Phase I Award Financial and Scientific Report

1. Activities

The goal of our GCE Phase I proposal was to develop an efficient, easy-to-operate, and novel waste treatment and bioenergy production system: mass cultivating a strain of algae using wastewater as the feedstock. *We have successfully constructed a multi-pond system that processes 200 L/day of municipal and agricultural waste, produces energy in the form of biogas, and generates valuable algae biomass that we have tested as an effective feed for various fish species.* As the target location for our waste treatment system will likely be urban areas of developing countries, we have developed our technology to be tailored for this application. As India and many other developing countries have a unique problem of livestock intermixed with humans in urban areas, their municipal waste tends to be mixed as well. Therefore, we are collaborating both with municipal plants (the North City Water Reclamation Plant and the Metro Biosolids Center) as well as with a local dairy farm (the Van Ommering Dairy of Lakeside, CA) to process both human and cow waste, both individually and combined, to ensure maximum flexibility of our system. In addition, we have developed a strategy for using the algae biomass directly as a feed for small crustaceans, such as fairy shrimp, which could be used to feed larger fish for consumption or sold to boost the local economy.

Our system would be ideally suited to serve as a "waste farm" on the periphery of a small Indian urban area, which would use mobile vacuum trucks to collect human feces from latrine pits and septic tanks as well as cow dung from urban cattle sheds to bring to the waste farm for processing and ultimately for the generation of energy and revenue. For our Phase I work, we have developed an experimental scale set up of such a "waste farm" (Fig. 1), and *our results support the hypothesis that such a set up would be a successful approach for a low-cost, low-maintenance waste treatment system that would generate energy and revenue for developing world communities.* We have also developed a sophisticated yet simple computational control system to enable remote monitoring of the treatment quality to minimize the need for on-site maintenance and service.

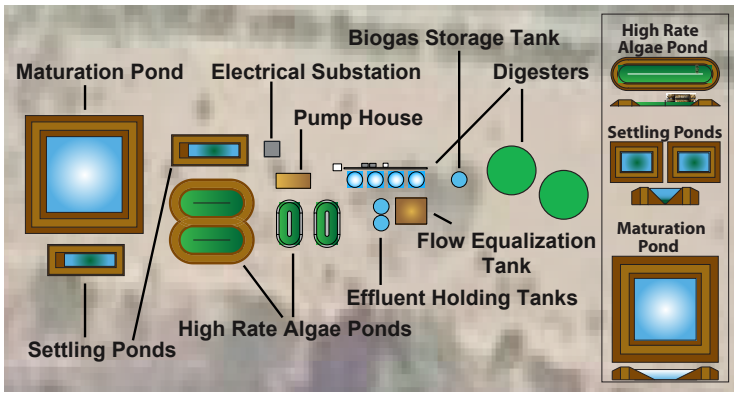

Figure 1: Layout of our advanced pond system. Magnified and profile views (right) demonstrate design features of each pond type. The waste starts in a set of anaerobic digesters, sealed to capture the biogas. The waste then passes to a set of raceway ponds that promote algal growth. Next settling ponds allow for algae setting and removal. Finally, a maturation pond enables grazers to reduce algae and micro-organisms.

We have set up a system that involves several independent and parallel components, so that we can experiment with conditions in both the digesters and the algae ponds. Our treatment system is composed of four primary environments:

1. **Anaerobic Digesters**: 5000 gallon tanks that promote sedimentation and anaerobic breakdown of waste and enable removal of the settled sludge. Tanks are sealed to capture the biogas produced during the waste breakdown.
2. **High Rate Ponds**: 25 cm deep, paddle wheel-mixed raceway ponds that promote algal growth for the uptake of nutrients and release of oxygen to reduce BOD (biological oxygen demand) and enhance disinfection of the water.

3. **Algae Settling Ponds**: 2 m deep, unmixed ponds for algae setting and removal. Growing algae in a paddlewheel mixed high rate pond promotes the cultivation of species which settle readily in the absence of mixing.
4. **Maturation Pond**: 1.5 m deep square pond for final treatment that enables grazers to reduce algae content and promotes removal of micro-organisms by solar radiation, sedimentation and protozoan grazing.

For the first stage of treatment, raw wastewater is transferred to two 5000 gallon anaerobic tanks (digesters) with a retention time of about 30 days, depending on the waste strength (these would be replaced by a covered earthen digester in a developing world context). Biogas is collected and stored in a floating gas storage tank for analysis. Mixing is performed intermittently at a 25% duty cycle using a flexible impeller pump controlled by a custom software routine on a Phidgets SBC2 microcontroller. The digester effluent is then continuously metered and transferred to a set of paddle wheel-mixed raceway ponds to begin the second treatment stage.

The nutrient rich effluent provides an ideal medium for growing algae. During the day the algae release high levels of oxygen into the water through photosynthesis. The oxygen transferred into the water allows aerobic bacteria to further break down the organic waste present in the water, while the robust growth of the algae depletes the water of polluting nutrients such as ammonia and phosphorus. When the nutrients have been fully removed from the wastewater, the effluent is transferred to settling ponds where the biomass is able to settle out. Finally, transfer to a maturation pond for final treatment enables grazers to reduce algae content and promotes removal of micro-organisms by solar radiation, sedimentation and protozoan grazing. Importantly, the extra time spent by the effluent in the maturation pond will greatly increase the destruction of pathogens in the treated water. The data in Figure 2 demonstrates the success of the treatment process for a sample batch over a week of treatment in the high-rate pond. Continuous monitoring of the pond shows results of the algae growth and water treatment: algae growth peaks with sunlight at about midday, causing dissolved oxygen to peak (a) and pH to increase due to CO_2 depletion (b), and ammonium is depleted over time as it is absorbed by the algae biomass (c).

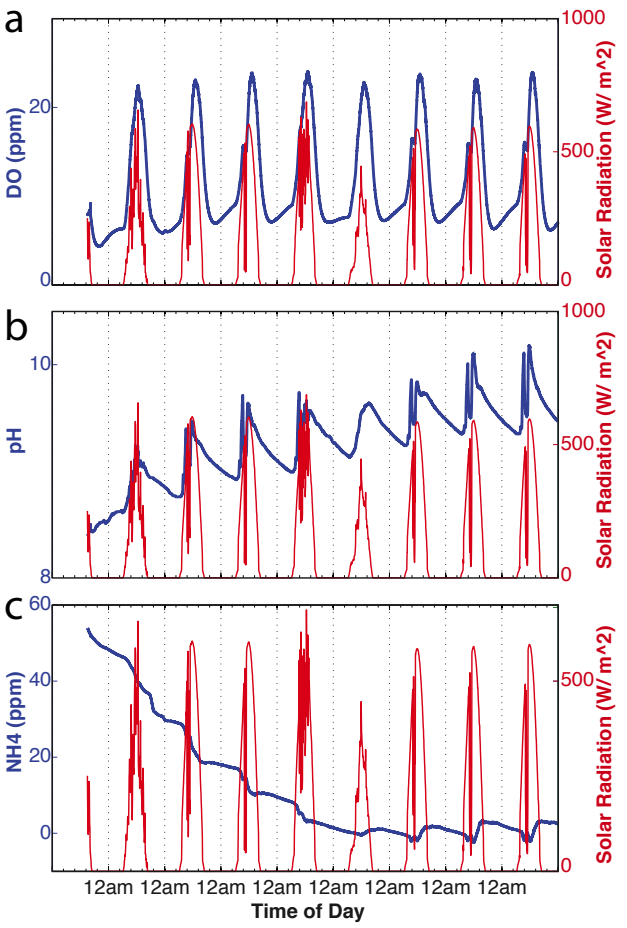

Figure 2: Continuously sampled data during algae growth and wastewater treatment in our advanced pond system. **(a)** Dissolved Oxygen (DO) rises and falls with sunlight, as the algae reach maximum photosynthesis with the peak of daylight. **(b)** pH rises over time as the algae consume carbon dioxide from the pond. **(c)** Total ammonia decreases over time, as the algae consume it and integrate it into biomass.

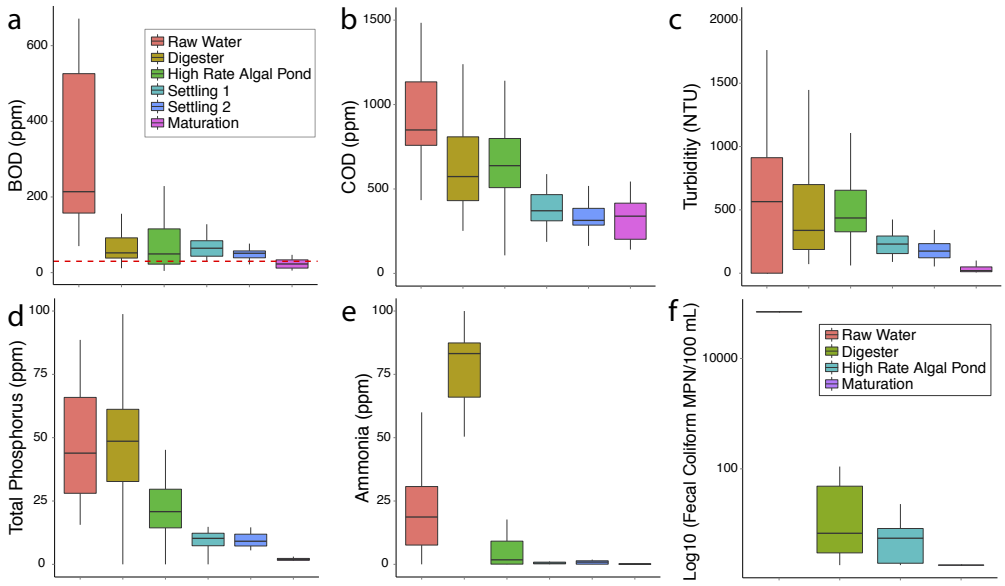

Figure 3: Critical water parameters measured at each stage demonstrate a high standard of treatment. The center dark line in each box is the 50% median. The top and bottom of each box represent the 75th and 25th percentiles, respectively. The top and bottom of the vertical gray lines are the 95th and 5th percentiles. **(a)** Biochemical oxygen demand (BOD) estimates the amount of oxygen required by bacteria to break down the organic matter. This drops drastically during digestion and ultimately falls below the threshold in California for recycled water (red dashed line). **(b)** Chemical oxygen demand (COD) estimates the amount of organic compounds in the water. **(c)** Turbidity measures the cloudiness of water. While not directly a health concern, high turbidity can interfere with disinfection and provide a medium for microbial growth. Phosphorus **(d)** and ammonia **(e)** are absorbed and recycled by the algae, a major benefit over standard treatment methods that often discharge these nutrients to local water bodies causing environmental problems. The ammonia increases after digestion due to the anaerobic bacteria breaking down organic nitrogen into ammonia. **(f)** The MPN test estimates the number of fecal coliforms in a water sample.

Treatment throughout the series of environments is monitored using standard probes coupled to an open source computer program for automation and analysis. As the ultimate goal is to reduce the organic material in the effluent, the organic matter must be quantifiable. Typically, it is measured in a 5-day test of biochemical oxygen demand (BOD_5), which estimates the amount of oxygen required by bacteria to consume it. In addition, every 3 days, levels of turbidity, total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD), total nitrogen, ammonia nitrogen, nitrate, nitrite, total phosphorus, reactive phosphorus and alkalinity are determined using Hach TNT test kits and a Hach DR 3900 spectrophotometer. Other parameters such as temperature, sunlight, dissolved oxygen (DO), pH, and ammonium are measured continuously. Also important is a "most probable number" (MPN) test, a statistics-based test which estimates the number of fecal coliforms in a water sample based on the degree of lactose fermentation by organisms in the sample. Importantly, our technology does an excellent job of reducing the amount of coliform bacteria in the waste stream. Figure 3 shows examples of the results of measuring many of these critical parameters as waste passes through our series of treatment environments over time, and it is clear that we are able to reduce critical parameters such as BOD, COD, nitrogen, phosphorus, turbidity, and fecal coliforms at each treatment stage.

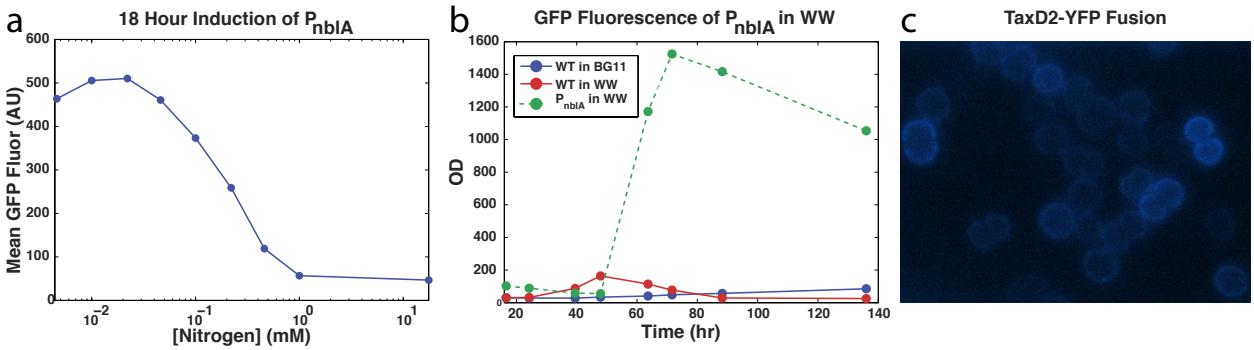

Figure 4: Fluorescence production by the cyanobacteria species *Synechocystis* after genetic transformation. **(a)** A genetic element designed to "turn on" in the absence of nitrogen produces the green fluorescent protein (GFP) in low nitrogen conditions. **(b)** This modified algae strain turns on after two days in wastewater, after the algae cells have consumed most of the nitrogen. **(c)** A GFP fusion to a membrane protein emits fluorescent light from the outer edge of the cell membrane.

Another aspect of our project has been to use synthetic biology to genetically modify algae cells, with the goal of generating a strain ideally suited for our application. Our aim was to develop a genetic "switch" that would activate in low nutrient conditions and cause the algae biomass in the treatment system to produce an aggregation-mediating molecule and settle to the bottom of the pond to facilitate its extraction. While the tools for synthetic biology in cyanobacteria (blue-green algae) are still in their infancy, we have had success inserting foreign DNA into a common lab strain of *Synechocystis* PCC 6803. We used standard molecular biology techniques to build plasmids (circular DNA elements capable of integrating into the host genome) which produce a protein of interest when the nitrogen level in the wastewater drops below a certain threshold. We tested the induction of this genetic element by using the nitrogen promoter (P_{nblA}) to produce the green fluorescent protein (GFP) as a marker and subjecting algae cells to various levels of nitrogen. Figure 4a demonstrates the success of this induction, as at low levels of nitrogen the cells were highly fluorescent, as measured by flow cytometry, while they were "turned off" in high nitrogen conditions. This system can be used to trigger a biological process of interest when waste treatment nears completion, as the algae cells will have depleted most of the nitrogen. To test that hypothesis, we grew these cells in wastewater for several days, and after about 48 hours we observed the cells begin to produce the GFP marker (Fig. 4b). We used flow cytometry to scan thousands of cells at each condition at various time points after introduction into the wastewater. Wildtype (WT) cells are used as a control for comparison.

We have also investigated various candidate proteins to use as anchors for the aggregation-mediating molecule that would cause the algae cells to form clumps and settle out of the wastewater. To this end, we selected over 30 candidate membrane proteins chosen from a literature search, and we fused these proteins to GFP. While many proteins were believed to be on the membrane based on a variety of other experimental techniques, none had been verified by optical means. Therefore, we used the nitrogen promoter described above to produce each of the candidates fused to GFP, so that we could visually localize each protein. We built a plasmid for each of these proteins and transformed them into *Synechocystis*, so that the cells would integrate this new element into their genomes. Scanning all of these candidates yielded a couple of promising options, including *TaxD2*, a methyl-accepting chemotaxis protein (MCP) required for the biogenesis of thick pili (hairlike appendages found on the cell surface). We observed this protein to be clearly localized to the membrane, as it could be seen forming distinctive rings around each cell (Fig. 4c).

2. Challenges

While we have had promising success with the synthetic biology aspect of this project, we have since evolved our idea for utilizing the algae biomass in a way that will optimize its benefit for the communities that we are targeting with this technology. First, after conducting thorough research of the market for algae biomass, we have determined that the most efficient and economical use of the algae will be as a feed for fairy shrimp or a similar organism that can serve as a live feed or can be sold to boost the local economy. By using the algae directly as an aquatic feed, it does not need to be harvested, which is the most expensive and time-consuming aspect of using high-rate algae ponds for waste treatment. If we can eliminate this expensive and energy-intensive step while producing a valuable product, we will take a huge step toward developing a system that can be self-sufficient, low-maintenance, and profitable for the target communities. In addition, the genetic modification of laboratory algae strains presents a few other challenges, including the regulatory hurdles likely to emerge when trying to get approval for the use of a genetically modified organism (GMO) in an outdoor pond and the difficulty of getting a laboratory strain to survive and thrive in a competitive environment.

Based on these developments, we have shifted the focus of the algae development aspect of this project to the "directed evolution" of natural wildtype strains for higher productivity. We are using a combination of cutting edge high-throughput sequencing technology along with our proven expertise with microfluidic devices to develop a process by which we can identify the most prominent native strains and rapidly evolve them to have the desired characteristics. We are using an Illumina MiSeq machine to analyze DNA samples extracted from the microbes present in the wastewater to identify the many species present in our system, and we plan to monitor how this natural ecology varies with time and to characterize the productivity and feed quality of the strains of interest. We will then use innovative microfluidic technology pioneered by our group to "evolve" these strains for higher productivity in wastewater conditions. While we have demonstrated the success of our approach for treating wastewater to a high standard and have used the native cultured algae strains as a feed for both tilapia and fairy shrimp, we believe that the application of these next-generation technologies will further enhance our technology and make it an economically viable solution on a large scale. For our Phase II proposal, we will develop a full-scale demonstration site, which will aim to directly mimic conditions that we would face in a developing world context.

3. Other Sources of Project Support

Since beginning work on our Grand Challenges Explorations project, we have received funding from two other organizations to support the development of our waste treatment technology. We have received $100,000 from the USDA NRCS "Conservation Innovation Grants" program. This program supports the development of innovative conservation approaches and technologies, and we were funded to develop algae-based waste treatment technology for a local dairy farm. We received an additional $1,500,000 for this same effort from the California Energy Commission's "Emerging Technology Demonstration Grant" program. We are working with the Van Ommering Dairy farm in Lakeside, CA to treat their entire 300-cow waste stream, and we plan to expand this to treat the dairy's human waste as part of the GCE Phase II work. As the two waste streams will be processed by the same downstream ponds, these two grants will serve as matching funds for our GCE project, enabling us to expand our Phase I work to a full-scale demonstration project. We will be able to develop and test the technology on a scale similar to that needed to treat a small urban community's waste, enabling us to fully prepare for a Phase III deployment into a real developing world community.