


LETTER

Clockwise and counterclockwise hysteresis characterize state changes in the same aquatic ecosystem

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Abstract

Incremental increases in a driver variable, such as nutrients or detritus, can trigger abrupt shifts in aquatic ecosystems that may exhibit hysteretic dynamics and a slow return to the initial state. A model system for understanding these dynamics is the microbial assemblage that inhabits the cup-shaped leaves of the pitcher plant *Sarracenia purpurea*. With enrichment of organic matter, this system flips within three days from an oxygen-rich state to an oxygen-poor state. In a replicated greenhouse experiment, we enriched pitcher-plant leaves at different rates with bovine serum albumin (BSA), a molecular substitute for detritus. Changes in dissolved oxygen (DO) and undigested BSA concentration were monitored during enrichment and recovery phases. With increasing enrichment rates, the dynamics ranged from clockwise hysteresis (low), to environmental tracking (medium), to novel counter-clockwise hysteresis (high). These experiments demonstrate that detrital enrichment rate can modulate a diversity of hysteretic responses within a single aquatic ecosystem, and suggest different management strategies may be needed to mitigate the effects of high vs. low rates of detrital enrichment.

Keywords

Aquatic ecosystem, ecosystem recovery, enriched ecosystems, hysteresis, model ecosystems, nonlinear dynamics, regime shifts, *Sarracenia purpurea*.

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INTRODUCTION

Anthropogenically enriched ecosystems often exhibit complex dynamics and regime shifts (Scheffer *et al.*, 2001). Such shifts occur when incremental changes in a driver variable suddenly tip these systems from one basin of attraction to another (Lewontin, 1969; Holling, 1973; May, 1977; Scheffer, 2009a,b, 2001). Aquatic ecosystems that collapse rapidly often recover slowly (Meijer, 2000; Diaz and Rosenberg, 2008; Sand-Jensen *et al.*, 2016) and may remain in an altered state long after enrichment has ceased. A general mechanism that might cause such a lag is hysteresis—a phenomenon in which the relationship between a response variable and driver variable depends on the state of the system (Scheffer, 2009b; Litzow and Hunsicker, 2016). In systems with hysteresis, changes in the response variable can lag behind those in the driver variable due to feedback loops between the response variable and other components of the system. Alternately, systems may display a reversal of the hysteresis loop (Grigg and Seebacher, 1999), such that changes in the state variable are proportionally larger than changes in the driver during recovery (Fig. 1). However, such idealized responses are rarely seen in nature, where it can be challenging to definitively isolate the effects of a single driver variable on regime shifts.

In spite of the popularity of models of hysteresis and alternative stable states (Beisner *et al.*, 2003; Schröder *et al.*, 2005), only a handful of studies have manipulated driver variables in controlled experiments to test for hysteretic responses (Faassen *et al.*, 2015; Garnier *et al.*, 2019). Even these studies have relied on highly artificial assemblages of only 2 or 3 species. In nature, the limitations to studying hysteresis are the long

time frame over which recovery occurs (Isbell *et al.*, 2013) relative to the generation times of the component species (Bestelmeyer *et al.*, 2011), the lack of constant background conditions (such as global temperature) over these time frames, and the challenge of replicating whole-ecosystem measurements (Schindler, 1998; Litzow and Hunsicker, 2016).

In this study, we asked whether hysteretic responses could be generated in a realistic multi-species assemblage by manipulating only a single driver variable and tracking the behavior of an ecosystem response as the state variable. We conducted a greenhouse experiment using the microecosystem that is found within the cup-shaped leaves of the northern pitcher plant, *Sarracenia purpurea*. This system is a good one for testing for hysteretic responses because it meets several of the criteria proposed for recognizing alternative stable states (Schröder *et al.*, 2005; Ellison and Gotelli, 2021). The pitchers of *S. purpurea* are cup-shaped leaves that fill with rainwater and capture arthropod prey (Cresswell, 1991), which forms the base of a ‘brown’ detrital food web (Butler *et al.*, 2008). Detrital enrichment is the driver variable and oxygen level is the state variable.

Like much larger aquatic ecosystems, the *Sarracenia* microecosystem is normally in an oligotrophic state, with high levels of dissolved oxygen (DO). But if it is loaded with excess prey or detritus, it will collapse quickly (1–3 days) to a low-oxygen (anoxic) state and then recover slowly (10 to *c.* 35 days). The low- and high-oxygen states have a bimodal frequency distribution (Sirota *et al.*, 2013), and protein expression and microbial diversity are very different in the two states (Northrop *et al.*, 2017). Except for the compressed time scale, this trajectory of enrichment, sudden collapse of DO, and slow recovery

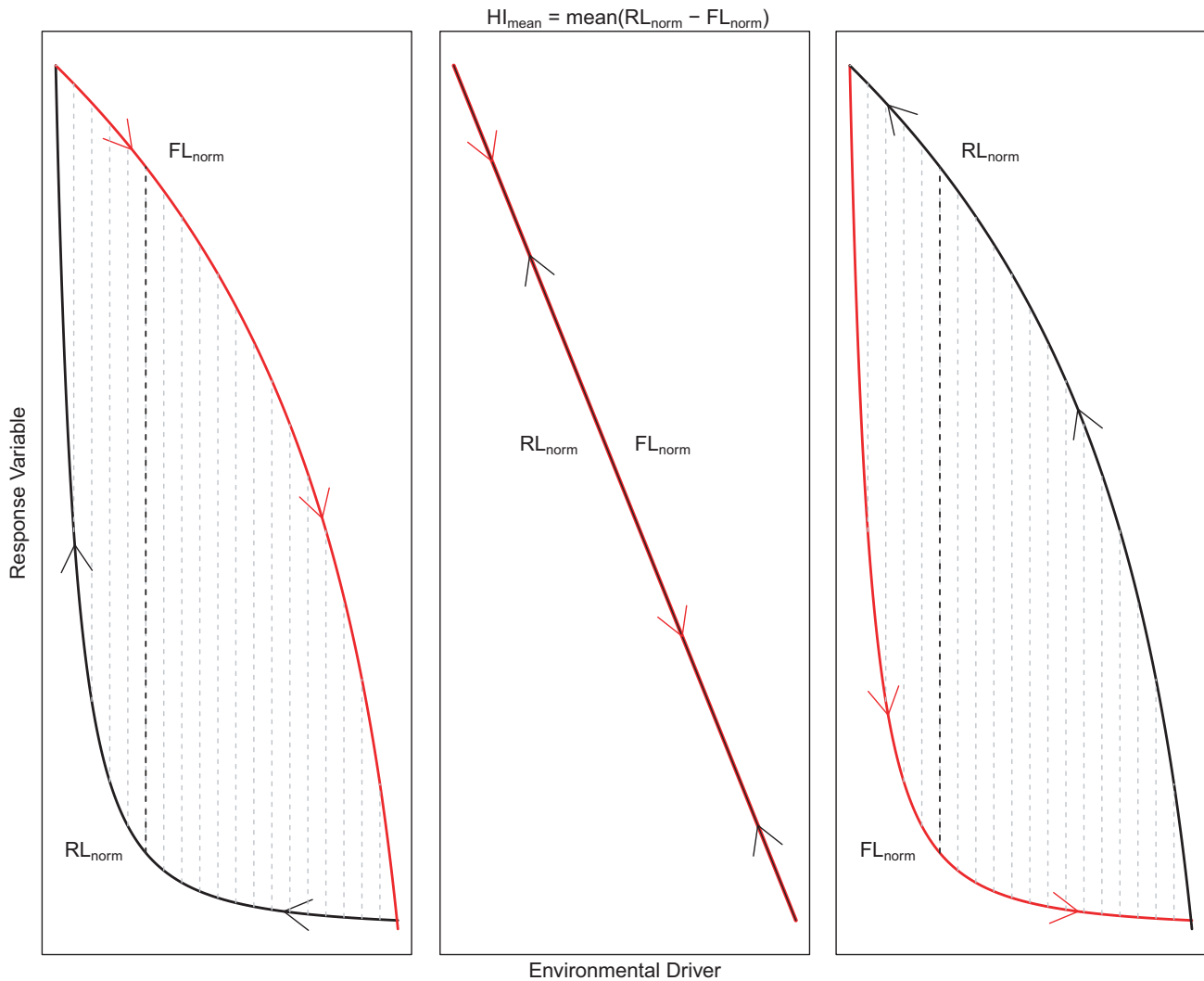


Figure 1 Idealized hysteresis loops in driver-response relationships during enrichment (red lines) and recovery (black lines). Clockwise hysteresis (left panel) ($HI_{MEAN} < 0$), counterclockwise hysteresis (right panel) ($HI_{MEAN} > 0$), and environmental tracking (center panel) ($HI_{MEAN} = 0$). HI_{MEAN} is the average of the difference between the rising limb and falling limb across measured intervals (dashed lines). Compare to empirical data in Figure 3.

characterizes both photosynthetic ‘green’ food webs that experience direct nutrient enrichment and tightly linked ‘brown’ food webs enriched with organic matter from the decomposition of primary-producer biomass (Zou *et al.*, 2016).

Our experiments revealed that differences in the nutrient enrichment rate can generate a surprisingly rich array of recovery dynamics in the same aquatic ecosystem.

MATERIAL AND METHODS

The *Sarracenia* microecosystem

Sarracenia purpurea occurs in peat bogs and sand plains of the eastern U.S. from northern Florida to Canada, and west to the Canadian Rockies (Ellison and Gotelli, 2021). Within this large geographic range, the rain-filled leaves of the plant support an aquatic food web that rapidly assembles, consisting of a prey detrital base, shredding and filter-feeding aquatic

arthropods, and a diverse array of protozoa and microbes. The drowned prey initially are shredded by aquatic larvae of sarcophagid flies and midges, but the complete breakdown and mineralization of the prey is predominantly the result of microbial activity (Butler *et al.*, 2008). The mineralized nutrients are quickly assimilated and translocated to plant tissues (Butler *et al.*, 2008). In the field, less than 1% of prey encounters result in successful capture (Heard, 1998; Newell and Nastase, 1998). With low detrital inputs and active photosynthesis by the plant, this aquatic microecosystem is normally in an oligotrophic state, with low prey abundance and dissolved oxygen (DO) close to 20% (where 21% = 100% air saturation). But with excessive loading of prey or detritus, DO collapses within 8 h to less than 5% (Sirota *et al.*, 2013). With no additional detrital enrichment, the system persists in this anoxic eutrophic state for many days or weeks until the excess prey is transformed and DO slowly recovers (Sirota *et al.*, 2013).

Experimental setup

Greenhouse experiments were conducted, starting 6 July 2015 (low and high enrichment treatments) and 3 July 2016 (intermediate enrichment treatment), at the University of Vermont's Biological Research Complex in a temperature-controlled greenhouse. The only difference in methods between the two years was that pitchers in 2016 were monitored until DO fully recovered, whereas the pitchers in 2015 were followed for only 35 days after enrichment. However, for our analyses, we used only the first 35 days of data from the 2016 plants, so the data from both years are strictly comparable. Moreover the critical result of clockwise and counterclockwise dynamics was obtained from the low and high enrichment treatments that were both conducted in 2015.

Recently fully formed pitchers greater than 8 ml in volume were randomly chosen and assigned to control and enrichment treatments. Plants were randomly placed on a greenhouse bench to account for spatial variation in greenhouse conditions. All pitchers were rinsed twice with reverse-osmosis water and allowed to dry overnight prior to the start of the experiment. Pitcher fluid was collected from Molly Bog (44.50°N 72.64°W), an ombrotrophic bog in Morristown, VT, USA. Fluid was collected randomly from multiple plants in the field using a sterile pipette and transported immediately to the greenhouse where it was passed through the 30- μ m frit bed of a chromatography column (BioRad, Hercules, CA) to remove microbes. The filtered fluid was mixed evenly and added to experimental pitchers. Although natural pitchers also contain a food web of macro-invertebrates that are controlled by both top-down (Gotelli and Ellison, 2006) and bottom-up (Kneitel and Miller, 2002) and bottom-up interactions, we did not include them in these experiments. Because the macroinvertebrate assemblage varies widely between pitchers and between times, we did not want to add additional heterogeneity to the system. More important, the rate of mineralization of detritus and prey is strongly controlled by the microbes, and the elimination of the entire macro-invertebrate assemblage has no effect on the rate of prey break-down and nutrient transfer to the plant (Butler *et al.*, 2008).

Organic matter loading

In this study, we used bovine serum albumin (BSA) as a molecular substitute for arthropod prey. BSA is a soluble protein that is well-studied, relatively inexpensive and has been used previously in experiments as a carbon and energy source for microbial communities (Tang *et al.*, 2005). Because BSA is water-soluble, we could easily monitor the concentration of unprocessed BSA with a non-destructive Bradford assay (Bradford, 1976). Moreover, BSA is colorless, so it does not introduce a confounding shading effect that is often associated with nutrient enrichment from sediment loading in natural systems. Pilot experiments confirmed that collapse in DO after BSA addition mimics collapse after arthropod prey addition (Fig. S1) and that BSA is stable in an aqueous solution and does not break down in the absence of microbial activity (Fig. S2).

We created a BSA cocktail that has the same nutrient content and stoichiometric ratios as natural insect prey (Northrop *et al.*, 2017). The BSA cocktail included the trace elements potassium (KCl), calcium (CaCl₂), sodium (NaCl), magnesium (MgSO₄) and manganese (MnCl₂) in the following ratio: 1: 0.115: 0.044: 0.026: 0.0001. This ratio is similar to the elemental composition of royal jelly and adult honeybees (Nation and Robinson, 1971; Stocker *et al.*, 2005) and most closely represents trace-element ratios in bald-faced hornets. We also added 1.5 μ g of DNA sodium salt from salmon testes (Sigma) per mg of BSA based on DNA yields between 1.4 and 1.5 μ g/mg from *Apis mellifera* (Reineke *et al.*, 1998). The BSA cocktail was pre-made for each pitcher, filtered through a sterile 0.2- μ m filter, stored in sterile 1-ml microtubes and frozen at -20 °C until loaded into pitchers. The BSA cocktail was introduced to pitchers using sterile 8-mL transfer pipettes and mixed into pitchers by drawing pitcher fluid in and out of the pipette three times. We were careful to limit the amount of air deposited by and introduced into the transfer pipettes during loadings so that DO measurements would be minimally affected. Control pitchers received sham loadings in which pitcher fluid was drawn into and deposited from a transfer pipette to account for the effect of mixing. All pitchers were topped off with reverse-osmosis water after enrichment/sham loadings.

Pitchers were enriched with 5.0 (high concentration) or 0.5 (low concentration) mg of organic matter per mL of pitcher fluid in 2015 and 2.0 mg/mL (intermediate concentration) in 2016. For each experiment, a set of control pitchers received no organic matter. These controls were not used to calculate hysteresis indices but to only determine when oxygen levels in experimental pitchers had recovered. Experimental pitchers were loaded with organic matter (between 9:00 am and 9:45 am) following pitcher-fluid sampling and DO measurement for the first four days of the experiment.

Data and sample collection

We measured DO twice a day each day—at 8:30 am and 5:00 pm (\pm 2 h)—from day 0 to day 20, once per day—at 8:30 am (\pm 2 h)—from day 20 to day 28, and once on Days 30, 31, 33 and 35 (8:30 am \pm 2 h). DO (expressed as a percent, where 21% = 100% air saturation), was measured using a D-166MT-1S microelectrode (Lazar Research Laboratories).

The microelectrode was calibrated prior to each sampling event according to the manufacturer's instructions. A single microelectrode was used to take readings from each pitcher and was rinsed twice with reverse osmosis water between readings. The order of readings from different replicates was randomized so that changes in temperature and sunlight over the sampling period were not confounding factors. During readings, the microelectrode was placed 2.5 cm below the surface of the pitcher fluid and swirled so that the more oxygen-rich pitcher fluid at the top of the pitcher was mixed and readings reflected average DO. Due to the sensitivity of the microelectrode, readings were taken as soon as the reader settled on a value for more than 10 s.

Pitcher fluid was sampled following each DO measurement for spectrophotometric analysis using a Bradford assay

(Bradford, 1976). Using sterile pipette tips, a 300- μ L aliquot was taken from 2.5 cm below the surface of each pitcher and placed in a sterile 1-mL microfuge tube. Sample tubes were immediately transported to the laboratory where they were centrifuged at 13 000 g for two minutes. The supernatant containing soluble BSA was removed, placed in a sterile 1-mL microfuge tube and stored at -80°C until analyzed.

BSA loading validation via SDS-PAGE

To validate that the majority of protein in extracted pitcher fluid was BSA, we ran a time series of pitcher fluid from two replicates each of the high- and low-concentration loading treatments on gels using SDS-PAGE next to known concentrations of BSA (0.1, 0.5 and 5.0 mg/ml) and a BenchMark Pre-stained Protein Ladder (Invitrogen). Ten- μ L aliquots of pitcher fluid from days 0, 2, 4, 6, 9, 12, 15, 18, 21, 24, 27 and 30 were added to 90 μ L of bromophenol blue sample buffer (150 mM Tris pH 6.8, 2% SDS, 5% beta-mercaptoethanol, 7.8% glycerol) and boiled at 95°C for five minutes. After centrifugation at 13 000 g for 30 s, 10 μ L of each sample was mixed with 10 μ L of sample buffer and loaded into separate lanes of a 10% polyacrylamide gel (37.5:1 acrylamide:bis-

acrylamide). Gels were subjected to SDS-PAGE and stained with Coomassie.

Bradford assay

We used a Bradford assay (Bradford, 1976) to determine the concentration of BSA. Bradford assays were done using diluted samples after generating a standard curve with known amounts of BSA. Absorbance was measured using a Biophotometer Plus (Eppendorf) at an optical density of 600 nm. To streamline 2016 BSA concentration data collection, assays were conducted on a 96-well plate. Absorbance was measured at 545 nm with a Synergy HTX (Biotek).

Data analysis

We used R software (version 3.4.3) running within RStudio (v1.1.442, RStudio, Boston, MA, USA) to plot and analyze the data. We plotted the hysteresis loops using the loess function to fit curves (span = 0.75) and 95% confidence intervals to each of the interpolated enrichment and recovery trajectories. For this study, we described loop directions as 'counter-clockwise' and 'clockwise' based on a response variable that

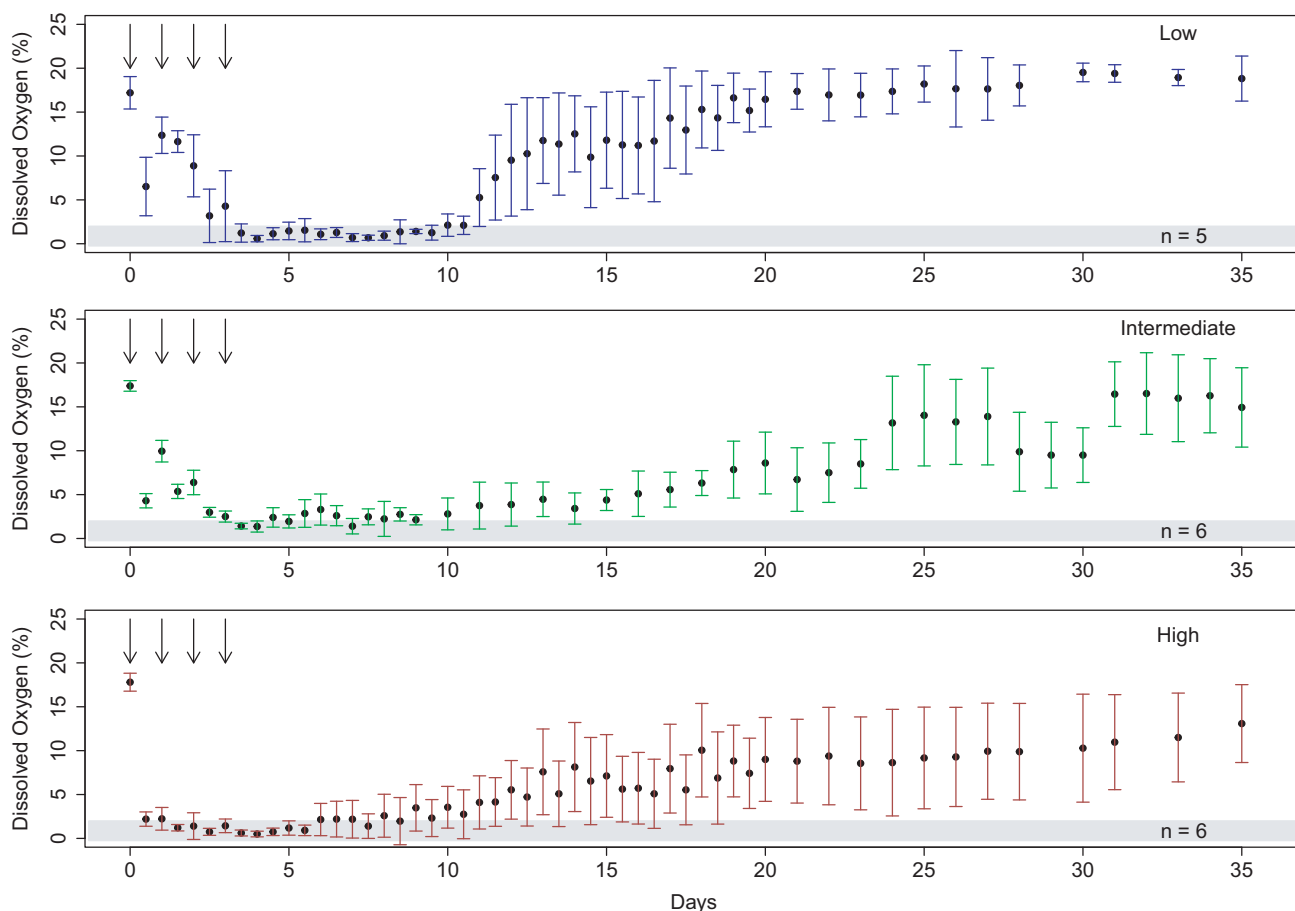


Figure 2 Addition of bovine serum albumin (BSA) to replicate pitchers causes hypoxia at all levels of enrichment greater than or equal to 0.05 mg BSA/ml pitcher fluid. Time series of average dissolved oxygen (DO) from all replicate pitchers in low, intermediate and high BSA addition treatments. Error bars represent one standard deviation of the mean. Vertical arrows represent the timing of BSA additions. Gray bands denote the range of DO considered hypoxic (<2%).

takes on high values at low levels of an environmental driver and decreasing values as the driver increases (Fig. 1). We calculated hysteresis indices (HI_{MEAN}) using the method proposed by Lloyd *et al.* (2016); however, we used loess-interpolated values. In our system, a negative value for Lloyd *et al.*'s (2016) index denotes a clockwise hysteresis loop while a positive value denotes a counter-clockwise loop. HI_{MEAN} was compared among treatments using a one-way analysis of variance (ANOVA), followed by a Tukey's *post hoc* test to compare all individual pairs of treatments. Results were qualitatively the same when we skipped the normalization step and plotted the hysteresis loops on untransformed axes (Fig. S3).

RESULTS

Within 5 d after the start of enrichment, DO in all but one enriched pitcher had collapsed to hypoxic levels ($\leq 2\%$) (Fig. 2). The average time to collapse differed significantly among the three enrichment treatments ($F_{2,14} = 14.38$,

$P = 0.0004$). Pitchers receiving low levels of enrichment collapsed more slowly ($\bar{x} = 60.0$ h) than pitchers receiving intermediate ($\bar{x} = 80.0$ h) and high levels of enrichment ($\bar{x} = 26.0$ h). After enrichment was halted, DO recovered gradually for another 30 days in treated pitchers until the experiment was halted on day 35 (Fig. 2).

There was variation in mean DO near the end of the experiment, especially in intermediate and high BSA treatments, due to the failure of pitchers in the intermediate (4 pitchers) and high (5 pitchers) BSA treatments to reach control DO levels by Day 35 ($\chi^2 = 8.30$, d.f. = 2, $P = 0.016$; Fig. 2 and Fig. S4). Using the terminal date of the experiment for pitchers that did not fully return to control DO levels (a conservative estimate), there was a significant difference ($F_{2,14} = 18.67$, $P = 0.0001$) among the treatments in recovery time. Pitchers enriched with low levels of BSA recovered more rapidly ($\bar{x} = 199.2$ h) than pitchers enriched with intermediate levels ($\bar{x} = 388.0$ h) and high levels ($\bar{x} = 426.0$ h) of BSA. In all treatments, there was a substantial reservoir of undigested

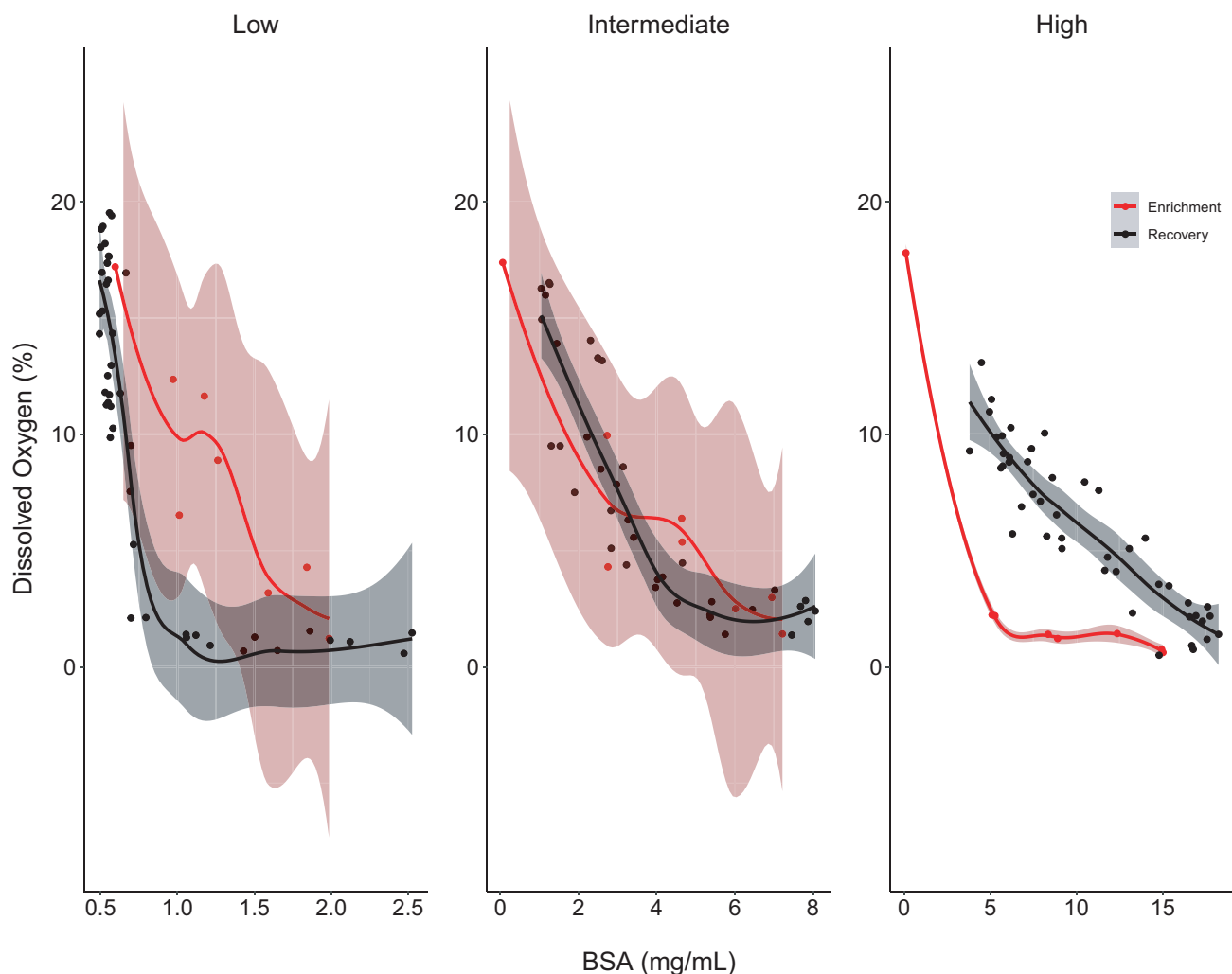


Figure 3 Altering a single driver variable, bovine serum albumin (BSA), elicits counterclockwise and clockwise hysteresis in an aquatic ecosystem. Local regression (loess) curves (span = 0.75) fitted to mean state-space plots in low, intermediate, and high BSA addition treatments with 95% confidence intervals. Red and black points, lines, and shading denote data from the enrichment and recovery phases respectively. Compare to idealized curves in Figure 1.

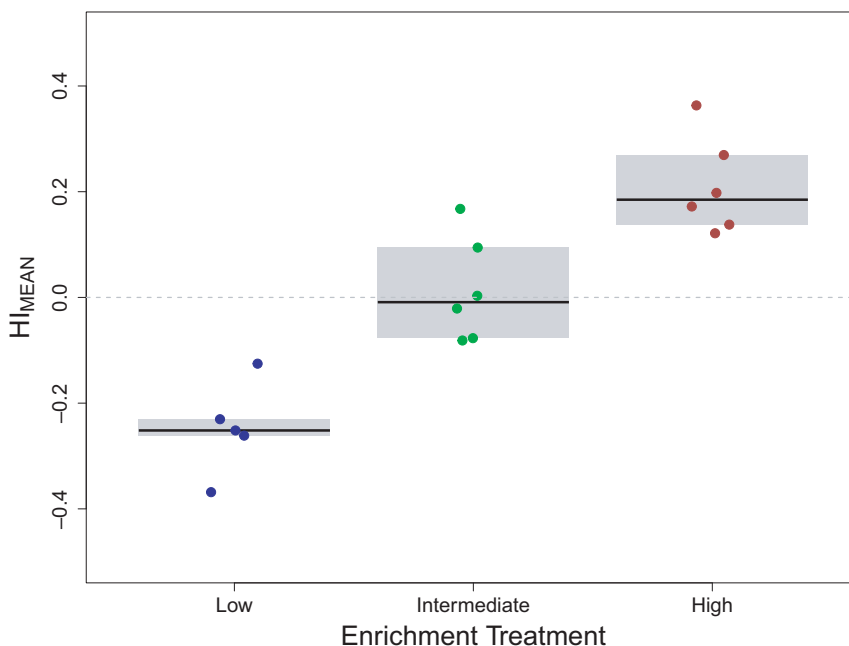


Figure 4 Strength and direction of hysteresis, indicated by HI_{MEAN} , was significantly different between all enrichment treatments (ANOVA, $F_{2,16} = 35.26$, $P < 0.001$; Tukey HSD, $P < 0.001$). HI_{MEAN} describes the magnitude of hysteresis (from 0 to 1, where 0 = no hysteresis) and direction (negative = clockwise, positive = counterclockwise). Blue, green and red dots represent HI_{MEAN} of replicates experiencing low ($n = 5$), intermediate ($n = 6$), and high ($n = 6$) levels of enrichment respectively. Solid horizontal lines represent mean HI_{MEAN} values in each treatment and the dotted horizontal line denotes where $HI_{MEAN} = 0$.

BSA remaining in pitchers after the cessation of enrichment that persisted for weeks (Fig. S5).

HI_{MEAN} was significantly different between all enrichment treatments (ANOVA, $F_{2,16} = 35.26$, $P < 0.001$). Moreover the hysteresis index differed significantly between each of the three unique pairs of treatments (Tukey's HSD, $P < 0.001$). Pitchers receiving a low rate of BSA enrichment displayed a clockwise hysteresis loop in the relationship between BSA concentration and DO, with a lag in DO recovery relative to decreasing BSA concentration during the recovery phase ($HI_{MEAN} = -0.247$; Figs 3 and 4, Figs S3 and S6). Hysteresis was absent at intermediate levels of enrichment: enrichment and recovery curves overlapped, suggesting a responsive tracking of BSA concentration by DO ($HI_{MEAN} = -0.014$; Figs 3 and 4, Figs S3, and S6). High levels of BSA enrichment yielded a counterclockwise hysteresis loop: DO changed faster per unit change in BSA concentration during recovery than enrichment ($HI_{MEAN} = 0.210$; Figs 3 and 4, Figs S3 and S6).

DISCUSSION

We have shown that an enriched aquatic ecosystem can display a diverse set of hysteretic responses modulated by changes in a single driver variable. At low levels of enrichment, there was a strong clockwise hysteresis loop in which recovery of oxygen lags behind changes in BSA. In contrast, high levels of enrichment yielded a counter-clockwise loop. Counter-clockwise hysteresis has been detected in some physical and biological (Grigg and Seebacher, 1999; Hu *et al.*,

2012; Louizos *et al.*, 2014) systems, but has rarely been documented in ecological studies. The few ecological studies with counterclockwise hysteresis loops quantify static patterns of hydrological relationships along a spatial gradient, rather than measuring ecosystem dynamics and changes through time (Whitfield and Schreier, 1981; Huang *et al.*, 2017).

It is worth noting that HI_{MEAN} describes hysteresis as a single number for each replicate ecosystem, but does not characterize variation among time points within a replicate. In the low enrichment treatment, confidence intervals for the loess fit are nearly overlapping; however, both high and low enrichment treatments differ in sign but have HI_{MEAN} values of similar magnitude. When all replicates are normalized and pooled within treatments (Fig. S7), a single HI_{MEAN} for each treatment still exhibits the same rank order as in the analysis with proper ecosystem replication: low < intermediate < high ($-0.021 < 0.063 < 0.184$).

An analytical systems model of the *S. purpurea* ecosystem predicts clockwise hysteresis as a result of smooth changes in photosynthesis coupled with an abrupt increase in biological oxygen demand (BOD) (Lau *et al.*, 2018). Indeed, the addition of organic matter to pitchers causes an abrupt increase in BOD resulting from decomposition (Sirota *et al.*, 2013; Lau *et al.*, 2018) by carbon-limited (Gray *et al.*, 2006) bacteria. Pitchers in all enrichment treatments saw a rapid decline in DO following the initial enrichment phase, suggesting that BOD increased rapidly (Fig. 2). This rapid change in BOD may have contributed to clockwise hysteresis at low levels of enrichment, but does not account for the counterclockwise hysteresis at high enrichment levels.

In other systems, counter-clockwise hysteresis is generally the result of positive feedback loops (Kéfi *et al.*, 2016). In our system, positive relationships exist between plant photosynthesis and DO, bacterial abundance and BSA, and potentially between the abundance of facultatively anaerobic bacteria and DO. A minimal model of the system would include such variables as the abundance of aerobic and anaerobic bacteria and the concentration of DO and BSA.

The magnitude of hysteresis is influenced by changes in the strength of feedbacks underlying the hysteresis response (Garnier *et al.*, 2019). We hypothesize that the changes in magnitude and direction of hysteresis in our system are a result of changing feedback strengths between bacterial abundance, DO and BSA concentration. Although we did not measure bacterial abundance, we suspect there were dramatic increases because pitcher fluid in enriched pitchers was cloudy and brown after enrichment. Complex hysteretic dynamics may also reflect changes in microbial composition and function; indeed, ongoing proteomic assays reveal increases in protein expression in facultative anaerobes as a result of BSA loading (in prep).

Hysteretic ecosystems may require larger restoration efforts to recover from a regime shift (Mayer and Reitkerk, 2004). For example, in eutrophic shallow lakes, a simple reduction in phosphorus input does not lead to a proportional recovery in macrophyte cover (Meijer, 2000) or community structure (Sand-Jensen *et al.*, 2016). Furthermore, these communities do not fully recover in the time frames in which they are studied (Sand-Jensen *et al.*, 2016) and may effectively remain permanently degraded. These examples and our work highlight the importance of applying a dynamic regime concept (Mayer and Reitkerk, 2004) to ecosystem management and restoration. Such an approach would include testing for hysteresis, characterizing feedbacks that maintain undesirable regimes, and identifying if and how system variables change as a result of a regime shift (Suding *et al.*, 2004).

In ecosystems where hysteresis is counter-clockwise, rapid reduction in a driver variable from high to low levels may be a successful restoration strategy. In contrast, systems that have experienced chronic low levels of enrichment may exhibit clockwise hysteresis that requires more extreme reductions of the driver variable, or alternative restoration strategies (Suding *et al.*, 2004), to restore. Our work highlights the importance of understanding how past histories of high versus low enrichment may dictate different restoration strategies for collapsed ecosystems.

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AUTHORSHIP

A.C.N., N.J.G, A.M.E. and B.A.B conceived the experiment. A.C.N. and V.A. conducted the experiments. A.C.N. analyzed the results. A.C.N. and N.J.E. authored the first draft and all authors contributed to revisions.

DATA ACCESSIBILITY STATEMENT

Data will be made available on Dryad upon publication. Additionally, all code and data generated during or analysed during the current study are available from the Harvard Forest Data Archive under ID Number HF334 (<https://doi.org/10.6073/pa/sta/ebf6b175a6f6e44d3e9747c13f0d376c>). Data DOI: <https://doi.org/10.5061/dryad.280gb5mnv>

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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