Seasonal succession influences the functioning of a host-associated microbial food web

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ABSTRACT

Ecosystem development theory predicts that successional turnover in community composition can influence ecosystem functioning. However, tests of this theory in natural systems are made difficult by a lack of independent replicates. Using the microbial digestive associates of a carnivorous pitcher plant, I tested hypotheses linking age-driven microbial succession to host functioning. Monitoring the yearlong development of independent digestive communities in two pitcher plant populations revealed a number of trends in community succession matching theoretical predictions. These included mid-successional peaks in bacterial diversity and metabolic substrate use, predictable and parallel successional trajectories among leaf communities, and convergence giving way to divergence in community composition and carbon substrate use. Bacterial composition, biomass, and diversity positively influenced the rate of prey decomposition, which was in turn positively associated with a host leaf's nitrogen uptake efficiency. These results highlight links between community succession and ecosystem functioning and extend succession theory to host-associated microbial communities.

Keywords: bacteria, biodiversity-ecosystem function, carnivorous plant, *Darlingtonia,* ecosystem development, stable isotope, Nitrogen, succession

INTRODUCTION

Although the capacity for community composition to mediate ecosystem processes is widely recognized (e.g., Hooper *et al.* 2005), surprisingly few theoretical (Finn 1982; DeAngelis 1992; Loreau 1998) and empirical studies (e.g., Fisher *et al.* 1982) have investigated community-ecosystem linkages along natural successional gradients. Ecosystem development theory (Odum 1969) seeks to explain temporal variation in ecosystem properties in terms of community successional turnover. Central to this theory is the prediction that successional turnover can influence elemental cycling rates leading to a coupling of community composition and ecosystem processes through time (Odum 1969; Huston & Smith 1987; DeAngelis 1992; Loreau 1998). It is worth noting that modern succession theory does not assume directionality toward a stable equilibrium (or 'climax'), but instead recognizes that the temporal trajectories of ecosystems can vary due to the

relative influences of general ecological processes (Meiners *et al.* 2015). Although these predictions have not been immune to critique on both theoretical and empirical grounds, adequately replicated tests in natural communities remain scarce.

The natural microcosms of host-associated microbial communities offer a number of unique advantages for testing ecosystem development hypotheses. First, microbiota can enable identifiable and measureable functions for their hosts (Bäckhed *et al.* 2005). Next, the habitats being colonized are often nearly identical among closely-related individuals, permitting repeated, independent observations of ecosystem development. Finally, the successional dynamics of host-associated microbiota frequently operate over time scales proportional to the host's lifespan, which can manifest as large shifts in community composition and function over relatively short time periods.

This study uses the microbial digestive communities in developing leaves of the pitcher plant *Darlingtonia californica* (Sarraceniaceae) to test the following hypotheses linking community succession to ecosystem function (Fig. 1): First, alpha diversity will either asymptotically increase or be unimodal over the host leaf's lifespan as taxa are recruited from the regional pool and subsequently persist or are excluded by superior competitors (Odum 1969; Loucks 1970; Auclair & Goff 1971; Connell & Slatyer 1977; Fierer *et al.* 2010). Consequently, trait diversity (e.g., biochemical pathways, C-substrate use) is also expected to increase as succession proceeds (Odum 1969). Second, productivity should decrease over time, as growth-limiting nutrients are lost from the system and/or stored in living biomass (Odum 1969; Vitousek & Reiners 1975; Fierer *et al.* 2010). Third, beta diversity will increase over time if environmental differences among pitchers cause spatially-variable selection or drift, or decrease over time if different leaves constitute similar selective environments (Christensen & Peet 1984; Dini-Andreote *et al.* 2015). Fourth, host ecosystem properties (e.g., nutrient cycling, decomposition) should increase monotonically or be unimodal, concomitant with changes in diversity and biomass (Cardinale *et al.* 2007; Weis *et al.* 2007; Armitage 2016). This leads to the prediction that biodiversity and biomass dynamics will help set ecosystem processes rates (e.g., decomposition), which will, in turn, set rates on host functioning (e.g., nutrient uptake rates) (Hooper *et al.* 2005).

To test these hypotheses, I followed cohorts of pitcher leaves over three years and quantified their associated digestive communities through time. In addition, I measured these communities' rates of decomposition, respiration, and their host leaves' nitrogen uptake efficiencies. These data were used to test whether host-associated digestive communities follow general, predictable successional patterns and whether their turnover can influence a host's ability to digest prey and sequester nutrients.

METHODS

Complete documentation of the field methods, laboratory sample processing, bioinformatics, and statistical analyses are provided in the supplementary materials and methods.

In situ isotopic labeling of pitcher leaves

A stable isotope pulse-chase experiment was used to measure rates of decomposition and nitrogen cycling by the pitchers' aquatic food webs. In early June 2013 and 2014, I identified and tagged 50 developing *Darlingtonia* pitcher leaves of equivalent age on different plants growing in the Plumas National Forest (Plumas Co., CA). On the day the pitcher leaves first opened, I fed gel capsules containing 20 sterile, ^{15}N-enriched fruit flies (*Drosophila melanogaster*) to five random leaves, which were then left undisturbed for 11 days. I returned

to the site to remove these ^{15}N-labeled pitcher leaves and to feed isotope-labeled flies to 5 additional leaves belonging to the same cohort. This process was repeated every 11 days up to day 88 (mid-September), and again on day 365 (June 2014) with 10 leaves. I repeated this experiment in 2014-2015 in a nearby population of *D. californica* and included an additional 166-day sample. The sampled leaves were placed on ice and quickly returned to the lab.

Quantification of pitcher leaf communities through time

In the lab, I dissected the pitcher leaves and categorized the state of fruit fly decomposition on an ordinal scale from 0 (no decomposition; flies undamaged) to 5 (completely decomposed; head capsules and wings only). I identified and enumerated all protists and living arthropods (primarily *Sarraceniopus* mites and *Metriocnemus* midge larvae) in each leaf's fluid and interior surface under a light microscope and used epifluorescence microscopy to enumerate SYBR-Gold (Thermo Fisher Scientific, Inc.) stained bacterial cells and virus-like-particles bound to 0.02 μm filters. All prey detritus in a leaf was oven-dried at 60° C and weighed.

Bacterial community sequencing

From each pitcher leaf I removed 700 mL of fluid for microbial community DNA extraction using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc.). DNA samples were stored at -80° C and sent for PCR amplification of the 16s SSU-rRNA genes (primer set 515f/806r) followed by multiplexed 2×151 bp paired-end sequencing on the Illumina MiSeq at the Argonne National Lab Core Sequencing Facility (Lemont, IL). The QIIME bioinformatics pipeline was used to assemble reads and cluster them into 97% operational taxonomic units (OTUs) (Caporaso *et al.* 2010). I calculated each community's alpha diversity (Shannon's H, phylogenetic) and beta diversity (Jensen-Shannon distance and weighted/unweighted UniFrac — a measure of community phylogenetic dissimilarity) using the *vegan* and *PhyloSeq* R packages (McMurdie & Holmes 2013; Oksanen *et al.* 2015; R Development Core Team 2015). Beta diversities for each sampling period were estimated using average inter-sample distances, and the results were unchanged when distances-to-centroid were used. I tested whether community composition changed with pitcher age using permutational analysis of variance (Anderson 2001) on samples' Jensen-Shannon distances (JSD) and UniFrac distances and visualized these results using PCoA plots.

To assess the generality of successional turnover in pitcher communities, I modeled OTU counts using a negative binomial generalized linear model (GLM) (Love *et al.* 2014). Models were fit using empirical Bayes and OTUs experiencing significant $\log_2$-fold change among time points were identified using Wald *p*-values. I defined the 'successional microbiome' as the subset of OTUs experiencing a statistically significant ($\alpha = 0.01$) ≥ 8-fold change in abundance between any two pitcher age classes and used these OTUs to construct an abundance-weighted heat map. The predictive accuracy of this subset of OTUs was assessed by training a random forest machine learning algorithm on OTU counts from the 2013 study population and using it to predict the age of samples from the independent 2014 study population. Model accuracy was evaluated using the coefficient of determination (R^2) for predicted vs. observed ages along a 1:1 line. The entire bioinformatic/analytical pipeline is illustrated in figure S1.

Estimating microbial community traits

The Biolog GN2 microplate assay (Biolog Inc., Hayward, CA) was used to measure the carbon substrate use patterns of the microbial communities from an independent collection of 11, 55, and 365 day-old pitchers (10 from each age in 2014). Plates were inoculated in

triplicate using the same dilute, filtered, starved communities described above, and incubated for 3 days at 25° C. I fit a negative binomial generalized linear model these data in order to determine whether the number of metabolized substrates differed among leaf community age. Jaccard distances between samples' substrate use profiles were calculated and plotted onto principal coordinate (PCoA) axes in R.

I used ancestral genome reconstruction implemented by the PICRUSt software (Langille *et al.* 2013) to predict the rRNA copy number and functional gene contents for the subset of OTUs in my samples present in the greengenes database. I estimated the mean weighted rRNA copy number of each pitcher sample (Nemergut *et al.* 2015) and then evaluated their temporal turnover using ANOVA. Pitcher samples were then ordinated based on their predicted level 3 KEGG pathway relative abundances (Kanehisa *et al.* 2016) using principal components analysis (PCA) and then hierarchically clustered. I filtered KEGG pathways using ANOVA *p*-values ($p \leq 0.01$) and effect sizes ($\eta^2 \geq 0.26$) in order to identify genes and pathways (focusing primarily on enzymes involved in protein degradation and nitrogen transformation) that were predicted to be differentially enriched across time points. The predictive nature of these data precluded them from being subjected to statistical falsification, and are instead treated as speculative hypotheses.

Quantification of pitcher ecosystem properties through time

Empty pitcher leaves were thoroughly rinsed, dried at 60° C, homogenized in a bead-beater, weighed, and analyzed for ^{15}N using an isotope ratio mass spectrometer at the UC Davis Stable Isotope Facility (Davis, CA). I used the fly and leaf ^{15}N measurements to estimate the total amount of fly-derived ^{15}N found in a leaf's tissue after 11 days, which is interpreted to be the host leaf's nitrogen uptake efficiency.

To estimate each pitcher microbial community's potential C-respiration rate, I inoculated starved, washed pellets of pitcher bacteria into deep-well plates containing 800 µL sterile medium comprised of M9 salt solution and ground cricket powder. I used the MicroResp^{TM} respirometry system to measure the rates of CO_2-C respired from cultures over three days at 25° C. These rates of CO_2 respiration reflect the potential respiration rates of each pitcher's bacterial community in a common environment.

I assessed temporal variation in pitcher ecosystem properties using ANOVA for N uptake efficiency/carbon respiration and a multinomial logit model for the fly decomposition category (Agresti 2013). To investigate whether bacterial community composition influenced host functioning, I ran a Mantel test to assess whether pairwise Euclidean distances among samples' N uptake efficiencies covaried with their pairwise JSD or UniFrac dissimilarity metrics.

Verifying the effects of community structure on host function

Pitcher leaves of differing ages might physiologically regulate nitrogen uptake independent of their associated food webs, which can obscure food web effects. To account for this, I ran a field experiment to separate the effects of the food web and host leaf age on rates of N uptake. During late July 2014 I identified 15 pitcher leaves aged 11 days, 55 days, and > 365 days (5 leaves of each age). The fluid from these leaves was removed and mixed in equal parts to form a homogenate. 5 mL aliquots of these homogenized communities were then returned to the host plants. Additionally, 20 ^{15}N-enriched fruit flies were delivered into each leaf. I returned after 11 days to harvest and process these pitchers for N-uptake efficiency as previously described. I used ANOVA to test whether the N-uptake efficiencies of these

pitchers with homogenized food webs recapitulated the N-uptake patterns from natural pitcher food webs of equivalent age from the same population.

RESULTS

Temporal changes in the *Darlingtonia* food web

The dynamics of dead and living biomass were qualitatively similar to the predictions in figure 1*A*. Prey biomass in pitcher leaves quickly increased after opening and remained relatively constant until prey capture diminished after the first growing season (Fig. 2*A*). Bacterial biomass also rapidly accumulated in young pitcher leaves and increased over time during the first growing season to a maximum of 1×10^{11} cells mL^{-1} before declining during the second growing season (Fig. 2*A*). Virus-like particles, *Sarraceniopus darlingtonae* mites, and *Polytomella agilis* flagellates also increased in abundance during the first growing season (Figs. 2*A*, S1). In addition to *P. agilis,* I detected numerous other eukaryotes, including *Bodo*, *Monas*, *Petalomonas*, *Rhynchobodo*, *Chilomonas*, *Colpoda*, *Philodina*, and *Chlamydomonas*, but these taxa were observed in 10 or fewer pitcher leaves with no apparent temporal trends in occupancy or richness (Fig. S2). Likewise, I did not detect a temporal trend in bacterivore beta diversity among time points until they diverged in year 2 (Fig. S2).

Composition and convergence of pitcher bacterial communities

After quality filtering of 16s amplicon sequences, the final OTU table consisted of 3642446 total reads representing 762 97% OTUs. The minimum and maximum number of reads per sample (n = 99) were 21983 and 83157, respectively (mean = 36972), and read counts did not differ among age classes ($F_{9,89} = 1.3$, $p = 0.26$). Of the top 50 most abundant OTUs detected across pitcher samples, the majority belonged to families Bacteroidetes (Fig. S3), Firmicutes (Fig. S4), and Proteobacteria (Fig. S5). As hypothesized in figure 1*A,* bacterial alpha diversities peaked at the end of the first growing season (Fig. 2*B*), whereas phylogenetic diversity increased over the entire study period (Fig. S2). In contrast with figure with figure 1*B,* however, community composition tended to converge (i.e., beta diversity decreased) during the course of the first growing season, and diverge again during the start of the second growing season, according to both taxonomic (Fig. 2*C*) and phylogenetic (Fig. S2) dissimilarity metrics. Furthermore, permutational ANOVA on Jensen-Shannon and UniFrac distances revealed a significant structuring of pitcher bacterial communities by age class (table S1) and parallel successional trajectories between years (Figs. 3*A*, S6).

I was able to identify a subset of OTUs that experienced particularly significant temporal turnover (Fig. S7). These taxa fell primarily into the phyla Proteobacteria (37 OTUs), Bacteroidetes (16 OTUs) and Firmicutes (14 OTUs). Using these OTUs to train a random forest classifier to predict the pitcher community's age resulted in a high classification accuracy for withheld data (observed vs. predicted $R^2 = 0.80$). Likewise, a random forest trained on data collected in 2013 was successful at predicting the ages of samples collected from an independent population in 2014 ($R^2 = 0.75$) (Fig. S8), implying that observed successional trajectories are likely parallel among leaves and generalizable between individuals and populations.

Temporal trends in the functional attributes of pitcher microbiota

Assays of pitcher communities' carbon substrate use patterns mirrored trends observed in taxonomic and phylogenetic alpha and beta-diversities — namely, early and late-stage pitcher communities both metabolized significantly fewer carbon substrates than did 55-day communities (Fig. S9). Furthermore, 11-day and 365-day pitchers' substrate profiles were much more variable than and clustered apart from the 55-day samples. (Figs. 6*B,* S9).

A PCA plot of samples' reconstructed metagenomes predicted pitcher samples to separate by age, with the greatest distances between the 11-day and 365-day communities (Fig. S10). The average number of rRNA gene copies per taxon was predicted to be greater in 11-day pitchers than in any other age class (Fig. S11). This trend was also observed in the relative abundances of a number of other predicted KEGG pathways, such as flagellar assembly, motility, chemotaxis, and ABC transporters (Fig. S12). Conversely, a variety of metabolic pathways were predicted to increase over time (Fig. S13). Likewise, the abundances of genes involved in nitrogen cycling (deamination, nitrogen mineralization, denitrification, and nitrogen fixation) were also predicted to increase over a pitcher leaf's lifespan (Figs. S14-S17).

Linking community dynamics and ecosystem properties

Prey decomposition was unimodal over leaves' lifespans, peaking at 44-88 days (Fig. 4*A*). This increased decomposition, however, did not herald similar temporal differences in common-garden community respiration rates, although there was still a positive, non-significant unimodal trend in mean respiration rates over time (Fig. S2). Multinomial logit models predicted bacterial diversity and bacterial and midge abundances to positively influence a pitcher's probability of having a higher decomposition score (Figs. 4*B* and *4C*, Table 1). Leaf nitrogen uptake efficiency also increased during the first growing season and subsequently declined at the start of year 2 (Fig. 4*D*) and was found to be positively associated with decomposition class (Fig. 4*E*, Table 1). Additionally, there was a weak but significant positive correlation between pitcher samples' JSD/unweighted UniFrac distances and their Euclidean distances in nitrogen uptake efficiencies (JSD Mantel $r = 0.08$, $p < 0.05$; UniFrac Mantel $r = 0.10$, $p < 0.05$). Finally, in contrast to natural pitcher samples collected in 2014, the nitrogen uptake efficiencies of experimentally-homogenized pitcher food webs did not differ between leaf age classes ($F_{2,12} = 0.98$, $p = 0.40$) (Fig. 5).

DISCUSSION

The biomass and diversity dynamics observed in developing pitcher plant microbial communities matched patterns previously observed across a variety of ecosystems undergoing primary succession. As predicted, community diversity and biomass were positively associated with rates of prey decomposition, and the extent of decomposition was positively associated with the fraction of prey-derived nitrogen removed from the food web by the host leaf. In concert, these results imply that the services these digestive communities provide their hosts are time-dependent — highlighting important, general linkages between the successional dynamics of communities and rates of ecosystem or host function.

Community convergence during succession

Whether or not replicate communities tend to converge toward a common 'climax' community is one of the oldest and most contentious successional hypotheses (Christensen & Peet 1984). Evidence for successional convergence is mixed (Meiners *et al.* 2015), which is likely due in large part to the varying nature of selective pressures acting on 'replicate' environments. Because pitcher plant leaves are similar in habitat structure and resource composition at a particular point in time, it is not surprising that bacterial communities converged over the first growing season. The assumption of homogeneous selection is supported by both the concurrent convergence in carbon substrate profiles between 11 and 55 day pitchers, and the parallel successional trajectories of pitcher leaves from two different populations. Successional convergence also occurs in non-bacterial communities in the pitcher plant *Sarracenia purpurea* (Miller & terHorst 2012), in other phyllosphere bacterial

communities (e.g., Copeland *et al.* 2015), in the human gut (e.g., Palmer *et al.* 2007), and more generally, across a variety of terrestrial (e.g., Christensen & Peet 1984) and aquatic ecosystems (e.g., Moorhead *et al.* 1998).

In contrast to the convergence observed during the first growing season, bacterivore and bacterial communities both diverged at the beginning of the second growing season. These changes coincided with increased variances of both bacterial alpha diversity and biomass. A similar pattern was also observed for metabolic substrate profiles. Divergence in these community properties can be explained either by heterogeneous selection or drift. Heterogeneous selection can occur because leaves capture far fewer prey items after their first growing season (*pers. obs*), which would increase heterogeneity in resource composition among leaves. If resource composition is a reasonably strong selective pressure, then inter-leaf differences should lead to community divergence and increased variation in leaves' alpha diversities (Dini-Andreote *et al.* 2015).

Alternatively, drift could also drive bacterial community divergence. As pitchers' prey contents are degraded into recalcitrant compounds (e.g., chitin), bacterial abundance (and presumably biomass) sharply decreases. Metacommunity theory predicts that as community size (i.e., the summed abundances/biomass of competing species) decreases, stochastic drift becomes an increasingly important determinant of community composition (Orrock & Watling 2010). Future studies are encouraged to employ experimental metacommunities for estimating the relative effects of variable selection versus drift as a function of community size.

Even if pitcher communities track immigration rates rather than local conditions, this convergence-divergence pattern can be explained by homogeneous rates of immigrant supply among leaves during the first growing season, and limited, heterogeneous supply over remainder of the first year. Because captured prey items constitute the major sources of bacteria and protists for leaves, a decrease in prey accumulation rates coupled to an increase in random capture events may provide the opportunity for heterogeneous immigrant supply to cause communities to diverge. A 55-year study of old-field communities observed similar patterns of convergence giving way to divergence (Meiners *et al.* 2015). The authors reasoned that early convergence was driven by common assembly rules selecting for compositionally-similar plots, while the subsequent divergence of these plots increased as dispersal-limited woody plants became dominant and developed patchy distributions. Nonlinear temporal trends in beta diversity have also been identified in host-associated microbial communities (e.g., Marino *et al.* 2014), though the processes governing these patterns remain vague. Pitcher plant microbial communities offer a tractable experimental system in which to assess the relative influence of deterministic vs. stochastic dispersal on temporal patterns of beta diversity.

Temporal trends in communities' functional attributes

Mirroring the results above, communities' carbon metabolic profiles also converged during year one and then diverged at year 2, implicating a link between community composition and metabolic functioning. However, microbial community sequences were not generated from the leaves used for Biolog assays, prohibiting a direct test of this hypothesis. Many of the genes predicted to be enriched in young pitchers (ribosomal RNA copy number, chemotaxis/motility genes) have previously been linked to a taxon's rate of response to unpredictable nutrient conditions (Livermore *et al.* 2014; Nemergut *et al.* 2015). These predictions are in accordance with successional tolerance and inhibition models, wherein

ruderal, fast-responders are eventually joined or outcompeted by more growth-efficient forms (Connell & Slatyer 1977; Huston & Smith 1987). While competition could not be directly measured in this study, a previous experiment using lab-reared bacterial strains isolated from individual *Darlingtonia* leaves over time found that competition among isolates was strongest during mid-succession (days 22-66) (Armitage 2016).

Metabolic pathways contributing to amino acid demamination, cadaverine and putrescine production, and N mineralization were predicted to be enriched during mid-succession — a pattern also detected during microbial succession on decomposing corpses (Metcalf *et al.* 2016). Although these patterns are based on indirect evidence obtained through ancestral state reconstructions, similar successional increases in metabolic genes have been documented in host-associated (Koenig *et al.* 2011) and aquatic (Teeling *et al.* 2012) bacterial communities. In concert with the community metabolic assays, these findings serve to demonstrate, in principle, how bacterial succession can influence rates of material cycling in ecosystems (Loreau 2001). Future work in this area is encouraged to combine field experiments with metatranscriptomic sampling to directly measure gene expression during succession.

Linking community dynamics to ecosystem function

Detrital processing rates by the pitcher leaf communities varied over time, and were positively associated with detritivore abundances (bacteria, midge larvae) and bacterial diversity — making this among the first biodiversity-ecosystem function relationships detected in a natural host-associated ecosystem. Loreau (2001) reasoned that microbial diversity would enhance decomposition only if the number of organic compounds able to be metabolized by the community increased with alpha diversity. This prediction is supported by observations of peak bacterial taxonomic diversity coinciding with peak carbon metabolic diversity during mid-succession (ca. 55 days). To date, the few studies to investigate microbial diversity and decomposition rates *in situ* have arrived at conflicting results (Hättenschwiler *et al.* 2011) but a positive relationship is common in the few experimental tests using bacteria (e.g., Bell *et al.* 2005), including in a lab experiment using bacterial isolates from the same *Darlingtonia* population studied here (Armitage 2016). More generally, microbial community composition is anticipated to set ecosystem process rates, particularly when the effects of environmental variation are minimal (Graham *et al.* 2016).

From a host plant's perspective, decomposition by its commensal biota should set limits on its rate of N sequestration. In my study plants, prey digestion explained a significant portion of variance in N uptake efficiency, and the best-fit model for host N uptake included information on both decomposition rates and microbial diversity and biomass (although the effects of the latter covariates were relatively weak). In a separate experiment, however, I failed to detect the same mid-succession peak in N uptake efficiencies among pitcher leaves containing experimentally homogenized bacterial communities. The related pitcher plant *Sarracenia purpurea* also relies primarily on its bacterial community, rather than its macroinvertebrates, for nitrogen processing (Butler *et al.* 2008). Furthermore, microbial community composition is an important determinant of nitrogen mineralization rates in soil (Strickland *et al.* 2009), and changes in N mineralization can track microbial community change over time, independent of environmental variation (Balser & Firestone 2005). In concert, these results highlight the potential for the pitcher microbial communities to mediate N transfer from prey to host — a function critical to the fitness of a host plant adapted to life in nitrogen-poor soils.

The rate of nitrogen loss from an ecosystem (e.g., due to leaching) is predicted to slow during mid-succession as it is immobilized in living biomass (Vitousek & Reiners 1975; but see Finn 1982; Loreau 1998). In *Darlingonia* leaves, however, maximal rates of N loss from the commensal food web occurred during periods coinciding with high standing biomass. This mismatch can be explained by differences between primary producer-controlled food webs, in which the N pool is growth-limiting and quickly immobilized in long-lived organic form, and donor-controlled food webs, which receive N subsidies at regular intervals (Fierer *et al.* 2010). Donor-controlled systems may therefore not experience severe N limitation during periods of rapid biomass production. This may be particularly true in *Darlingtonia* and other host-associated digestive communities for two reasons. First, rapid bacterial turnover (e.g., via viral lysis & protozoan grazing) serve to increase the concentration of bioavailable N. Second, pitcher leaves' continuous accumulation of low C:N detritus (relative to plant-based food webs) may buffer the food web from nitrogen losses to the host plant. If bacteria outcompete host plants for bioavailable N, then the relatively large fraction of prey-derived N found in pitcher foliar tissue suggests that it is not growth-limiting for a leaf's digestive associates. Future studies are encouraged to utilize model axenic plant-microbe systems to experimentally quantify their competition for nutrients and determine whether N limitation can divert the natural successional trajectories and functioning of pitcher digestive communities.

Succession or seasonality?

Because study leaves belonged to the same cohort, their temporal dynamics may reflect the effects of seasonal forcing rather than succession. In particular, annual temperature fluctuations have the potential to influence rates decomposition and propagule supply into leaves. Although minimum air temperatures at the study sites dipped below freezing on several occasions, temperature fluctuations in pitcher leaves' digestive zones were likely buffered by peat cover, plant cover, occasional snowpack, and most importantly, by 4-8 °C spring water flowing over the base of the plants throughout the year. Because of this, pitcher leaves removed from underneath snow cover in Dec - Feb contain active populations of mites and midge larvae (*pers. obs*). Furthermore, seasonal forcing implies that community composition will be cyclical over an annual cycle, and communities from different leaf cohorts should be similar to one another when sampled at a fixed point in time. I observed a distinct separation between 11-day and 365-day communities despite the fact that they were collected within a few days of each other, implying that these communities do not 'reset' at the start of a new growing season. Additionally, the pitcher communities used for carbon metabolic assays belonged to different cohorts of leaves collected on the same day in July, yet had unique substrate utilization patterns. This implies that even under similar external environmental conditions, communities show measurable age-related differences. Therefore, although seasonality undoubtedly influences rates of resource and propagule accumulation by pitcher communities, it may not be as critical a determinant in community turnover as age-driven succession.

Cross-system considerations

The rapid generation times and adaptive capacities of microbial populations should not preclude them from being used to test ecosystem development hypotheses (Fierer *et al.* 2010). Although succession theory originated from observations of plant communities, its modern conceptual models are agnostic to taxonomic group or biome and do not necessarily assume convergence to stable 'climax' states. It is now recognized that community and ecosystem dynamics are shaped by unique combinations of disturbances, competition, and dispersal (Meiners *et al.* 2015). And although succession is most frequently defined in terms

of species turnover, it is entirely reasonable to redefine it as the change in average trait values or gene frequencies within a community. Thus, even if pitcher leaf communities were entirely closed to colonization after the arrival of their initial occupants, the process of succession could still occur as microbial lineages adapt or perish over time. Such evolutionary changes could influence the functioning of the host plant if, for instance, selection favored a more efficient processing or storage of nitrogen by commensal organisms. The potential for rapid evolutionary change to influence ecosystem properties has been documented previously (e.g., Harmon *et al.* 2009), yet theory integrating ecosystem development and evolution is scarce (but see Loreau 1998). In doing so, care must be taken to avoid ascribing adaptive properties to ecosystems (i.e., treating ecosystems as 'super-organisms') (Odum 1969). However, it is worth noting that because many host-associated systems actually serve functions critical to their hosts' fitnesses, such ecosystems more closely fit Odum's 'super-organism' concept, and their dynamics may therefore be expected to align with his oft-discounted predictions for successional increases in stability and productivity. Tests of these predictions using existing quantitative frameworks (Finn 1982; DeAngelis 1992; Loreau 1998) would be difficult but valuable contributions toward a unified theory of communities and ecosystems.

Conclusions

By combining a ^{15}N stable isotope pulse-chase experiment with observations of community dynamics, I have confirmed a number of successional hypotheses in natural, host-associated digestive communities. In particular, my data support and extend the hypotheses of parallel successional trajectories and mid-successional peaks in functional and taxonomic diversity to host-associated bacterial communities. In concert, these results represent a step towards integrating host-associated communities into classical conceptual models of ecosystem development and hint at the coupling of community dynamics and host functioning. Looking ahead, far more theoretical and experimental work is needed before we can identify definitive links between community succession and ecosystem functions, and I believe that the continued experimental use of replicated, natural host-associated communities offers a productive path forward.

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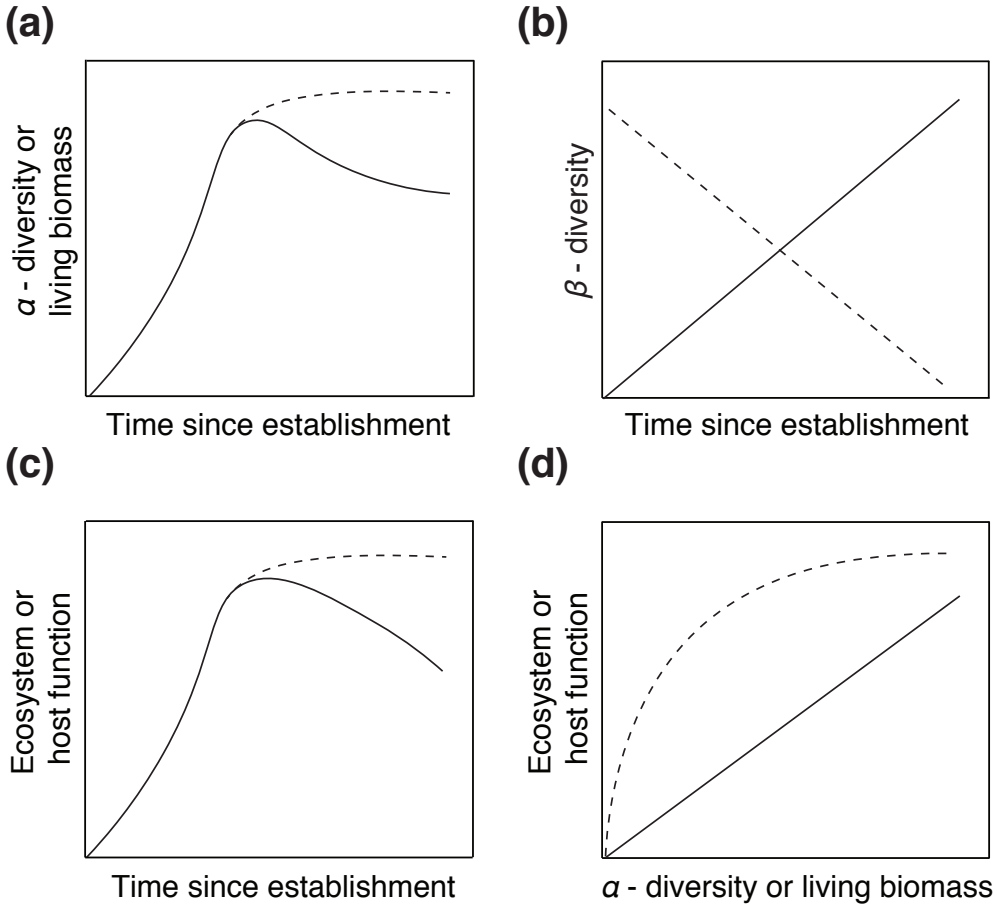

Figure 1. Predictions for successional patterns in *Darlingtonia* leaves. *(A)* α-diversity and living biomass are predicted to increase in pitcher leaves after opening, consistent with observations made across a variety of ecosystems (Odum 1969; Loucks 1970; Auclair & Goff 1971; Vitousek & Reiners 1975; Connell & Slatyer 1977; Peet & Christensen 1988; Alday *et al.* 2011). *(B)* Compositional differences among leaf communities (*β*-diversity) may either decrease or increase depending on whether selection is homogenous or variable among leaves (Christensen & Peet 1984; Dini-Andreote *et al.* 2015; Meiners *et al.* 2015). *(C)* Ecosystem or host function is anticipated to be unimodal or saturating over a successional gradient (van Ruijven & Berendse 2005; Cardinale *et al.* 2007; Weis *et al.* 2007; Armitage 2016), — a pattern predicted to be influenced by *(D)* the positive effects of *α*-diversity and living biomass on ecosystem function (Bell *et al.* 2005; Hooper *et al.* 2005). Dashed lines denote alternative hypotheses.

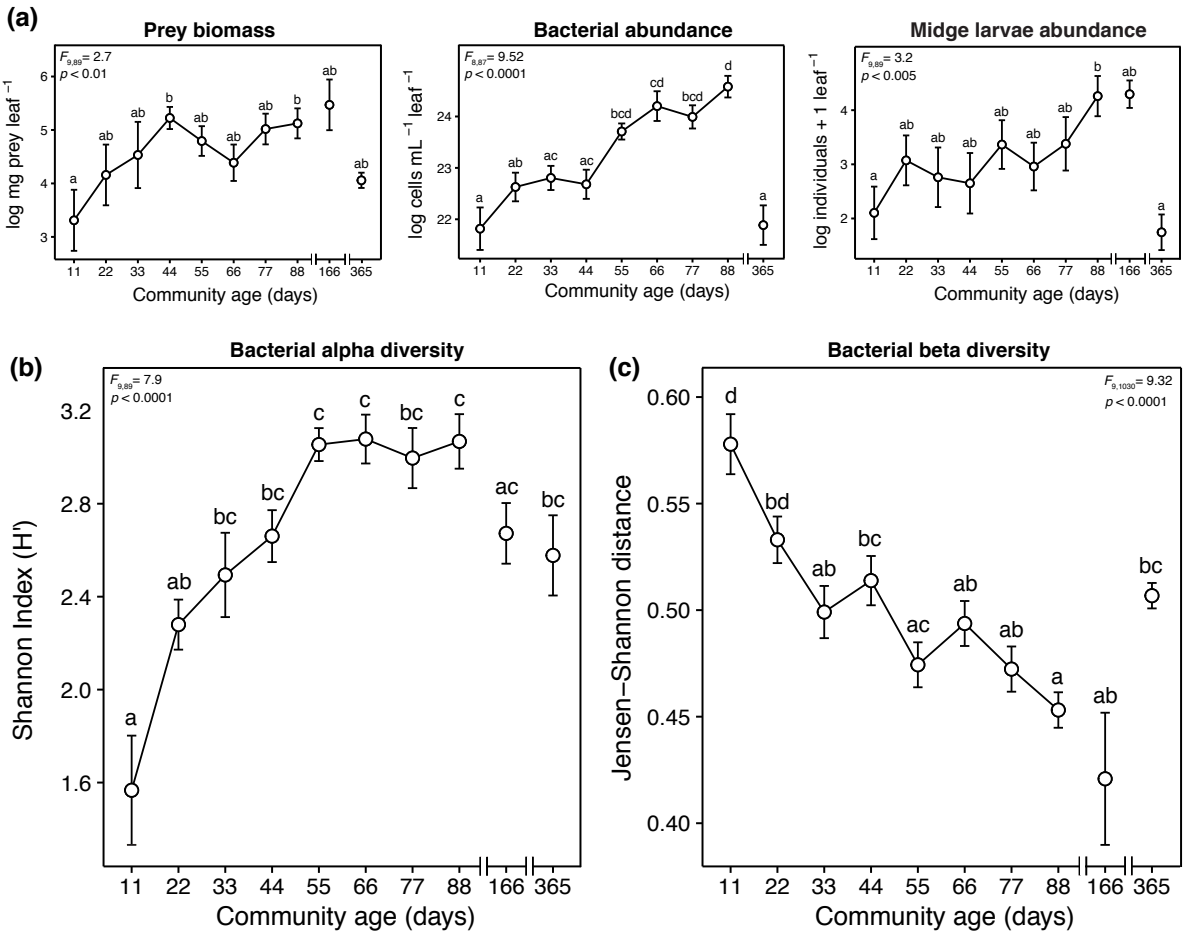

Figure 2. Trends in community composition during succession. (*A*) Insect prey biomass rapidly increased in leaves after opening and remained relatively steady throughout the remainder of the leaf's lifespan, while bacterial and midge larval abundances steadily increased throughout leaves' first growing season, and then sharply declined after the first year. (*B*) Bacterial alpha diversities increased and then leveled off in middle-aged pitcher communities, dropping slightly during year 2. (*C*) Conversely, leaf bacterial beta diversities decreased during the first growing season and increased at the beginning of year 2. In each graph, shared letters above groups indicate no significant pairwise differences ($p > 0.05$). Points denote mean values ± SEM.

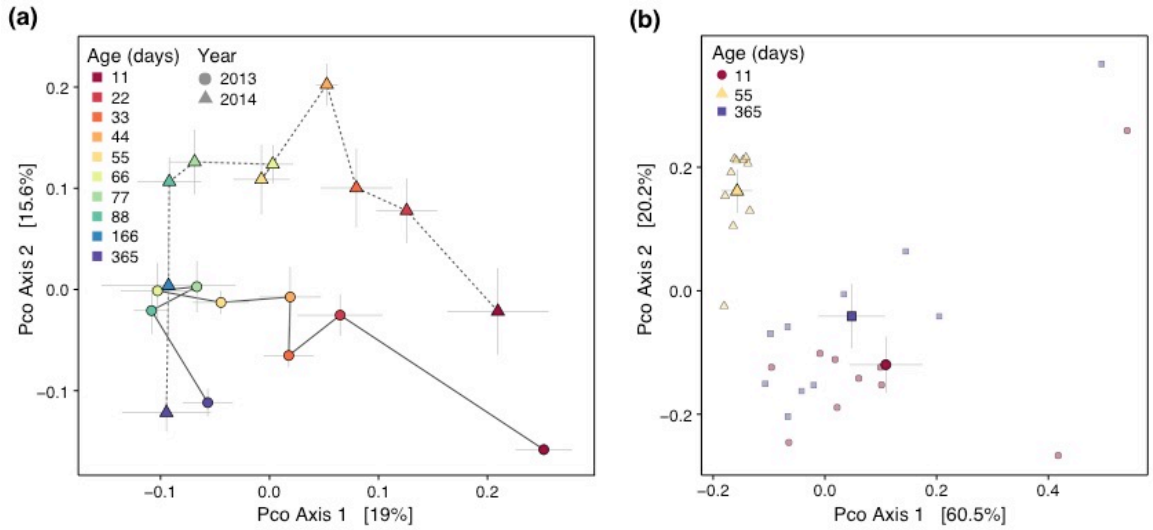

Figure 3. Principal coordinate (PCoA) plots for (*A*) Jensen-Shannon distances between samples, demonstrating convergence and approximately-parallel successional trajectories in between-population community structures over time, and (*B*) Jaccard distances between Biolog ™ plates for communities of different ages, demonstrating the convergence of metabolic profiles in mid-successional pitcher leaves and overlapping metabolic profiles for young and senescing leaves. The percentages of variance explained by the principal coordinates are displayed on each axis. Points denote yearly centroid values ± SEM.

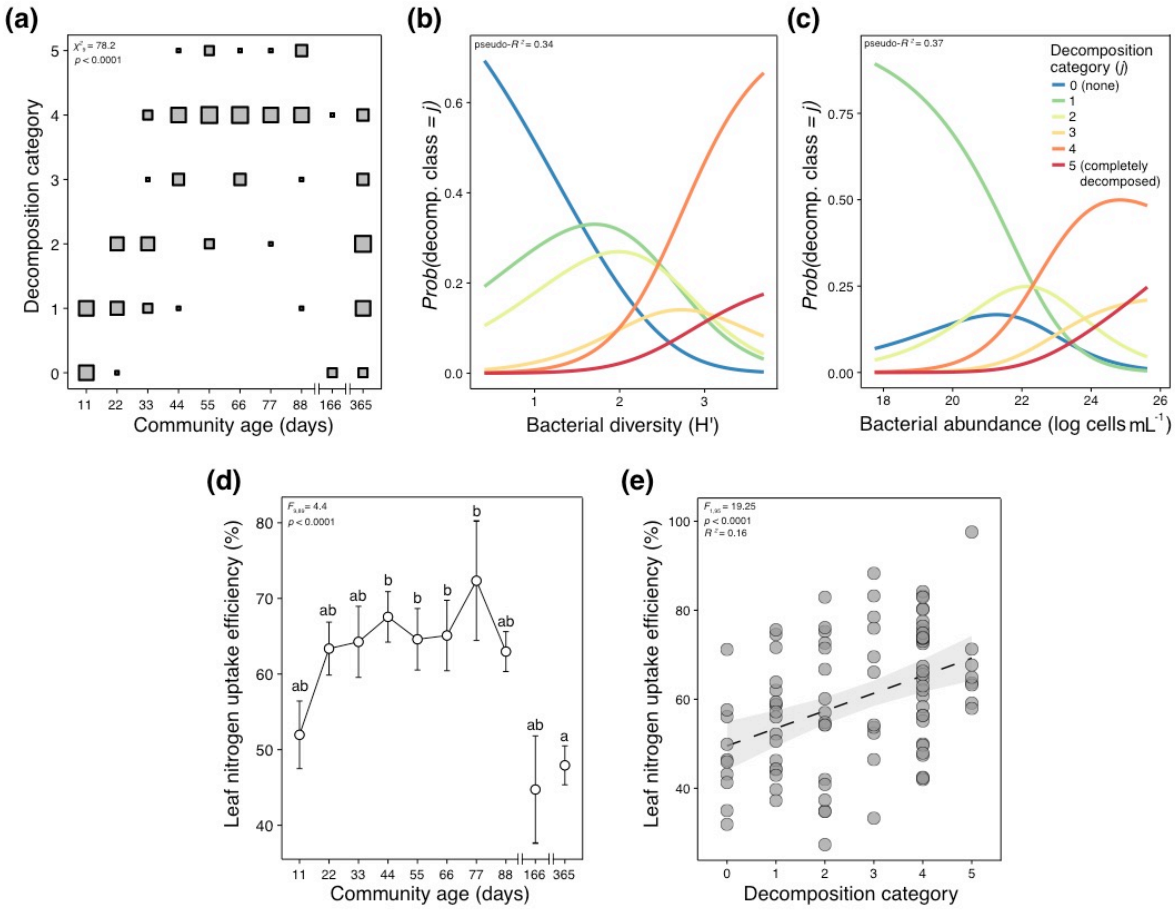

Figure 4. Trends in ecosystem properties during succession. (*A*) The frequencies of decomposition classes for pitchers of different ages. Square size is proportional to relative frequency of a particular decomposition category for that age class. χ^2 is the likelihood ratio test statistic for the effect of pitcher age on the fit of a multinomial logit distribution to predict decomposition categories. (*B & C*) The probabilities of observing high decomposition rates increases with both bacterial diversity and bacterial biomass. Curves represent fitted proabilities of multinomial logit models, and individual curves can be interpreted as logistic regression fits for each decomposition category. (*D*) Pitcher leaves' nitrogen uptake efficiencies change over time, and are significantly lower in late-stage pitcher leaves. Points denote mean values ± SEM. (*E*) The extent of prey decomposition is positively associated with the percentage of prey-derived nitrogen found in the host leaf's foliar tissue. The dashed line denotes the best-fit linear model ± 95% CI.

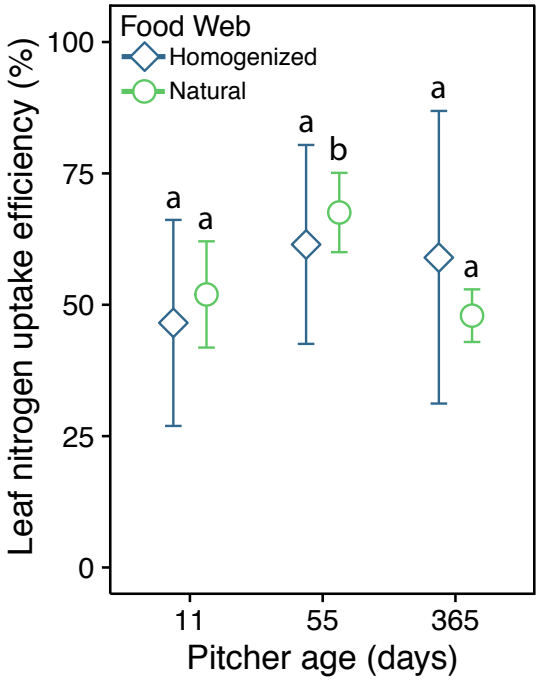

Figure 5. Homogenizing the food webs of 11, 55, and 365 day pitchers and placing them back into the plants removes the significant differences observed in natural pitcher communities of the same ages. Letters above the groups represent the within-treatment contrasts. Points denote mean values ± SEM (n = 5).

Table 1. Model selection results of multinomial logit and linear regression models for decomposition category and nitrogen uptake efficiency, respectively. Bolded values indicate the best-performing models based on AIC and R^2 values. AIC values falling within 9 units of one another were considered equally parsimonious.

Decomposition Category			**Nitrogen Uptake Efficiency**		
Predictor Variables	ΔAIC	Pseudo-R^2	Predictor Variables	ΔAIC	R^2
~ *Community age (A)*	32	0.71	~ *Community age (A)*	22	0.24
~ *Bacterial abundance (B)*	23	0.37	~ *Bacterial abundance (B)*	27	0.04
~ *Bacterial diversity (D)*	26	0.34	~ *Bacterial diversity (D)*	36	0.05
~ *Bacterivore richness (R)*	61	0.05	~ *Bacterivore richness (R)*	42	0.00
~ *Log midge abundance (M)*	45	0.20	~ *Log midge abundance (M)*	41	0.01
~ *Log mite abundance (N)*	60	0.05	~ *Log mite abundance (N)*	41	0.00
~ *B + D*	**0**	0.56	~ *Decomposition category (C)*	9	0.16
~ *B + D + M*	**3**	0.59	~ *A + C*	**5**	**0.26**
~ *A + B + D + M*	24	**0.82**	~ *A + B + D + C*	**0**	0.23
~ 1 (intercept-only null)	55	0.00	~ 1 (intercept-only null)	40	0.00