for connecting system level evaluation criteria to lower-level process and organism design including (a) conventional sustainability analysis, and (b) sustainable system design methods

7. Impact

The objective of the proposed work is to provide a renewable source of fuels and chemicals previously derived from crude oil. This work will directly lead to a scalable, photosyn-

thetic process that directly converts CO_2 and sunlight into fuels and chemicals in a sustainable way.

The transformational nature of this work is encompassed by the fact that we use a multifaceted approach to tackle fundamental problems associated with strain development and photobioreactor design identified and evaluated by using LCA. Understanding the dynamics of mixing within a large scale photobioreactor and being able to mimic these dynamics at the bench scale will allow us to better predict the performance of our strains during scale-up. This is a major improvement over what is currently standard practice in the literature where engineered strains are grown under continuous low light levels that in no way mimics industrial scale processes. The use of systems biology approaches to understand the physiological effects of mixing which correlates to the frequency a cell will see high light stresses will allow us to rationally design cyanobacteria strains for high productivity of biofuels and biochemicals. Life cycle analysis will help us to focus our design and research efforts on the areas that will have the biggest impact on designing a sustainable system. The insights gained through this research will feed back into all parts of our research to guide us to a sustainable process. This process will produce both biofuels and biochemicals. The progress described here can only be accomplished through our diverse team of experts working together to develop a sustainable process for biofuel and biochemical production.

Our broadening participation plan includes various activities aimed at different demographical segments of the population with an effort to involve underrepresented groups. We will work to develop teaching/hands-on educational modules that are appropriate for K-12 students. We will work closely with the Colorado State University STEM outreach center to develop this initiative. We also plan to open our labs to high school students and teachers and may apply for additional funding through the RET program. We will continue to host REU students as part of our involvement in the Colorado Center for Biorefining and Biofuels (http://www.c2b2web.org/). This REU was awarded to the University of Colorado as the lead institution (Award Abstract #1005238) with participation by NREL, CSU, and CSM. Two of the PI's have hosted students in the past. The PI and co-PIs also work closely with the CSU's Sustainable Bioenergy Development Center (http://sbdc.colostate.edu/) and CSU's NSF IGERT program in Multidisciplinary Approaches to Sustainable Bioenergy (http://bioenergy-igert.colostate.edu/) (PI Reardon is a Director of both), which offer access to diverse graduate and undergraduate students and a network of bioenergy researchers and equipment. In addition, the PIs are committed to mentoring all post-docs, graduate students, and undergraduate students who work in our labs to help them develop into independent thinkers and researchers.

8. Project Relevance to Air Force Interests

This proposal is relevant to the Air Force interest in that we are developing strains of cyanobacteria to efficiently produce "drop-in ready" biofuels in the form of long-chain hydrocarbons (bisabolene) that are produced in a sustainable way from CO_2 and sunlight. We are also actively analyzing PBR technology and new LCA approaches to ensure the sustainable production of these fuels.

9. Results of Prior NSF Support

Reardon and Dandy: Project title: "Biosensor arrays based on DNA shuffling and chemometrics for measurement of chlorinated solvent mixtures" PI: Reardon; co-PIs: Dandy and Lear (NSF-CBET-0529048; $725,270; 10/2005-09/09). Biosensors based on monooxygenase enzymes were developed for simultaneous measurements of the concentrations of multiple, chemically similar analytes. The research focused on the measurement of mixtures of chlorinated ethenes in groundwater, and used a multidisciplinary approach to develop biosensors, optoelectronic hardware, and software. This was the first development of oxygenase-based biosensors. Training: Iris Yi, (MS, 2010), Zhong Zhong (PhD 2011), and three undergraduate researchers. Publications: Three refereed journal article published and three in preparation. Outreach & Education: A new graduate-level course on biosensors was developed.

Reardon: Project title: "Workshops on Basic Research Needs for Biofuel Production from Photosynthetic Microorganisms" PI: Reardon (NSF-CBET-1144433; $34,994; 8/1/11–7/31/12). Two workshops, one in the US (November 2011) and one in Germany (TBD, 2012) will be held to discuss basic research needs at the level of the biochemistry, biophysics, ecology, and systems and synthetic biology of photosynthetic microorganisms that must be addressed to develop biofuel production. Training: N/A. Publications: Workshop reports and a manuscript for journal publication are in preparation. Outreach & Education: Representatives of US government agencies were invited to attend the US workshop.

Drs. Bradley, Peebles, Peers have not had NSF support within the past five years.

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