Section II: Detailed Proposal Information

1. EXECUTIVE SUMMARY

Biofouling, the result of extensive colonization of substrates by communities of bacteria, algae, and barnacles, causes considerable damage to human-made structures in marine environments, especially ships. Biofouling of a ship's hull increases drag and dramatically reduces energy efficiency, leading to excess fuel consumption and greenhouse gas emission. Biofouling also threatens global biodiversity by providing a means of transport for invasive species. Thus, control of biofouling will have significant benefits to society and the environment[1,2].

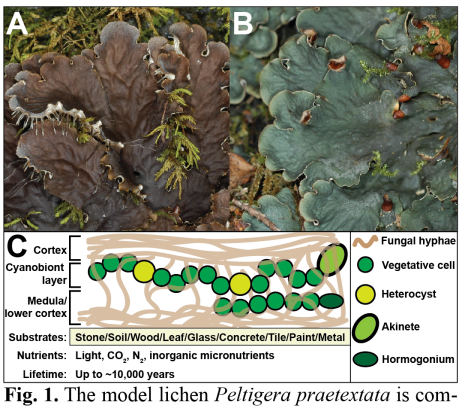

Fig. 1. The model lichen *Peltigera praetextata* is comprised of a photosynthetic cyanobacterium (*Nostoc*) in a matrix of fungal mycelium. **(A)** Dry and **(B)** hydrated P. praetextata. **(C)** Structure of a lichen and specialized *Nostoc* cell types.

Traditionally, biofouling has been treated using chemical agents such as toxic metals, surface coatings and textures, pharmaceuticals, and through mechanical disruption. However, current solutions are costly, time consuming, and potentially damaging to the environment.

We propose the development of an intelligent "armor" based on naturally occurring multispecies communities such as lichens (**Fig. 1**) that can be programmed with biological-based control systems to sense and respond with protective/defensive measures to specific biological agents responsible for biofouling of ships and other marine military installations (**Fig. 2**). Lichens, symbiotic organisms comprising a cyanobacterial or algal photobiont in a matrix of fungal mycelium (mycobiont), are extremophiles found in diverse alpine, desert, tropical, and maritime environments, and have been shown to survive exposure to outer space[3]. In nature, lichens cover large surfaces and can survive for hundreds to even thousands of years, making them ideal biological templates for robust coatings. Moreover, naturally occurring cyanobacterial compounds have already been shown to inhibit biofilm and biofouling[4]. **Developing a synthetic lichen presents a unique opportunity to harness the synergy that has evolved between cyanobacteria and fungi over billions of years.**

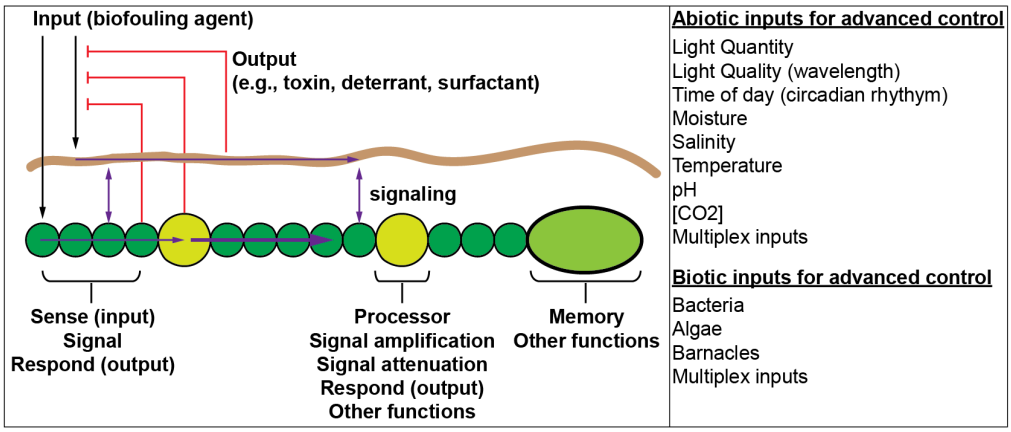

Fig. 2. Biological closed-loop control system embedded in a synthetic lichen. This responsive, living armor will function to prevent biotic biofouling of surfaces in a wide variety of environments.

Initially, the biological control circuits (**Fig. 3**) will be genetically encoded within the filamentous, nitrogen-fixing, cyanobacterial photobiont (*Nostoc* sp.) naturally embedded in the fungal matrix (**Fig. 4**). We will employ the model lichen, *Peltigera praetextata* (*P. praetextata*), because it has many properties amenable to these studies including the ability to re-synthesize the lichen from separately cultured photobiont and mycobiont cells[5]. Each cell will have the capacity to sense a biofouling "agent" and respond by producing a toxin or deterrent molecule as

well as a mobile signal to activate additional response pathways in neighboring cells. Once the agent has been neutralized, the initiating signal will dissipate and the control loop will reset to the ground state.

This responsive armor is innovative because it will be able to **sense and respond to threats simultaneously and at multiple scales** (microns to meters, seconds to years) as determined by the strength of the input stimulus, rate of signal diffusion/propagation, and system-level memory. For example, we propose to encode memory circuits that have the potential to increase the effectiveness of the system to detect and ameliorate a range of threats (e.g. agents found in specific environments such as harbors vs. open ocean). The material could also be designed to have desirable surface characteristics that prevent adhesion by external agents as well as to reduce frictional drag. For instance, the material could be engineered to be hydrophobic, hydrophilic, or to secrete surfactants. Increased functionalities will be encoded into the system by taking advantage of the differentiated photobiont cell types: vegetative, heterocyst, and akinetes (**Fig. 1**) and through mixtures of multiple "sensor" strains in a single coating. There are many challenges that have to be overcome to realize our goals. First is the cultivation and genetic manipulation of lichens. In Phase I, we will engineer the cyanobiont and characterize the control system in simplified systems while we simultaneously study and model signaling pathways in native lichens. These efforts will make use of our extensive resources and world-class collection in the CU Lichen Herbarium.

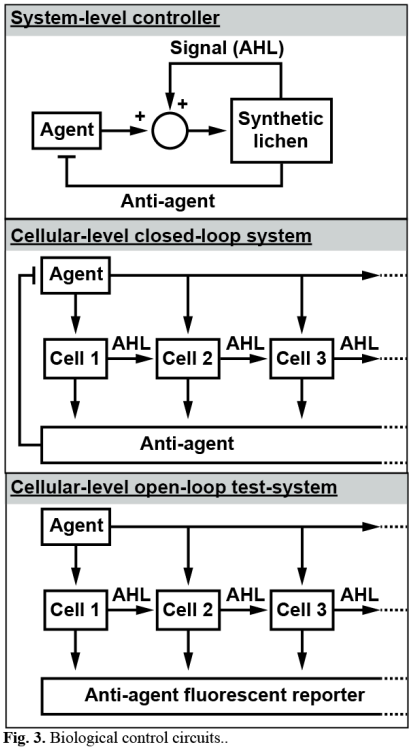

Fig. 3. Biological control circuits..

This archetypal, symbiotic natural system provides an ideal test bed with built-in multi-scale control and adaptive response, wherein the fungus provides mechanical support, moisture, nutrients, a defensive barrier, and long-range communication, and the cyanobacteria provides energy through photosynthesis and fixed nitrogen from N_2. In this project, we will leverage and augment the control systems manifested in natural lichens with tailored biological controllers suited toward the particular application of biofouling. **However, the broader aim is to develop a smart, responsive, synthetic lichen paradigm capable of addressing a wide variety of military and civilian applications through inherent control of biological complexity.**

Our team has extensive experience in biochemistry, microbiology, ecology, synthetic biology, material science, control systems, and mathematical and computational biology. With support from DARPA, we will leverage our technical expertise and extensive resources at the University of Colorado Boulder, the Renewable and Sustainable Energy Institute (RASEI), and the National Renewable Energy Laboratory (NREL), to achieve our objective outlined in this proposal of developing a living, responsive armor to prevent biofouling.

2. GOALS AND IMPACT

In this proposal, we aim to harness the natural properties of lichens to develop a living armor that protects against micro- and macro-scale biofouling of military and civilian infrastructures in marine environments. Lichens are typically comprised of two species, a cyanobacterium and a

fungus, but are among the most robust organisms on earth, surviving in extreme environments for thousands of years. The fungal matrix provides support to the cyanobacterium, which in turn performs photosynthetic CO_2 assimilation and N_2 fixation to support growth and maintenance using only light, air, and micronutrients present in the environment. Using an existing synthetic biology toolkit and the underlying principles of control theory, we will integrate a closed-loop biological controller into the embedded cyanobacterium to rapidly detect a biofouling agent, signal the threat to neighboring cells that an agent has been detected, and respond by producing a deterrent, toxin, or other system-level behavior to prevent agent attachment and colonization. This armor will be able to locally respond to diverse threats across a multiple time and distance scales and will exhibit long-term system stability, memory, and self-repair. Success of this project will revolutionize our ability to develop biological controllers that function in real-world and extreme environments, including potentially extraterrestrial applications. Developing a synthetic lichen paradigm will also have major impacts in other areas important to the mission of DARPA including the "Biological Robustness in Complex Settings" and "Battlefield Medicine" programs, due to lichen's innate physical robustness and ability to synthesize an incredible diversity of bioactive natural products. In summary, this project will develop a highly novel and multi-functional biocontroller embedded in a synthetic lichen, along with the tools, theory, and testbeds to optimize and generalize this paradigm for a variety of crucial military and civilian purposes.

3. TECHNICAL PLAN

The controllers in this project will consist of a feedback loop of agent→signal→anti-agent→agent **(Fig. 2, 3, and 4).** In the fully functioning target system, the agent will be the initiator of the fouling agent, for instance the attachment of Mussel Adhesive Proteins (MAPs). In the beginning phases of the project, *E. coli* will serve as a proxy for the biofouling agent (e.g. MAPs), to afford control of local perturbation of the synthetic lichen network through titration at different concentrations and subsequent activation with IPTG, a common transcription inducer (**Fig. 5**). The signal will be carried by n-Acyl Homoserine Lactone (AHL), a common autoinducer responsible for quorum sensing in bacterial populations[6,7]. The anti-agent will be either a surfactant that inhibits the adhesion of the agent or a toxin that eliminates the agent before contact with the synthetic lichen surface. **Importantly, none of the controller components require fundamental discovery - they are each well-established tools of synthetic biology that will be incorporated into higher levels of system organization for the project**.

A multitude of control parameters will be utilized. These include: (i) the AHL synthesis rate, which depends on the expression levels of *luxI* and *luxR* transcription regulators[8–14], (ii) the AHL diffusion rates, which depend on the length and polarity of the ligands used, (iii) the frequencies and spacings of the various cell differentiations (vegetative, heterocyst, and akinete), (iv) the logical operations carried out by the heterocyst cells, (v) the morphological structure of the microbial community, which includes cyanobacteria filament length, density, and arrangement and relation to the fungal support matrix, and (vi) environmental parameters of sunlight, temperature, nutrient level, and pH.

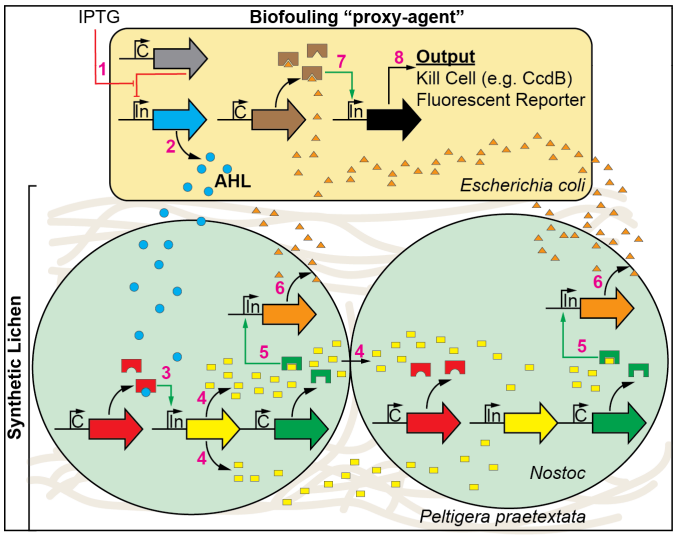

Fig. 4. Detail of closed-loop biological controller. Signal propagation through orthogonal n-acyl-homoserince (AHL) autoinducer pathways. Inducible (In) and constitutive promtoers (C).

Initial phases of the project will focus on control system identification and verification, in which each controller component is quantified in isolation. These include measurements of the time dynamics of gene expressions and measurements of signal transport rates within and between cyanobacterial filaments (**Fig. 2**). These low-level control components will then be combined to yield full controller functionality in closed-loop operation. **Phase I** of the project will conclude with demonstration of the synthetic lichen "armor" with *E. coli* as a proxy for the biofouling agent (**Fig. 4**). **Phase II** will move on to higher levels of complexity by incorporating multi-agent deterrence via multiplexing of *Nostoc* strains and by integrating more complex logic functions in the heterocysts (**Fig. 2 and 5**). **Phase III** will focus on tailoring the controllers toward specific applications with true biofouling agents (e.g. MAPs) and on tailoring the microstructure and mechanical properties of the material system toward its end use in marine or other environments.

Minimizing signaling crosstalk, developing orthogonal cell-communication systems, and quantifying 3-dimensional signal propagation in intact lichen are major challenges of the project. We will address these challenge in several ways. First, spatial separation of cognate promoter/activator pairs between cell types will reduce the crosstalk (**Fig. 4**). CRISPR based genome editing with mutation tracking capabilities and high-throughput screening/selection procedures developed by the Gill lab will be used to generate sets of orthogonal signaling systems. Many biological parts do not function as expected when introduced into different host systems. To overcome these challenges, we will generate libraries of promoters and ribosome binding sites in order to identify parts with predictive outputs in the three biological systems of interest. Imaging cyanobacteria can be technically challenging due to phototoxicity of the microscope light source[15]. The Cameron lab has extensive experience in quantitative long-term imaging and synchronization of sub-cellular and population-level processes in cyanobacteria. His previous work combined synthetic biology and time-lapse fluorescence microscopy to uncover the process by which cyanobacteria assemble their carbon-fixing machinery; this work was published in *Cell*[16]. Another potential challenge will be in the genetic manipulation of the cyanobiont (*Nostoc*) and the mycobiont (*P. praetextata*). We anticipate we can overcome these challenges, since multiple strains of *Nostoc* are routinely engineered[17]. For example, we could use *Nostoc punctiforme* or related Anabaena PCC 7120, as these model cyanobacteria have fully sequenced genomes and available methods to manipulate gene expression. While the *P. praetextata* genome has not been sequenced, a close relative, *P. membranacea*, has been. Co-PI Tripp has already been awarded funding by the NSF to sequence the *P. praetextata* genome.

3.2 Task Area II: Testbed

During Phase I, we will design an adaptive, responsive, biological-armor by introducing a control system into a lichen (*P. praetextata*) such that it can 1) sense the attachment of a

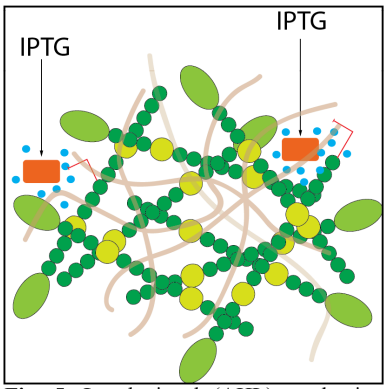

Fig. 5. Local signal (AHL) production using *E. coli* "proxy-agent" (orange).

biofouling agent (e.g. bacteria, algae, or barnacle), 2) signal to neighboring cells that an agent has been detected, and 3) respond with an output that kills the agent or disrupts its ability to attach. This system will comprise a closed-loop since detachment or death of the agent will remove the input signal, resetting the system. This control loop will be created using well-characterized biological parts that function in native bacterial cell-communication systems for quorum sensing (**Fig. 4**) to control system-level behavior. For initial characterization and quantification of the system, we will utilize a strain of *E. coli* engineered to secrete a signaling molecule as a proxy for the biofouling agent in a localized manner (**Fig. 5**). Sensors and signal-generators will be introduced into the cyanobacterial and used to quantify rates and dynamics of intercellular signal transduction in a range of testbed configurations (**Fig. 6**). Phase II and Phase III testbeds will be developed to examine a range of inputs and outputs relevant to maritime biofouling environments (**Fig. 2**).

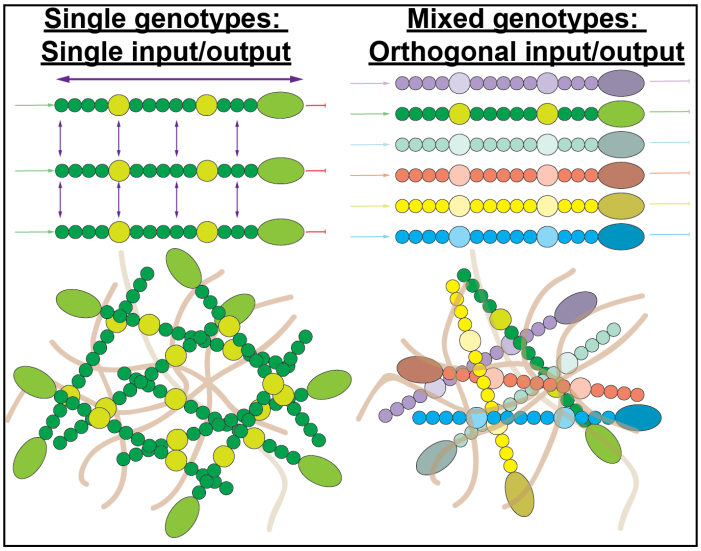

Fig. 6. Testbed configurations using single and mixeg genotypes.

3.3 Task Area III: Theory and Modeling

To design and validate the biochemical control loop, at each phase of this research program we will model the system on the microscale, the single-cell level, and finally on the macroscale complex-networked multi-cell level, where the vegetative and heterocyst cells are interconnected through physical connection as well as signaling pathways. At the single cell level, we will quantify each cell's static and dynamic input-output behavior. In all phases, Task 1 (T1) will focus on modeling and verification of the steady-state input-output (phenomenological) behavior of the subsystems and the concatenated systems; Task 2 (T2) will develop and validate mechanistic, dynamical models for the open-loop controller and interconnected subsystems (**Fig. 3**); and Task 3 (T3) will focus on the study, design, and validation of the full closed-loop, spatially coupled system (**Fig. 4**).

Phase I: Study of the steady-state single input system: In this phase of the study, we develop a prototype of a complex networked system containing vegetative cells.

Task 1: This task will focus on developing the input-output characteristics of the input (agent, signal) to output (anti-agent, signal) behavior of vegetative bacterial cells. The following diagram (**Fig. 7**) represents the phenomenological, systematic viewpoint of the vegetative cells

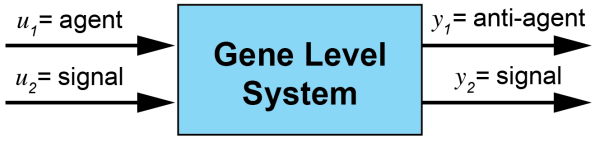

Fig. 7. Input-output system parameters.

as a subsystem with two inputs, agent and signal concentrations, and two outputs, anti-agent and signal concentrations.

In this task, we will formulate, quantize, and verify the steady-state behavior model of the above input-output system. In this open-loop (i.e., no feedback) system, the anti-agent does not affect the input agent's concentration. For experimental verification and validation, we will use genetically encoded fluorescent reporter genes as the output such that they won't affect the input. Experimental protocols (needed number of replicates and temporal/spatial resolution) will be created using a robust experimental design methodology. As a step towards the dynamic modeling of the system, we will identify the steady state behavior of this building block of our system. To do this, we will quantify the anti-agent and signal concentration at the steady-state for fixed input concentrations. This will give rise to the following simplistic model: $y = F_\theta(u)$,where θ is the vector of the design parameters of the system, and $F_\theta: \Re^2 \to \Re^2$ is the transfer function for the steady-state behavior of the gene-level system.

Once this relationship is characterized for the single cell, we will use this working system as a basis for a mechanistic model as well as the interconnected system.

Task 2: The focus of Task 2 is to formulate the dynamic model for the system. In this task, we develop a mechanistic model and identify the input to output behavior of the system through a set of possibly nonlinear dynamics:

$$\dot{x}(t) = g(x(t), u(t))\; y(t) = h(x(t), u(t))\,.$$

This is a standard nonlinear control framework where $x(t)$ is the vector of internal states (representing concentrations of transcriptional regulators at time t, $u(t) \in \Re^2$ is a control-input vector representing the concentration of the agents and input signal at time t, and $y(t) \in \Re^2$ is a vector of concentrations of the anti-agent and signaling material at time t. Initially the function g will simply be an input-output response, but it will be refined to include a more biochemically mechanistic modeling. At the multi-cell level, an interconnected system of cells is formed as their concerted input to each of the agents is the aggregate signal that is present in the environment.

Task 3: The final step towards designing our first prototype is to close the control loop. For this, we will characterize the dynamics of the feedback loop, i.e. we will model the interaction between the agents and anti-agents. After modeling and identification of the signaling and spatial interaction, we consider the dynamics of the closed-loop system, and we will optimally design the closed-loop system's parameters such that the closed-loop system is asymptotically stable (to zero) with a sufficiently large region of attraction. Asymptotic stability of the closed-loop system ensures that with the introduction of agent in the environment (i.e. small perturbations in the input), the closed-loop system is capable of returning the concentration to zero. However, at the same time we intend to minimize the anti-agent concentration (over time). So, for the optimal design of the closed-loop system, we will minimize the integral of the anti-agent concentration in the environment, subject to the local stability of the closed-loop system that contains a prescribed region of attraction.

Phase II: We will study the dynamics of the multiplexed system of vegetative and heterocyst cells. As in Phase I, first we will model the static and dynamic input-output behavior and then, by modeling the feedback loop, we will characterize and design the closed-loop system. Our research plan in Phase II follows the research plan in Phase I (with the addition of taking into account the presence of heterocyst cells (**Fig. 2**)).

Task 1: We will develop static (steady-state) models of a heterocyst cell as a subsystem with many input and outputs. We will validate this model using lab experiments. Once the static

model of the heterocyst cells are developed, we will develop the static model of the (open-loop) interconnected system whose input is agents' concentration and whose output is anti-agent (or fluorescent reporter) secretion into the local environment. We will design experimental protocols using a robust design method to obtain this model.

Task 2: In this task, we will develop and verify the dynamic model of the system by first characterizing the dynamic model for the heterocyst cells. Once the dynamic model is developed, we develop a model for the interconnected system that consists of many vegetative and heterocyst cells (**Fig. 5 and 6**).

Task 3: After modeling the whole system and also by taking into account of the dynamics of the closing loop, we will characterize the closed-loop behavior of the system and the optimal design of the controller parameter. For this design, we seek to minimize the anti-agent production of the system subject to local stability (to zero) of the closed-loop system, where the local-stability (on sufficiently large domain of attraction) ensures that with perturbations in the input (i.e. introduction of some agent), the output of the closed-loop system (agent's concentration) still returns to zero.

Phase III: We will study the dynamics of the multiplexed system in a laboratory-simulated marine biofouling environment. With the insights from the previous phases of this project, we identify, verify, and design the closed-loop system to prevent biofouling. The work plan in this phase follows closely the flow of the work plan outlined in Phase I and II but will be performed in the laboratory-simulated marine biofouling environment (**Fig. 8**).

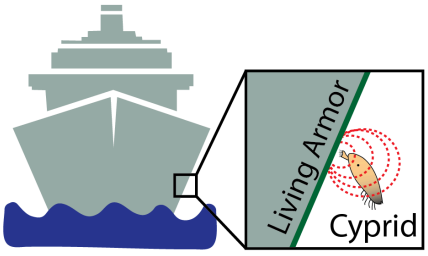

Fig. 8. Living armor to prevent biofouling.

Task 1: This task will address the static model of the vegetative, and heterocyst cells in the marine environment. Once the model of these individual cells is developed, the static model of the interconnected open-loop system will be developed.

Task 2: The dynamic model for the open-loop components (vegetative and heterocyst) will be developed and the model of the interconnected system will be derived.

Task 3: Finally, the dynamics of the feedback-loop will be identified and verified, and subsequently the dynamics of the interconnected closed loop system will be characterized. Once this is achieved, we will design the control parameters such that we minimize the anti-agent production of the closed-loop system while zero state (no agent) is an asymptotically stable equilibrium for the closed-loop system with a region of attraction that is guaranteed to contain large enough perturbations in the agent concentration of the environment.

Table I : Commitment from team members in terms of percent effort

Team Member	Affiliation	Year 1	Year 2	Year 3	Year 4	Team Member	Affiliation	Year 1	Year 2	Year 3	Year 4
Jeffrey Cameron	CU/RASEI	8.33%	8.33%	8.33%	8.33%	Postdoc 4	CU/RASEI	100%	100%	100%	100%
David Bortz	CU	21.60%	21.60%	21.60%	21.60%	Postdoc 5	CU/RASEI	100%	100%	100%	100%
Carrie Eckert	NREL/CU	8.33%	8.33%	8.33%	8.33%	Postdoc 6	CU/RASEI	100%	100%	100%	100%
Ryan Gill	CU/RASEI	8.33%	8.33%	8.33%	8.33%	Postdoc 7	CU/RASEI	100%	100%	100%	100%
Sean Shaheen	CU/RASEI	8.33%	8.33%	8.33%	8.33%	Postdoc 8	CU/RASEI	100%	100%	100%	100%
Behrouz Touri	CU	8.33%	8.33%	8.33%	8.33%	Postdoc 9	CU/RASEI	100%	100%	100%	100%
Erin Tripp	CU	8.33%	8.33%	8.33%	8.33%	Postdoc 10	CU/RASEI	100%	100%	100%	100%
Admin. Asst.	CU/RASEI	100%	100%	100%	100%	Postdoc 11	CU/RASEI	100%	100%	100%	100%
Postdoc 1	CU/RASEI	100%	100%	100%	100%	Postdoc 12	CU/RASEI	100%	100%	100%	100%
Postdoc 2	CU/RASEI	100%	100%	100%	100%	Postdoc 13	CU/RASEI	100%	100%	100%	100%
Postdoc 3	CU/RASEI	100%	100%	100%	100%						