Demonstration of transgressive overyielding of algal mixed cultures in microdroplets†

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Algae are ubiquitous in natural ecosystems and have been studied extensively for biofuel production due to their unique metabolic capabilities. Most studies to date have approached biofuel optimization through synthetic biology and process engineering with few industrial scale projects considering algal community interactions. Such interactions can potentially lead to increased productivity and reduced community invasability, both important characteristics for scalable algal biofuel production. It is estimated that over a million species of algae exist such that elucidating the interactions that might be beneficial for biofuel production remains extremely resource and time intensive. Here we describe a strategy for rapid, high-throughput screening of algal community combinations using a microfluidic platform to generate millions of parallel, nanoliter-scale algal mixed cultures for estimation of biomass accumulation. Model communities were first studied in a bench scale flask experiment and then examined using microfluidic droplets. These experiments showed consistent results for both positively interacting algal bicultures that increase biomass when together, and negatively interacting bicultures that decrease biomass. Specifically, these included enhanced performance of two bicultures, \textit{Ankistrodesmus falcatus} and \textit{Chlorella sorokiniana}, \textit{Chlorella sorokiniana} and \textit{Selenastrum minutum}, and reduced performance of a biculture consisting of \textit{Selenastrum capricornutum} and \textit{Scenedesmus ecornis}. While the ultimate techno-economic feasibility of algal bioproducts hinges on an amalgamation of scientific fields, rapid screening of algal communities will prove imperative for efficiently discovering community interactions.

Introduction

Microalgae show promise in their viability for the production of cleaner, renewable sources of energy with respect to their fossil fuel analogues. They also provide an attractive opportunity for industrial development due to the inherent compatibility of algal lipid derived fuels with the current petroleum-based infrastructure. However, the methods with which algae may be engineered competitively for market operation remain especially challenging. A dominant approach in algal biofuel synthesis involves the development of genetically modified organisms (GMOs) to optimize laboratory yields. Researchers have demonstrated the ability to manipulate seemingly innocuous target microbes to create a product with industrial viability and the potential to meet market demands through metabolic engineering and increased lipid production. Novel processing techniques such as the hydrothermal liquefaction of algal biomass have complemented these advances with the production of up to 64% biocrude per unit biomass.†
 Nonetheless the dominant approach has been hampered by a variety of problems, including horizontal gene transfer in which species naturally evolve and change their properties, functional trade-offs in metabolism that make it difficult to simultaneously optimize multiple fuel properties, and the inherent instability of monoculture cultivation with respect to disturbances and invaders.\textsuperscript{3,4} These effects have been identified as major barriers for maximizing the long-term productivity and stability of open pond biofuel systems.\textsuperscript{5} A multifaceted approach is required to address the enormity of this challenge. Engineering specific strains for production capacity, while useful, cannot be considered as the standalone approach to algal biofuel research. An approach to reduce the disparity between laboratory and outdoor cultivation must integrate biological and process engineering developments within an ecological context.

Ecological engineering is an emergent approach in algal biofuel optimization that addresses limitations in yield and stability through the selection of algal consortia with a predisposition for increasing productivity. Such productivity is intimately linked to interspecific interactions involving competition, niche partitioning, or facilitation. Competition involves organisms contesting the same limited resources, leading to a change in fitness that negatively impacts one or both organisms. Niche partitioning occurs when species are limited by different resources, or divide the same resource in space or time.\textsuperscript{6} Complete niche partitioning indicates the absence of interspecific interactions altogether such that individual species perform equally well in monoculture as in polyculture. Facilitation is a positive interaction in which at least one of the constituent organisms within the community benefits, quantified by an increase in overall production. If an interspecific interaction is sufficiently strong, then a net change in biomass may be substantially measured through transgressive overyielding (TO).\textsuperscript{7} In this context, TO occurs when the biomass production of a community of algae species exceeds that of the most productive constitutive species. The degree to which TO arises is dependent on the nature of the community, specifically the relative carrying capacities and growth rates of subspecies.\textsuperscript{8} In a meta-analysis of plant species, Cardinale et al. showed that polycultures produce an average of 1.7 times more biomass than their constituent species grown in monoculture, with increased productivity in 79% of 96 polyculture experiments.\textsuperscript{7} However, only 12% of these polycultures demonstrated TO with respect to the most productive constitutive monoculture and the ratio of polyculture to constitutive highest producing monoculture was 0.87 on average.\textsuperscript{7} In algal polycultures, Shurin et al. conducted an experiment with 119 species pairs, of which 25% produced more biomass than the average monoculture.\textsuperscript{9} In these experiments, the ratio of polyculture biomass to constitutive highest producing monoculture was 0.82 on average.\textsuperscript{9} While TO relationships certainly arise in algal polycultures, combinations that generate more biomass with respect to the dominant species are elusive. The prevalence of TO relationships is further convoluted by contradictory research and varied experimental conditions considering the effects of species richness on biomass and biocrude yield.\textsuperscript{10,11} While tending to overlook the mechanisms through which these interactions occur (metabolite exchange, niche partitioning, microbial symbionts, etc.), these studies are nonetheless promising in their relation to scaled algal production. Algal ponds typically facilitate a single species in its fundamental niche, relatively independent of interspecific interactions. The singularity of this approach thus ignores potentially lucrative combinatorial overyielding relationships. Yet prospecting for these overyielding relationships can be tedious, expensive, and extremely time consuming particularly given that over 44 000 species of algae have currently been identified with a maximum of 1 million more estimated to exist.\textsuperscript{12,13} Studies have been limited in practice to several hundred combinations. Such interactions are also difficult to model and predict due to the limiting density dependence or resource provisions of the individual species.\textsuperscript{8,9} The diversity of algal species necessitates the development of a high-throughput platform by which algal polycultures can be quickly analyzed, with relationships elucidated and considered on the basis of productivity (e.g. measured by biomass accumulation). Selected species combinations could then be considered as candidates for thermochemical processing, by which the entire algal biomass is converted into a bio-crude product.\textsuperscript{14}

Algal experiments in the laboratory are conventionally conducted at the bench scale, consuming large quantities of resources. At this scale, it is impossible to screen tens of thousands of species combinations for overyielding. Screening of algal communities might, therefore, benefit from microfluidic droplet generators (MFDG), which have been ameliorated and incorporated within a broad range of research topics. Aqueous droplets separated by a surrounding continuous medium form parallel independent mini-compartments, providing an effective means for high-throughput screening.\textsuperscript{15} Additionally, the miniaturization of fluidic systems facilitates conservation of reagents, experimental expenses, and increases general efficiency.\textsuperscript{16} Tunability and controllability of each droplet allows for parallel processing such that large datasets are quickly and easily generated with extremely high resolution.\textsuperscript{16,17} Studies have succeeded in encapsulating and culturing various species of algal monocultures for the study of growth kinetics with regards to pH, nitrogen concentration, and resource consumption.\textsuperscript{18} Park et al. demonstrated mutualistic interactions in synthetic E. coli communities in microdroplets on a platform readily applicable to algae.\textsuperscript{19} The aqueous phase may also be readily substituted with many target media including wastewater sources to study algal polyculture growth for bioremediation.\textsuperscript{20} Adaptation of this platform would enable rapid creation of highly parallel algal communities for high-throughput spectroscopic analysis of the fluorescent molecule chlorophyll-a, which provides a metric by which growth, and hence production, may be compared between monocultures and polycultures for the elucidation of TO.\textsuperscript{21}

In this work, we have demonstrated the feasibility of using microfluidic droplet co-cultivation and microscopic fluorescence analysis for detecting TO of algal polycultures. The overall concept is depicted in Fig. 1A and B and a real droplet generation device is shown Fig. 1C. First, a set of flask experiments was performed to provide benchmark data. Then, a microdroplet generation device was fabricated using the design illustrated.
Algal cultivation in Erlenmeyer flasks

Transgressive overyielding (TOc) can be quantified using the natural log of the ratio of biculture biomass with respect to the most productive monoculture within the combination as follows:

$$\text{TOc} = \ln \left( \frac{B_{\text{biculture}}}{B_{\text{monoculture}}} \right)$$

Higher biomass in biculture will lead to a positive value for transgressive overyielding whereas higher biomass in monoculture yields a negative value. Algal cultures were initially studied in a flask experiment to identify overyielding combinations for translation into the droplet co-cultivation framework (Supplementary Information 1). Of the combinations explored, two bicultures demonstrated transgressive overyielding with statistical significance (two sample one-tailed Student’s t-test).

These bicultures were *A. falcatus* and *C. sorokiniana* (*p* = 0.0014) and *C. sorokiniana* and *S. minutum* (*p* = 0.0004), which demonstrated TO values of 0.59 ± 0.1 and 0.59 ± 0.09, respectively. Given the logarithmic nature of TO, this means the bicultures achieved approximately 1.8 times the biomass of the most productive monoculture. A third biculture of particular interest was *S. ecornis* and *S. capricornutum*, which demonstrated a statistically significant underperformance (*p* = 0.014) with a TO value of −0.42 ± 0.41 or 0.66 times the biomass of the most productive monoculture. These bicultures were then used as model systems for the validation of the microfluidic droplet co-cultivation framework.

Microdroplet generation and incubation

Fig. 1A illustrates the set-up for droplet generation and storage. A PFTE tube (1.5 mm inner diameter) was used to connect the droplet generation device to a sterile 1.7 mL Eppendorf tube. After generation, droplets were stored within the Eppendorf tube with the addition of generic mineral oil. As a less dense fluid, the mineral oil provided an insulating layer above the microdroplets, avoiding evaporative losses. Microdroplets rested above excess oil used in droplet generation. This method provided a sterile environment for bulk droplet storage from which droplets were extracted and visualized on disposable hemocytometers (iNCYTO C-Chip DNC-N01) for point reads over the algae growth cycle.

Fig. 1B illustrates the set-up for droplet generation and storage. Microdroplets (2) are cultivated and stored above a layer of excess HFE oil (3) and below a layer of mineral oil (1) (not to scale). (B) The microdroplets were transported to a 1.7 mL Eppendorf tube with a syringe tip for sampling. (B) The microdroplet generator from this work.

Results and discussion

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were $F_{\text{oil}}/F_{\text{aqueous}} = 1.4/1.6\ \mu \text{L min}^{-1}$. Microfluidic devices with 250 μm (for oil) and 200 μm (for aqueous cell suspension) inlet widths connected to 150 μm orifice widths, were used for larger droplet of 250 and 500 μm diameters. The flow rate for 500 μm diameter droplets was $F_{\text{oil}}/F_{\text{aqueous}} = 3.3/5.4\ \mu \text{L min}^{-1}$. It was observed that as the droplet diameter (thus volume) expanded, the maximal number of algae cells each droplet could sustain also increased (Fig. 2D).

**Algal cultivation in microdroplets**

Droplets were stored under identical growth conditions as flasks. Growth curves were constructed using fluorescent point reads. *A. falcatus* and *C. sorokiniana* (AC), as well as *C. sorokiniana* and *S. minutum* (CE), were used as model systems that exhibited high level of transgressive overyielding in flasks. The biculture *S. ecornis* and *S. capricornutum* (JL) was selected to represent an apparent negative relationship. Each biculture study consisted of three Eppendorf tubes, one from a 1:1 biculture feedstock and two with its respective monocultures as controls. Theoretically, each experiment requires only a single biculture feedstock to generate all possibilities, however *C. sorokiniana* and *S. minutum* are morphologically indistinguishable under the microscope at 10× magnification. Further magnification becomes analytically challenging. Therefore, bicultures were lumped in bulk and compared to individual monocultures. While flask experimental fluorescence was determined using an M5 spectrophotometer, droplet fluorescence was calculated and normalized as a product of pixel intensity and fluorescent area using ImageJ.

As summarized in Fig. 3, AC biculture droplets outperformed monoculture droplets containing A or C only, which was consistent with results from flask experiments (Fig. 3A, $TO = 0.59 \pm 0.1$). All droplets from the biculture feedstock were regarded as containing bicultures and transgressive overyielding was estimated to be $TO = 0.55 \pm 0.09$ (Fig. 3B). Alternatively, when A and C were distinguished based on morphological differences, it was estimated that $TO = 0.26 \pm 0.05$ (Supplementary Information 1).

This lower TO estimate based on morphological distinction was repeatedly observed for the *A. falcatus* and *C. sorokiniana* biculture. Reduced cell growth was noted when comparing...
A. falcatus monoculture droplets in biculture and strict monoculture conditions. One possible explanation is that bulk biculture cultivation negatively affected A. falcatus cell growth because of the presence of the more abundant C. sorokiniana, reducing light availability. This observation should be examined more carefully in a future investigation to determine any plausible interdroplet interactions.

A subsequent study involved C. sorokiniana (C) and S. minutum (E), which are morphologically indistinguishable in this work. Fig. 4B indicates that the droplet trends mimic those of the flasks (TO = 0.58 ± 0.09). Fig. 4A, with the biculture reaching a higher fluorescence even with the underestimation (TO = 0.29 ± 0.14) and thus indicating higher biomass accumulation in the biculture with respect to the most productive monoculture.

Lastly, S. ecornis and S. capricornutum were examined to study whether this analysis could also distinguish negative relationships. In Fig. 4D, S. capricornutum outperforms the combined biculture in droplets over their respective growth cycles (TO = −0.42 ± 0.30). Despite a poor logistic fit, this trend was in good agreement with the flask experiment (TO = −0.22 ± 0.11) depicted in Fig. 4C, indicating lower biomass accumulation within the biculture. It should be noted that as in the CE experiment, the two species J and L were not distinguished morphologically here.

The three droplet experiments described above validate the microfluidic platform in its ability to identify transgressive over-yielding of algal co-cultures. In the meantime, this work suggests an analytical bottleneck that hinders application to more complex polycultures if the identification of TO combinations in droplets would rely on morphological differences between species. Another issue observed is algal quiescence where cells continue to fluoresce but fail to reproduce. While the microdroplet co-cultivation method described here provides a foundation for high-throughput screening, algal combinations demonstrating TO in droplets would need to be further studied. In addition to more accurate TO quantification, these follow-up experiments would also reveal underlying mechanisms such as niche partitioning and metabolic cross-feeding.

**Experimental**

**Algal species selection**

Species selected from the Cardinale Lab's algal library and previously investigated in a mesocosm experiment were utilized in this study. Experiments were conducted using BOLD 3 N liquid medium, which is common for freshwater xenic microalgal cultures. The BOLD 3N medium contains 8.82 mM NaNO3, 0.17 mM CaCl2·2H2O, 0.30 mM MgSO4·7H2O, 0.43 mM K2HPO4, 1.29 mM KH2PO4, and 0.49 mM NaCl. Additionally, a P-IV metal solution is used to provide 2.01 mM Na2EDTA·2H2O, 0.36 mM FeCl3·6H2O, 0.21 mM MnCl2·4H2O, 0.037 mM ZnCl2, 0.0084 mM CoCl2·6H2O, and 0.017 mM Na2MoO4·2H2O. Of these, sodium nitrate and potassium phosphate are the sole sources of nitrogen and phosphorus with atmospheric CO2 as the sole carbon source.

Seven species, *Ankistrodesmus falcatus*, *Pediastrum duplex*, *Clorella sorokiniana*, *Scenedesmus acuminatus*, *Scenedesmus ecornis*, *Selenastrum capricornutum*, and *Selenastrum minutum* were cultivated as monocultures and selected bicultures in quadraplicate flask cultures at an initial concentration of 1 × 10^4 cells per mL in 50 mL BOLD 3N medium. These were shaken at 120 RPM under constant luminous flux of 1375 lumens at 25 °C in a refrigerated incubator (Isotemp 2000, ThermoFisher Scientific Inc. Fife, Washington, USA). In terms of photosynthetically active radiation, light intensity was measured to be approximately 100 µmol photons m^-2 s^-1 (Spectrum Technologies, Inc). Daily fluorescence readings were taken using 200 µL sample aliquots over a period of approximately 45 days on a Spectramax M5 spectrophotometer (Molecular Devices). Every five days 1 mL of fresh medium was added to account for culture volume reduction with evaporative losses assumed negligible. Flasks were removed after 2 or 3 days of declining fluorescence or significant changes in colour for measurement of biomass in the
stationary phase. Fluorescence data were fit to the Verhulst logistic growth model through the MATLAB curve fitting program. It is worth noting that in general the biculture experiments exhibited biphasic growth curves, causing worse fits of the model. This likely arose in large part due to the complex interactions and dynamics in these bicultures. The carrying capacity and growth rate for each culture were determined with fluorescence based carrying capacities used in the calculation of transgressive overyielding. A similar strategy was employed for droplet analysis. The overall shapes of growth curves in droplets do not exactly resemble those in flasks due to the smaller number of time points and larger variations from the inherent stochasticity of small cell numbers in droplets. Nevertheless, logistic fits tended to fall within acceptable confidence intervals with some variability (Supplementary Information 1). A comparison of fluorescence and dry weight biomass was conducted by centrifugation, removal of supernatant, and oven drying at 55 °C for 72 hours per conical flask. This allowed for physical measurement of endpoint biomass of each culture at their estimated carrying capacity. Selected species combinations AC, CE, and JL demonstrated similar relationships between fluorescence and biomass overyielding, asserting the validity of chlorophyll-a fluorescence as a proxy for biomass. Explicitly, these biomass TO values were $0.47 \pm 0.15$, $0.38 \pm 0.38$, and $-1.51 \pm 1.67$ for AC, CE, and JL, respectively. While chlorophyll-a has some limitations as a pigment based method for approximating biomass accumulation, it remains a common and realistic method of analysing cellular growth on the microscale. Method validation has considered intercellular variations in chlorophyll-a concentration within each species and over their growth cycles. Such intraspecific and temporal chlorophyll-a variations are well-known caveats when using this fluorescent proxy. Furthermore, growth was terminated at the beginning of stationary phase to avoid the inherent nonlinearity between biomass and chlorophyll-a concentrations during stationary phase. Variations in chlorophyll-a concentration between species were accounted for via aforementioned

![Fig. 4](image-url) Comparison of bicultures in flasks and microdroplets. Growth curves of *C. sorokiniana* and *S. minutum* monocultures and their biculture in flasks (A, $n=4$) and in microdroplets (B, $n=20$). Growth curves of *S. ecornis* and *S. capricornutum* monocultures and their biculture in flasks (C, $n=4$) and in microdroplets (D, $n=20$). Flask error bars represent standard deviation while droplet error bars represents standard error. Curves represent a best fit logistic regression for each experiment.
normalization methods. Microdroplets were generated at an expected lambda of 1.5 to account for flocculation and the cylindrical approximation for an initial concentration of 3.82 × 10^6 cells per mL per culture. Although S. ecornis forms small coenobia, this did not significantly affect image analysis or droplet generation. Analysis of larger coenobia, as common with Pediastrum sp., is possible through the generation of much larger microdroplets that are able to accommodate these larger particles.

**Microfabrication of microdroplet generator**

The flow focusing droplet generating device was microfabricated using standard photolithography with a poly(dimethylsiloxane) (PDMS) substrate. Firstly, a photomask was designed using L-Edit software (ver 12.1) and translated to a Heiderberg μPG 501 Mask Maker with a chrome in glass substrate. Channels were designed with widths of 100 μm and an orifice width of 50 μm. SU-8 2025 (MicroChem) negative photoresist was spin-coated in a uniform 50 μm layer on a 4-inch silicon wafer. The droplet generation design was transferred to the coated wafer by photolithographic UV exposure with a mask aligner. The exposed wafer was then developed with SU-8 developer (MicroChem). The wafer was then baked and developed using SU-8 developer and subsequently washed with acetone and isobutyl alcohol. After developing, this photomask was treated with trichloro(1,1,2,2-perfluorocyclo)silane in a vacuum chamber to form a monolayer on the silicon surface, reducing surface energy and increasing hydrophobicity. A 10:1 (w/w) mixture of pre-polymer Sylgard 184 and cross-linker (Dow corning) was made and poured onto both the patterned wafer and a blank wafer in weigh dishes. Mixtures were degasified in a vacuum chamber for 1 hour until all bubbles were absent and then cured at 80 °C overnight. After curing, the PDMS layers were removed from both wafers and dishes, cut into manageable slabs with a razor blade, and cleaned with 3M Scotch tape. Inlet and outlet holes were punched to facilitate insertion of 1.5 mm PET tubing and syringes for droplet generation. The layers were treated with oxygen plasma for ten seconds per layer and bonded. Devices were baked for 6 hours to further increase hydrophobicity.

**Microdroplet generation and incubation**

The droplet generation process employed dual pneumatic pumps that used LabView programming to control voltage and hence forced air flow rates of the respective fluid phases. The system was set up at a microscope station equipped with a Nikon Eclipse Ti-S and Nikon Intensilight C-HGHI, allowing for real time analysis not only of droplet generation but also of fluorescence of chlorophyll-a within generated droplets. These pneumatic pumps were connected to a dual 2.5 mL syringe system for the aqueous and continuous phases. The aqueous phase for these experiments involved BOLD 3N medium as was used in the flask experiment, with the continuous phase consisting of 3M Novec HFE 7500 Engineered Fluid with 2% (w/w) fluorosurfactant (008-fluorosurfactant, RAN Biotechnologies, Inc.) to maintain droplet integrity over the growth cycle. Syringes were connected with PTFE microtubing to Norson precision tips designed for dispensing engineered fluid. The tips were then inserted into the microfabricated PDMS device on a glass slide. The glass-PDMS contact was sealed with epoxy for stability.

**Image collection and data analysis**

A SpectraMax M5 microplate reader (Molecular Devices) with 96-well microplate (Greiner) was used to monitor fluorescence of cell suspensions in Erlenmeyer flasks. 435 nm excitation and 685 nm emission wavelengths were used to detect chlorophyll-a fluorescence, measured in relative fluorescence units (RFU). Microdroplet generation and cell growth over time were monitored with camera module (EXi blue, Q imaging) of Nikon eclipse Ti microscope. Both bright and fluorescent field images were taken with Endow GFP Longpass Emission filter. Cell counts in droplets were performed using Adobe Photoshop CS5.1. Droplet fluorescence was calculated as a product of pixel intensity and fluorescent area using ImageJ with consistent thresholds, labelled as arbitrary units (a.u.). Monoculture and biculture droplets were visually characterized through morphological differences. For each read, a 10 μL sample of droplets was taken from each Eppendorf tube and added to a disposable hemoctometer C-Chip. Twenty random droplets of each monoculture or biculture were analyzed and averaged. The C-Chips and samples were discarded after imaging.

**Conclusions**

The work described above successfully demonstrated the translation of flask-size overyielding experiments to microfluidic droplets displaying both positive relationships between A. falcatus and C. sorokiniana as well as C. sorokiniana and S. minutum and a negative relationship between S. ecornis and S. capricornutum. Validation of this strategy provides a foundation for applying microdroplet co-cultivation to high-throughput screening of massive numbers of algal combinations from a complex algal library. Extrapolation of this framework to higher order polycultures will allow for swift elucidation of productive communities thereby maximizing overall biomass accumulation and, ultimately, facilitating more efficient bioproduction strategies. Given the low resource inputs, it could be similarly dispatched to quantify algal growth on varied media such as municipal and industrial wastewater sources for bio remediation strategies.

Two major avenues for future optimization pertain to droplet storage and droplet analysis. Recent research has explored monolayer droplet tracking systems by which individual algal droplets are studied for photoautotrophic growth within a PDMS microdroplet storage device. Creating a highly controlled, humidified environment in which droplets may be arranged and stored in such a device would allow for not only increased experimental precision, but for rapid and predictable screening of droplet fluorescence in MATLAB. Arranging droplets in this manner facilitates high-throughput computational analysis. A second opportunity involves high-throughput droplet sorting. This can be achieved by using fluorescence-activated microfluidic droplet sorters based on total fluorescence intensity or the number of
fluorescent particles in a droplet. Alternatively, double emulsion droplets can be employed, where the oil phase is emulsified in an outer aqueous phase to generate droplets compatible with standard FACS instruments for drop-in sorting. In either case, high-throughput droplet sorting would allow automated isolation of particularly productive communities without the laborious bottlenecks of image analysis, thereby accelerating the elucidation of interspecies interactions.

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