TITLE

Dynamics, consequences, and protein biomarkers of state changes in the *Sarracenia purpurea* microecosystem

SUMMARY

The microecosystem within the water-filled, cup-shaped leaves of *Sarracenia purpurea*, the Northern Pitcher Plant, has been put forth as a model system for studying shifts between alternative ecosystem states in larger aquatic ecosystems. However, it is not clear if states in the *S. purpurea* system represent alternative stable states (as they do in lakes and ponds), how state changes impact the food web inhabiting *S. purpurea* pitchers, and how these state changes can be predicted. In this project, I propose to investigate state change dynamics, consequences, and predictors in the *Sarracenia purpurea* microecosystem. I will use controlled greenhouse experiments and metaproteomic technique to explore five broad questions: Q1) Do oxic and hypoxic states in the *S. purpurea* microecosystem represent alternative stable states? Q2) How do state changes alter food web structure? Q3) How does food web structure influence timing of and recovery from state changes? Q4) How does microbial protein expression change as an ecosystem undergoes a state change? Q5) Can changes in microbial protein expression serve as a reliable biomarker for predicting state change in aquatic ecosystems? This work will further develop the *S. purpurea* microecystem as a model for studying state changes and will explore a molecular approach to defining ecosystem states and predicting state changes using metaproteomics.

INTELLECTUAL MERIT

In a time where climate change threatens to drastically alter ecosystems, it is becoming increasingly important to understand the dynamics of catastrophic state changes, their effects on ecosystems, and how to predict them. My work in the *Sarracenia purpurea* model system will give insight into the effects of state changes on natural ecosystems, the importance of food web structure in preventing state changes, and the utility of molecular biomarkers for predicting state changes in aquatic ecosystems. My work will represent one of the first attempts to identify protein biomarkers for state changes in a natural ecosystem using metaproteomics. The application of metaproteomics to predicting state changes in the *S. purpurea* ecosystem could be expanded to larger aquatic systems and give insight into the changes in functional pathways that underlie state changes.

BROADER IMPACTS

My work in the *S. purpurea* ecosystem has the potential to inform conservation efforts in larger aquatic systems. The pipeline I have developed for metaproteomic analysis of microbial communities can be used in any aquatic system. Additionally, the pipeline uses only open-source software. All of my data will be publicly available via the Harvard Forest Data Archive. My work spans multiple fields and I have mentored students interested in public health, biochemistry, and ecology. I have mentored two student apprentices, one undergraduate researcher, and have aided two undergraduates in preparing proposals for the UVM Office of Undergraduate Research Summer Award (one was successfully funded). I will continue to involve undergraduate students in my research as student apprentices and as student researchers.

BACKGROUND

Ecosystems may shift abruptly from one state to another in response to perturbations or slow changes in environmental conditions (Lewontin 1969, Holling 1973, May 1977, Scheffer et al. 2001). For example, gradual enrichment of a clear water lake with phosphorous can cause a sudden increase in phytoplankton, loss of submerged plants and higher trophic levles, and depletion of dissolved oxygen, switching the lake to a turbid state. Alternatively, large perturbations in turbidity can also push the system from a clear state to a turbid state (Carpenter 2005). State changes occur in a variety of ecosystems including coral reefs (Knowlton 1992, Dudgeon et al.), marine communities (Petraitis et al. 2004, Petraitis et al. 2009), mesic grasslands (Ratajczak et al. 2014), and lakes and ponds (Scheffer et al. 1997, Scheffer 2001, Carpenter 2005). Often, these changes occur as a result of human activity and have negative consequences for an ecosystem (Dodds et al. 2009).

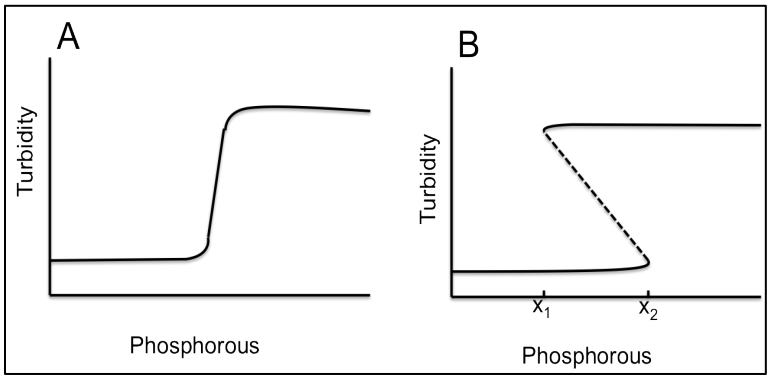

Figure 1. *A)* Non-linear, continuous change in turbidity in response to phosphorous enrichment. *B)* Hysteretic, discontinuous change in turbidity in response to phosphorous enrichment. For phosphorous values between x_1 and x_2, the system can be in a clear or turbid state.

State changes can occur in a continuous or discontinuous fashion and discontinuous changes may be more difficult to manage because they represent changes between alternative stable states (Figure 1)(Scheffer et al. 2001). States are considered alternative stable states when a change in a driver, such as phosphorous enrichment, causes discontinuous changes in a state variable, such as turbidity (Figure 1). These discontinuous changes are the result of a phenomenon called "hysteresis" and are evidenced by the backwards folding of a response curve (Figure 1B) (Scheffer et al. 2001). In hysteretic systems, the dynamics of the system depend on the former state of the system. For example, in a clear shallow lake, the switch to a turbid state occurs when phosphorous levels are higher than they are for the switch to a clear state from a turbid state (Figure 1B). Because systems with alternative stable states are hysteretic, it can be difficult to reverse state changes once they have occurred (Kéfi et al. 2013); therefore, research concerning the management of state changes has focused primarily on identifying early warning signals rather than managing changes after they occur (Scheffer et al. 2009, Drake & Griffen 2010).

Many systems at the threshold of a state change exhibit "critical slowing down," or a decrease in the recovery rate of a state variable in response to small perturbations (Wissel 1984, Scheffer et al. 2009). Symptoms of critical slowing down, such as rising variance and autocorrelation in the time series of a state variable, may serve as early warning indicators of state changes (Carpenter & Brock 2006, Dakos et al. 2012). These indicators have been identified in simplified ecosystems (Dai et al. 2012) and by retroactive analysis

of state variable time series (Bestelmeyer et al. 2011). However, the utility of these indicators in natural ecosystems is not well studied. Simulations indicate that they do not provide enough lead-time for successful mitigation (Contamin & Ellison 2009, Biggs et al. 2009) and may not be detectable in noisy systems (Perretti & Munch 2012). An alternative approach is to use molecular biomarkers, which may provide more lead-time than traditional indicators ultimately driven by changes in underlying biological processes (Sirota et al. 2013). In recent years, advances in molecular techniques have allowed researchers to scan the entire genome and proteome of microbes and other organisms in various marine and freshwater ecosystems (Venter et al. 2004, Morris et al. 2010, Lauro et al. 2011, Sowell et al. 2011, Thureborn et al. 2013). Such molecular approaches have resulted in the identification of taxonomic and molecular biomarkers for aquatic ecosystem function (Zhang et al. 2010). Nevertheless, limits on manipulation and replication of natural ecosystems act as barriers to identification and validation of such biomarkers as predictors of state changes.

The multi-trophic level microecosystem within the pitcher fluid of *Sarracenia purpurea*, the Northern Pitcher Plant, is an established model system for studying food web dynamics and has recently been put forth as a model for studying state changes in aquatic ecosystems (Sirota et al. 2013). *S. purpurea* is a carnivorous plant found in wetlands throughout North America and Canada. The plant has cup-shaped leaves, or pitchers, that fill with rainwater and trap and drown insect prey. Each pitcher harbors a multi-trophic food web that includes bacteria, protists, rotifers, and invertebrate larvae (Figure 2). This microecosystem is easy to manipulate and replicate and can be forced to switch from an oxic state to a hypoxic state with organic matter loading (Sirota et al. 2013), making it an ideal natural ecosystem for studying state changes.

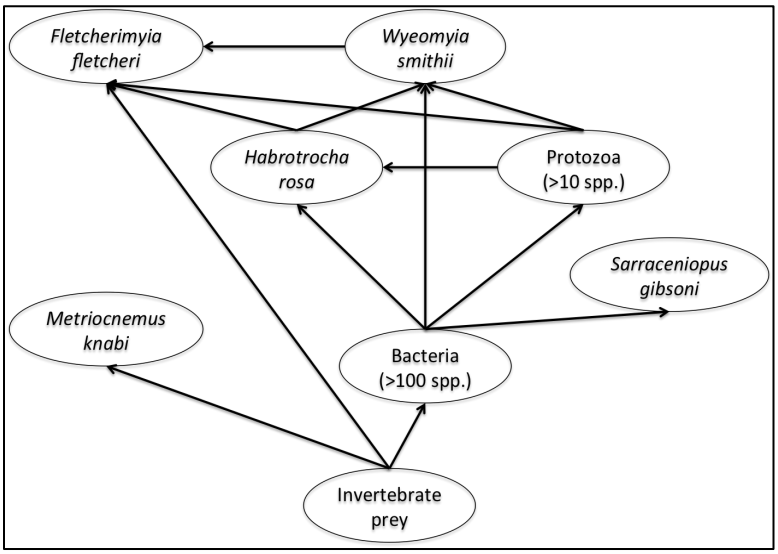

Figure 2. *Sarracenia purpurea* food web. Invertebrate prey is shredded by the midge larva *M. knabi* and the fly larva *F. fletcheri*. Shredded prey is then mineralized by bacteria. Bacteria are preyed upon by *F. flecheri*, the rotifer *H. rosa*, mites (*S. gibsoni*), protozoa, and the keystone predator mosquito larva, *W. smithii.* Adapted from Baiser et al. 2013.

My work in the *S. purpurea* ecosystem has demonstrated that state changes alter the function and structure of the microbial community. Betaproteobacteria are more abundant in the hypoxic state and the community switches from predominantly aerobic bacteria to predominantly facultative bacteria in oxic to hypoxic states, respectively. These shifts in taxa are also accompanied by shifts in metabolic pathways. Metaproteomic analysis of

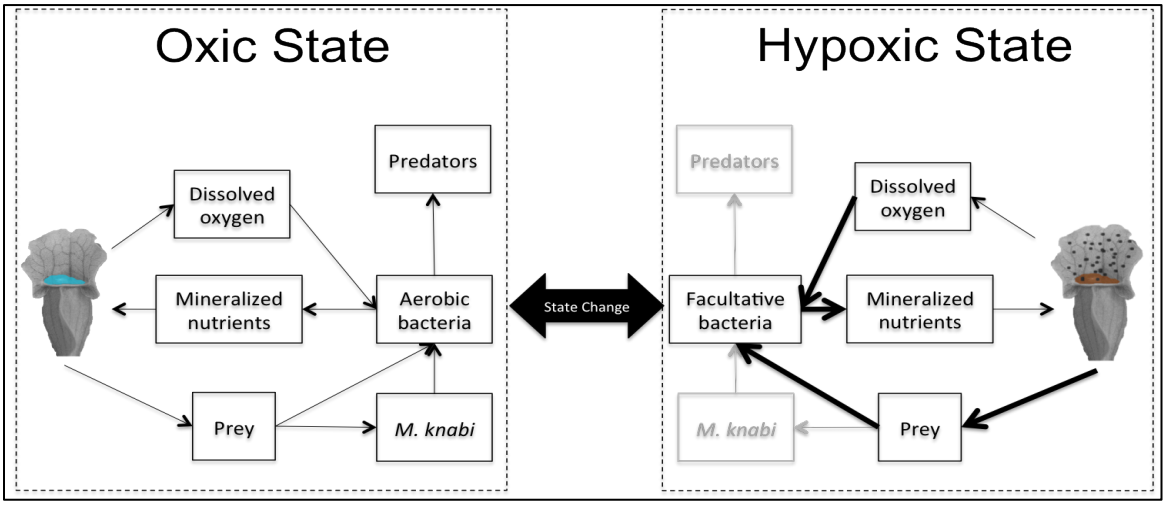

Figure 3. State change in the *S. purpurea* ecosystem. In the oxic state, insect prey drowns and is shredded by midge larvae, (*M. knabi)*. Aerobic bacteria decompose the shredded material and the plant takes up the resulting mineralized nutrients. Top predators prey on bacteria and each other. A single arrow is shown for dissolved oxygen, though all organisms consume oxygen, because bacteria are the main drivers of oxygen during the state change. During a state change, excess prey addition causes an increase in bacterial productivity and oxygen demand and oxygen is consumed more rapidly than it is returned to the pitcher fluid. The resulting hypoxia may cause the loss of *M. knabi* and predators.

the microbial communities in both states revealed several metabolic pathways that differ significantly in representation between states, including fatty acid biosynthesis and degradation, carbon fixation, and various amino acid metabolism pathways (unpublished data). Changes in microbial community structure and function indicate that it is possible to identify microbial proteins that can serve as biomarkers of state changes in the *S. purpurea* system.

The state change in the *S. purpurea* system is similar to state changes in larger aquatic systems in that an external driver causes increased productivity at the base of the food web, ultimately leading to the depletion of dissolved oxygen. As organic matter is added to pitcher fluid, microbial productivity, decomposition rates, and biological oxygen demand increase, depleting dissolved oxygen and switching the system to a hypoxic state (Figure 3). At low concentrations of regular organic matter loading, the dissolved oxygen time series "flickers" but returns to baseline; however, at high concentrations of regular organic matter loading, there is a dramatic decline in the concentration of dissolved oxygen to hypoxic levels (<1%)(Sirota et al. 2013). Though some of my preliminary experiments demonstrated that dissolved oxygen concentrations return to baseline if the addition of organic matter is halted, pitchers in those experiments received only intermediate-sized perturbations and dissolved oxygen never dropped below 5%, indicating that a state change did not occur (Figure 4 - unpublished data). We do not yet know if pitchers that undergo a state change recover from the hypoxic state, if the state change represents a shift between alternative stable states, and how the state change in alters the *S. purpurea* food web structure.

RESEARCH APPROACH

My general research approach is to use controlled greenhouse experiments and state-of-the-art proteomic techniques to investigate dynamics and identify protein biomarkers of state changes in the *Sarracenia purpurea* microecosystem. I will determine whether or not oxic and hypoxic states represent alternative stable states by testing for hysteresis and lasting changes in dissolved oxygen concentration and food web structure (Q1 & Q2). I will investigate how food web structure impacts the timing of and recovery from a state change using previously collected data from an experiment manipulating top predator counts (Q3). Finally, I will quantify changes in microbial protein expression and identify potential microbial protein biomarkers in a time series of protein samples using SDS-PAGE and quantitative proteomics methods (Q4 & Q5). Greenhouse experiments and field collection will be conducted at the University of Vermont (UVM) greenhouse and Molly Bog (Morristown, VT), respectively. All proteomic analyses will be conducted at UVM in collaboration with the Vermont Genetics Network Proteomics Facility.

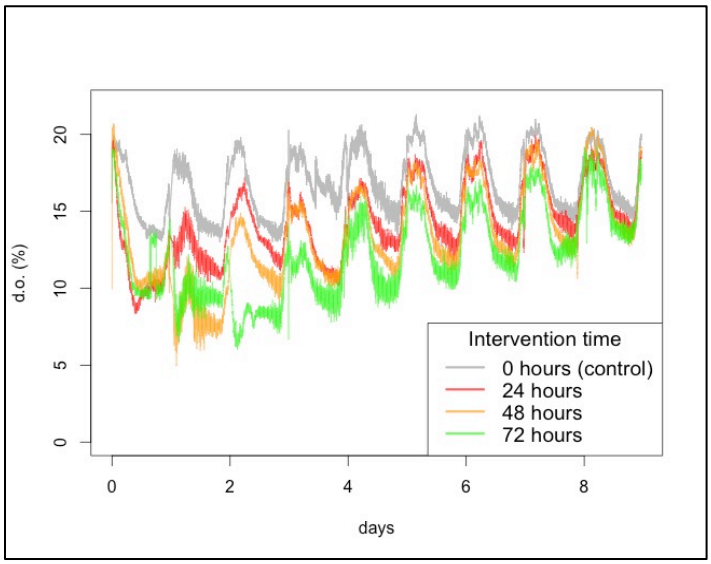

Figure 4. Dissolved oxygen time series in *S. purpurea* pitchers loaded with 0.50 mg/ml/day of organic matter as a function of intervention time, or the time at which organic matter enrichment was halted. Pitchers receiving no organic matter (gray line) show natural diurnal cycles of dissolved oxygen. Pitchers receiving organic matter and subsequent intervention show a decline in dissolved oxygen within 24 hours and a recovery to initial oxygen levels after 10 days.

Q1 & 2) Do oxic and hypoxic states in the *S. purpurea* ecosystem represent alternative stable states and how do state changes alter food web structure?

Although the theory behind alternative stable states is well established, there has been little direct evidence for alternative stable states from manipulation experiments in both natural and artificial ecosystems because the majority of studies fail to satisfy the criteria for demonstrating alternative stable states (Schröder et al. 2005). These criteria are that alternative states 1) occur at the same site, 2) have different, self-replacing communities, and 3) are caused by natural instantaneous (pulse) perturbations (Peterson 1984, Connell and Sousa 1983, Petraitis et al. 2009). The requirement for self-replacing communities implies that experiments must be carried out at least as long as the life span of the organisms comprising the studied ecosystem (Schröder et al. 2005). Furthermore, if experimental manipulations use continuous (press) perturbations to tip the system from one state to another, then the press perturbations should cease after the system has changed from one state to another so that alternative states are not externally maintained (Petraitis and Dudgeon 1999). Previous experiments in the *S. purpurea* system used press

perturbations of organic matter addition and assumed no organic matter loading in pitchers in the oxic state (Sirota et al. 2013). In the field, *S. purpurea* pitchers experience sporadic addition of organic matter throughout a season (Heard 1998, Newell & Nastase 1998).

Previous experiments in the *S. purpurea* system were conducted using a simplified version of the food web that included only the microbial community and excluded higher trophic levels. Though early theoretical ecology studies had conflicting results regarding the impact of diversity and connectivity of food webs on ecosystem stability (MacArthur 1955, May 1972), recent work has shown that predator-prey relationships stabilize ecosystems (Allesina and Tang 2012). Furthermore, positive feedbacks between trophic levels are known to stabilize alternative states in aquatic systems (Scheffer 2001). Therefore, experiments aimed at determining the stability of alternative states in the *S. purpurea* ecosystem should include as much of the food web as possible, especially predators.

A test for changes in food web structure and dissolved oxygen concentration.
To explore food web structure and stability of the oxic and hypoxic states, I will experimentally induce a state change in *S. purpurea* pitchers by enriching them with organic matter (driver) and determine ecosystem state by measuring dissolved oxygen concentration (state variable) in each pitcher in a greenhouse experiment over the course of 30 days. Unlike previous experiments, all pitchers will include a complex food web with multiple trophic levels and a baseline addition of organic matter to mimic natural conditions. Organic matter loading will serve as a treatment and levels will include the following: 1) no organic matter loading (unmanipulated control), 2) baseline organic matter addition (unperturbed), and 3) baseline organic matter loading with a pulse input of organic matter (perturbed). The unmanipulated treatment will act as a reference to ensure that the unperturbed treatment does not undergo a state change and remains in the oxic state. The perturbed treatment should result in a change to the hypoxic state and the pulse input will ensure that the hypoxic state is not externally maintained. To ensure similar starting conditions in all pitchers, pitcher fluid containing bacteria, protozoa, rotifers, and invertebrate larvae will be collected from pitchers at Molly Bog (Morristown, VT), filtered to remove macroinvertebrates, and homogenized and added to experimental pitchers. To control for the abundance of higher trophic level organisms, macroinvertebrates will be added to experimental pitchers in counts consistent with average counts for pitchers in Molly Bog. Pitchers will be randomly assigned to treatments and all pitchers in the unperturbed and perturbed treatments will receive 0.10 mg of autoclaved ground wasp per milliliter of pitcher fluid per day for the duration of the experiment. Pitchers loaded with 0.10mg/ml/day did not undergo a state change in previous experiments. Pitchers in the perturbed treatment will receive a pulse of 1.0 mg/ml of pitcher fluid of autoclaved ground wasp on day seven. Dissolved oxygen and temperature will be measured in pitchers continuously throughout the course of the experiment. To quantify changes in food web structure, I will count rotifers (*Habrotrocha rosa)* and insect larvae (*Wyeomyia smithii, Fletcherimyia fletcheri,* and *Metriocnemus knabi*) at the beginning and end of the experiment. I will calculate species richness of the non-microbial organisms in all replicate pitchers, and use t tests to determine if species

richness is significantly different between the two states. If there are alternative stable states, dissolved oxygen in perturbed pitchers should plummet to near 0% and remain close to 0% for the duration of the experiment and there should be significant and lasting changes to food web structure.

A test for hysteresis.

There is some argument as to whether or not hysteresis is a necessary condition for alternative stable states; however, the presence of hysteresis is strong evidence for alternative stable states (Scheffer et al. 2001, Beisner et al. 2003). To test for hysteresis in the *S. purpurea* system, I will compare the relationship between the mass of organic matter in pitchers and dissolved oxygen concentration for forward and reverse shifts between states (Figure 5). I will experimentally manipulate pitchers by enriching them with whole ants in a press experiment and measure dissolved oxygen of the pitcher fluid in each pitcher over the course of three, 20-day blocks. At the start of the experiment, 20 pitchers containing intact food webs will be randomly assigned to an unmanipulated (no organic matter loading) or enriched treatment (organic matter loading). Pitchers in the enriched treatment will receive a single autoclaved ant each day for the first ten days and no ants for the last ten days. Every two days, I will sacrifice two pitchers, remove any ants, and record dissolved oxygen in each pitcher. Removed ants will be air-dried for two days and weighed to determine the mass of organic matter left in each pitcher (see Baiser et al. 2011). To statistically test for differences between forward and reverse shifts between states, I will fit smoothed sigmoidal curves to the data comprising the two shifts, calculate the time corresponding to their inflection points, and use t-tests to determine significant differences in inflection point times. If there is hysteresis in the system, data collected during the reverse shift (after day ten) should show a response curve significantly shifted to the left of the forward shift (before day ten) response curve.

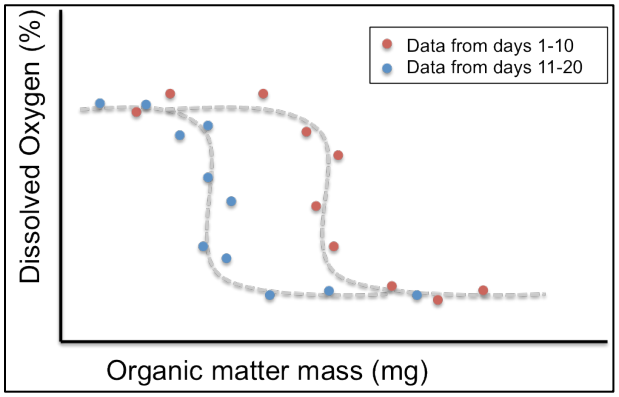

Figure 5. Expected response curves for pitchers in forward and reverse shifts between oxic and hypoxic alternative stable states. Red dots represent data from the forward shift and blue dots represent data from the reverse shift.

Q3) How does food web structure influence timing of and recovery from state changes?

While a state change may alter food web structure, it is also possible that food web structure can alter the dynamics of state changes in the *S. purpurea* system. In larger aquatic systems, the removal of higher trophic levels can reduce the resilience of an ecosystem, making it more vulnerable to state changes (Folke 2004). Studies in the *S. purpurea* system show that top-down regulation by the keystone predator *Wyeomyia smithii* (mosquito larva) negatively affects the density of rotifers and protozoa (Hoekman 2007); however, these studies show conflicting results regarding the impact of *W. smithii* on the abundance of bacteria (Kneitel & Miller 2002, Hoekman 2007). Nevertheless, these studies suggest that the presence of *W. smithii* may buffer against state changes in

the *S. purpurea* system by limiting the density of intermediate and lower trophic levels and therefore limiting biological oxygen demand. Preliminary results from a pilot study in which I varied counts of *W. smithii* in pitchers suggest that the predator may impact state change dynamics. Though the timing of the state change was not altered, there was a trend for post-state change dissolved oxygen concentration to increase with increasing *W. smithii* density, indicating that *W. smithii* abundance could alter recovery time (Figure 6). However, the pilot experiment excluded intermediate trophic levels, which may be important in top-down regulation of the bacterial community. I propose to repeat this experiment with an intact food web, altering only the abundance of *W. smithii* in pitchers (0, 5, 10, 15, or 20 larvae per pitcher), to determine the impact of keystone predator abundance on the timing of and recovery from a state change in the *S. purpurea* system. I will use the cpt.mean and cpts functions of the *changepoint* package in R to determine the timing of the state change in all replicates and use an ANOVA test to determine if the timing of state changes differs between treatments. Additionally, I will fit linear models to the post-state change portions of the dissolved oxygen time series and use an ANOVA test to determine if the slope of the models, a proxy for recovery time, differs between treatments.

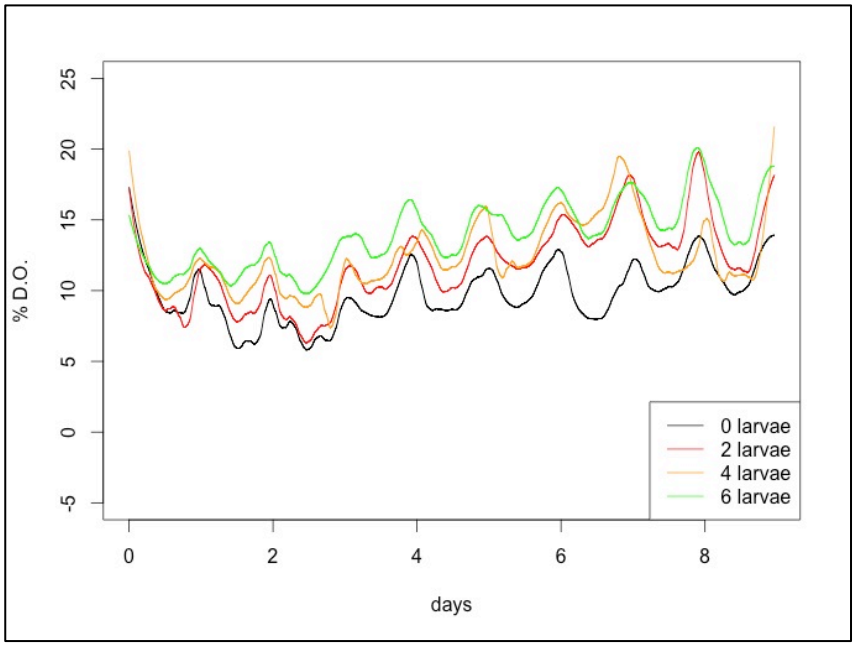

Figure 6. Average dissolved oxygen time series for pitchers loaded with organic matter containing 0, 2, 4, or 6 *W. smithii* mosquito larvae. There is a trend after ~24 hours for pitchers containing higher abundances of *W. smithii* to have higher concntrations of dissolved oxygen, indicating top-down control of bacteria and therefore biological oxygen demand of bacteria.

Q4 & Q5) How does microbial protein expression change as an ecosystem undergoes a state change and can changes in microbial protein expression serve as reliable biomarkers of an impending state change in aquatic ecosystems?

Successful identification of reliable biomarkers requires three major steps: 1) Screening for candidate proteins that change in abundance between states, 2) Quantification and statistical verification of changes in abundance in candidate proteins, and 3) Validation of the biomarker as useful for prediction (Rifai et al. 2006). As noted above, protein expression differs between microbial communities in oxic and hypoxic states in the *S. purpurea* ecosystem (unpublished data). However, the data are based on samples taken from multiple pitchers in the field and the microbial community of pitchers can vary

between plants and among pitchers on the same plant (Gray et al. 2012). To control for differences in microbial communities, I conducted an experiment in which pitchers were rinsed with deionized water and filled with homogenized pitcher fluid from multiple plants in the field. These pitchers were enriched with organic matter every 8 hours for 24 hours or not enriched and the microbial community was sampled every 2 hours. I will use SDS-PAGE to identify microbial proteins in the enriched pitchers that appear to increase or decrease in abundance relative to the same proteins in unmanipulated pitchers, especially in the first 8 hours. In pilot experiments, the state change occurred within 24 hours (unpublished data). Once I have identified candidate proteins, I will use tandem mass spectrometry and AQUA peptide analysis (Gerber et al. 2003) to quantify their change in expression. Finally, I will conduct a proof-of-application experiment to validate candidate biomarkers in which pitchers are enriched with organic matter while the abundance of candidate proteins is monitored using SDS-PAGE and comparison to known standards such as bovine serum albumin. If candidate proteins are reliable biomarkers, then intervention (halting organic matter loading) based on candidate protein expression should be successful and a state change should not occur in enriched pitchers.

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