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Time-variant species pools shape competitive dynamics and biodiversity–ecosystem function relationships

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Biodiversity–ecosystem function (BEF) experiments routinely employ common garden designs, drawing samples from a local biota. The communities from which taxa are sampled may not, however, be at equilibrium. To test for temporal changes in BEF relationships, I assembled the pools of aquatic bacterial strains isolated at different time points from leaves on the pitcher plant *Darlingtonia californica* in order to evaluate the strength, direction and drivers of the BEF relationship across a natural host-associated successional gradient. I constructed experimental communities using bacterial isolates from each time point and measured their respiration rates and competitive interactions. Communities assembled from mid-successional species pools showed the strongest positive relationships between community richness and respiration rates, driven primarily by linear additivity among isolates. Diffuse competition was common among all communities but greatest within mid-successional isolates. These results demonstrate the dependence of the BEF relationship on the temporal dynamics of the local species pool, implying that ecosystems may respond differently to the addition or removal of taxa at different points in time during succession.

1. Introduction

The rates at which ecosystems cycle nutrients are predicted to be set predominantly by the actions of their constituent organisms [1–3]. Over the past two decades, this conceptual unification of communities and ecosystems has been empirically evaluated using the biodiversity–ecosystem function (BEF) framework [4–6]. This research commonly reports a positive covariance between species richness and community biomass production and is hypothesized to be jointly driven by community members' differential contributions to ecosystem properties (selection effects) and their degree of niche overlap (complementarity effects) [7].

The relative importance of these effects is in large part a function of resource competition among community members [8]. Many ecosystem functions are enabled by a single guild of competitors. If taxa within a guild vary in their contributions to ecosystem function, then turnover resulting from interspecific competition should result in shifting BEF relationships. Communities, however, are naturally dynamic and can experience both gradual successional turnover and rapid state transitions [9,10]. Such turnover is predicted to result, in part, from temporal variation in species interactions—particularly competition—as new taxa arrive and changing local conditions lead to fitness differences among competitors [11]. Because the strength of resource competition among community members is predicted to vary over the course of primary succession [1,12,13] and also influence the magnitude and drivers of the BEF relationship, it stands that the BEF relationship should vary along a successional gradient. Thus, a comprehensive theory linking biodiversity to ecosystem function

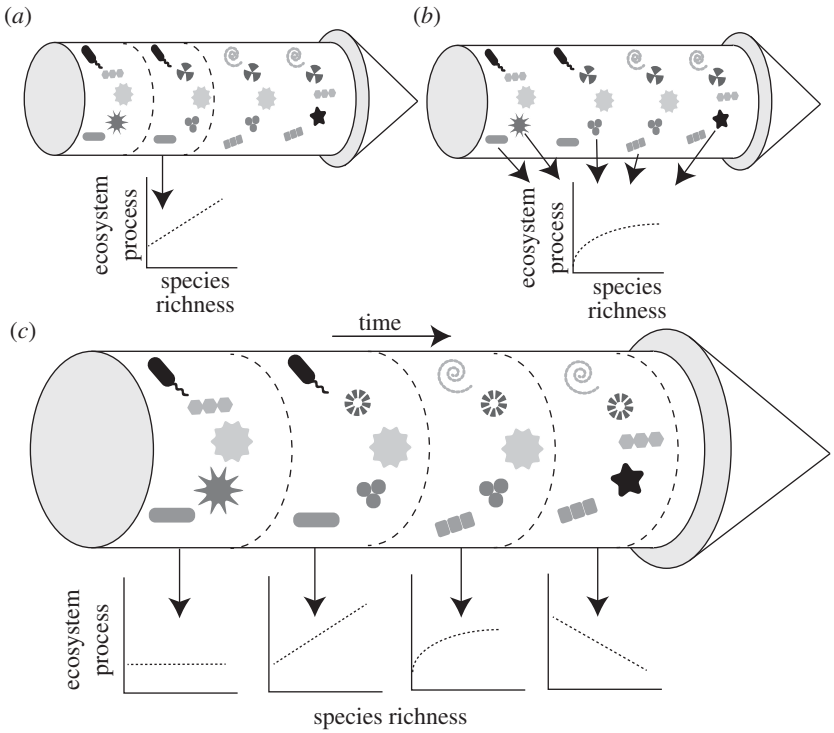

Figure 1. Species pools for BEF experiments are typically chosen either by sampling a community at a single point in time (*a*) or from a group of taxa that may not co-occur at a particular time point (*b*). Far fewer studies have taken the approach of measuring BEF relationships over a temporally dynamic species pool (*c*).

must explicitly account for the effects of community turnover through time [14,15].

The majority of BEF experiments track the productivity of monocultures and polycultures assembled from taxa randomly drawn from a natural biota or from *ad hoc* combinations of tractable organisms such as algae or protists. In these experimental communities, the magnitude and drivers of the BEF relationship are often found to change over time [16–26]. While these experiments have contributed fundamental insights into the temporal dynamics of BEF relationships, they do not account for a dynamic species pool. In other words, the groups of species used to seed these communities represent either a *snapshot* of a natural community at a particular point in time (figure 1*a*) or a collection of species that may be differentially distributed across time such that two species added into a community do not necessarily co-occur under natural settings (figure 1*b*). Communities assembled from a dynamic species pool, however, may show different BEF relationships over time owing to the shifting identities and interactions of the constituent taxa (figure 1*c*).

Whereas BEF experiments are most commonly conducted using primary producers, the framework has also been successfully extended to other groups. In particular, bacterial communities have been the subject of numerous BEF studies, owing to both their experimental tractability and importance in regulating ecosystem processes [18,24,27–30]. Because natural bacterial communities often exhibit marked turnover through time [31,32], they provide an opportunity to investigate the strength and drivers of the BEF relationship over a temporal gradient.

Carnivorous pitcher plants in the family Sarraceniaceae are a group for which bacterial communities provide a particularly critical function. These plants have evolved to capture arthropod prey by means of a conical leaf in which trapped insects are drowned by fluid secreted by the host [33,34]. Digestion is facilitated both by enzymes produced by the plant and by a dynamic community of bacteria residing in the fluid [32,35–37]. The pitcher plant *Darlingtonia californica* (Torr.) is hypothesized to rely heavily on bacteria for prey digestion [35]. The pitcher leaves of this species are produced at regular intervals throughout the June–October growing season and are sterile prior to opening [32]. Once the leaves fully develop, they quickly begin trapping insects, and bacterial biomass skyrockets to over 10^9 cells ml^{-1} [32]. After approximately two months, a leaf ceases prey capture but remains photosynthetically active for a second growing season. Bacterial diversity in *Darlingtonia* pitchers changes predictably over time, as has been documented by both culture-independent molecular approaches as well as among bacterial cultures isolated from different aged leaves [32,38]. These temporal isolates provide a unique opportunity to measure BEF relationships along a natural microbial successional gradient.

My goals for this study were twofold. First, I investigated whether the contribution of bacterial richness to rates of carbon mineralization changed over time along a natural successional gradient in *Darlingtonia* leaves. Second, I used these data to estimate the relative influences of individual strains and their interspecific interactions (such as competition) on the BEF relationship [39]. The strength of interspecific competition among bacterial strains growing in a polyculture can be approximated as the difference between the community's predicted respiration in the absence of any interference (i.e. the sum of the strains' monoculture respiration rates) and the community's realized respiration rate, given the mono and polycultures have equal total starting densities [40,41]. If strains in a polyculture do not inhibit one another through resource competition or direct antagonism, then the community's rate of carbon respiration will not significantly differ from the additive monoculture expectation [40]. This measure of competitive inhibition is anticipated to increase over time if, for instance, a competition–colonization

trade-off results in the dominance of early pitcher leaves by less competitive, ruderal taxa which are later excluded by superior competitors [11,13]. Alternatively, the bacterial taxa dominating late-stage pitchers may be specialists on recalcitrant carbon resources and therefore may not contribute significantly to respiration, compared with early, fast-growing colonists [42]. In this case, I anticipated a negative trend in competitive inhibition over time. In order to experimentally test these hypotheses, I assembled synthetic microbial communities, using pools of bacterial strains isolated from a cohort of pitcher leaves at regular intervals and measured their rates of carbon mineralization.

2. Material and methods

(a) Sample collection and strain isolation

In the field, I tagged five unopened *Darlingtonia* pitcher leaves of the same approximate age at the beginning of the growing season and tracked them over their first year. I visited this cohort of leaves every 11 days from June to September 2014 and once in June 2015 to remove 0.5 ml of pitcher fluid from each leaf. This fluid was diluted and spread on R2A agar plates and incubated at 25°C, and bacterial colonies expressing unique colony morphologies, cellular morphologies and pigmentations were isolated in pure culture. The 10 most abundant bacterial strains isolated from each pitcher age class were then used to inoculate experimental microcosms (electronic supplementary material, table S1). Extended discussion of the sampling and isolation methods can be found in the electronic supplementary material accompanying this article. Electronic supplementary material, figure S1 provides a graphical walkthrough of the experimental procedure.

(b) Microcosm experiment

I combined the 10 strains isolated from each time point into 1-, 2-, 5- and 10-strain communities using the random partitions design introduced by Bell *et al*. [39]. My experiment consisted of four partitions (P), each containing four strain richness treatments (R) and $10/R$ randomized communities within each $P \times R$ treatment (electronic supplementary material, figure S2). Every experimental community was replicated three times. This experimental design ensures that all species are equally represented within and among richness levels, giving each one an equal opportunity to contribute to selection and complementarity effects and weakens statistical artefacts such as the 'variance reduction effect' [43]. It also permits the statistical separation of species effects and richness effects on ecosystem processes without the need for measuring the contribution of an individual species to the properties of the polyculture, as is traditional in BEF studies using plant biomass as a response. This enables the user to estimate species' contributions to emergent ecosystem properties (e.g. carbon mineralization rates) that cannot be attributed to individual taxa in polyculture. Furthermore, it relaxes the requirement for a full-factorial experimental design, which becomes intractable as the number of species increases. In total, I assembled 216 communities per time point, resulting in a total of 1944 cultures spanning nine source community ages and four levels of richness.

The bacterial microcosms consisted of 1.2 ml 96-well plates containing a sterile artificial pitcher medium comprised M9 salt solution and ground cricket powder. Individual bacterial strains were grown to mid-log-phase in R2A broth, washed of their medium and starved for 2 h. Each strain was introduced into its community at the volume required to keep the total number of cells across richness treatments equal (100 µl, or approx. 10^4 colony-forming units). Once assembled, plates were clamped onto 96-well MicroResp™ (James Hutton Institute, Inc.) respirometry plates containing a colorimetric CO_2 indicator solution [44]. All replicate communities for a single time point were incubated simultaneously at 25°C for 3 days, after which time I estimated rates of CO_2–C entering each agar well on the MicroResp™ plate from its absorbance at 590 nm on a microplate reader. I measured the carbon metabolic profiles of each 10-strain community using the GN2 microplate (Biolog, Inc.), which assays a community's potential to metabolize 95 different carbon compounds. Each Biolog assay was run in triplicate at 25°C for 3 days, and only substrates scoring positive for metabolism across all replicates were scored as positive. I used ANOVA to test for differences in the mean number of compounds used between community ages and principal coordinates analysis to ordinate samples' metabolic profiles based on their Jaccard distances. Additional experimental procedures are detailed in the electronic supplementary material.

(c) Statistical analyses

To assess how drivers of the BEF relationship differed among time points, I fitted a linear model to community respiration rates [39]. This model took the form

$$y = \beta_0 + \beta_{LR}x_{LR} + \beta_{NLR}x_{NLR} + \left(\sum_i^S \beta_i x_i\right) + \beta_Q x_Q + \beta_M x_M + \varepsilon, \quad (2.1)$$

where y is a community or ecosystem process (e.g. respiration rate), β_{LR} is the effect of strain richness measured on a continuous scale (linear richness, x_{LR}), β_{NLR} is the effect of strain richness measured on a categorical scale (nonlinear richness, x_{NLR}), β_i is the impact of an individual strain's presence on the productivity of its community, β_Q is the effect of the particular taxon pool used in each $P \times R$ treatment, β_M is the effect of a particular community composition within each taxon pool, β_0 is the intercept and ε is the error term.

Importantly, by estimating the linear richness term prior to the nonlinear richness and strains' impact terms, the latter two terms become orthogonal. The species impact (β_i) terms sum to zero and reflect the relative influence an individual strain exerts on the community's respiration. The nonlinear richness term (β_{NLR}) can be interpreted as the magnitude of deviations from linear richness effects. Non-zero values of β_{NLR} reflect the influence of facilitative and competitive interactions on ecosystem processes. I used least-squares to estimate the model coefficients and an F-test to determine the statistical significance of each variable. The denominator term for the F-statistics of the β_{NLR} and β_i parameters were the partitioned mean-squares from the species pool (Q) or species composition (M) factors, respectively. Model terms were entered in the order in which they appear in equation (2.1): nonlinear richness (β_{NLR}) and species impacts (β_i) were estimated from the residuals of the model containing the linear richness (β_{LR}) term.

I estimated the effects of the source pitchers' ages and experimental communities' richness on the rates of CO_2 respiration using linear regression. To aid in the interpretation of interactions, predictors were centred to their mean values prior to model fitting. I assessed the pairwise differences among community ages using Tukey's range test ($\alpha = 0.05$). Community age was treated as an ordinal, discrete variable to account for the absence of sampling between days 88 and 365.

I estimated the extent to which strains inhibit one another's potential CO_2 production in polyculture by calculating the difference between a community's predicted and observed respiration rates. The predicted values were calculated by summing all community members' average monoculture respiration rates. The difference between a polyculture's predicted and observed respiration values will equal zero if there are no inhibitory effects between members of the community (i.e. all taxa in a polyculture perform as well they do in monoculture) [40,41]. Alternatively,

Table 1. ANOVA and ANCOVA results for total respiration and respiration differences (i.e. interspecific inhibition). (Respiration rates were log-transformed to satisfy homoscedasticity. Richness was treated as a continuous variable and age as a categorical variable with contrasts summing to zero. Marginal (type 3) sums-of-squares (SS) are presented.)

response	covariate	d.f.	SS	*F*	*p*(<)	R^2
log respiration rate	intercept	1	17.35	317.35	0.001	0.17
	source community age	8	12.89	12.89	0.001	
	species richness	1	47.03	47.03	0.001	
	interaction term	8	14.52	3.311	0.001	
	residuals	1926	1056			
expected – observed respiration	intercept	1	689	154	0.001	0.88
	source community age	8	1050	29.3	0.001	
	species richness	1	7481	1675.0	0.001	
	interaction term	8	1133	31.7	0.001	
	residuals	270	1206			

direct antagonism (e.g. antibiotic production) or resource competition is anticipated to result in respiration rates less than the additive prediction. I used ANCOVA to test the null hypothesis that the mean differences between predicted and observed respiration rates were equal among community ages, controlling for richness effects. Pairwise differences between centred predictor variables were assessed using Tukey's range test. All models were fitted using R v. 3.1 [45].

(d) Pairwise antagonism assay

I performed spot assays to determine whether a particular bacterial strain directly inhibits the growth of a co-occurring strain. I created lawns of focal strains by spreading log-phase broth cultures onto two plates containing R2A agar onto which I spotted 2 µl log-phase broth culture of each co-occurring isolate. Each spot was replicated four times on the same plate, resulting in eight cross-inoculations per strain pair (excluding sterile blanks). After 24 h at 25°C, I searched for zones of clearing surrounding a colony. I considered the spotted strain to be inhibitory to the focal strain if unambiguous zones of clearing surrounded at least six replicates.

3. Results

I found an average of 6.9 (s.e. = 0.18, range = 5–9) bacterial strains remaining in each 10-strain community, and there were no significant differences in the proportions of surviving strains among source community ages ($F_{8,27} = 2.3$, $p = 0.06$). Thus, although the strains' relative abundances changed throughout the incubation period, no single, dominant strain was able to exclude the majority of others. I detected significant differences between the mean respiration rates of bacterial communities isolated from pitcher leaves of different ages (table 1 and electronic supplementary material, figure S3). *Post hoc* analysis revealed respiration rates to be greatest among bacterial communities isolated from pitcher leaves between 22 and 55 days old (electronic supplementary material, figure S3). This pattern was consistent under all four richness treatments, although there was a general tendency for variance in respiration rates among treatments to increase when more strains were present. Bacterial richness had a significantly positive effect on overall respiration rates, ($\beta_R = 0.05 \pm 0.007$; table 1 and figure 2), although there was a significant interaction between richness and source community age (table 1).

The effect of linear richness (β_{LR}) on respiration rates was significantly positive for all source community ages except those from days 88 and 365 (figure 2). This positive effect of richness on respiration was greatest for isolates from pitcher leaves between 22 and 66 days old, and tended to increase from days 11 to 22 and then slowly decrease towards zero throughout the rest of the pitchers' lifespan (figure 3*a*). For each bacterial isolate pool, I detected individual nonlinear richness effects (β_{NLR}) and individual strain (β_i) effects significantly greater or less than zero, but these effects were not significant overall (electronic supplementary material, table S2). Despite this, the relative influence of nonlinear richness effects was greater than overall strain effects for the majority of time points (figure 3*b*).

The average differences between expected and observed respiration rates initially increased between samples collected from 11- and 22-day pitchers, and then declined with source community age (figure 4). There were a number of instances where the observed respiration rates of two-strain mixtures were greater than their predicted values, but overall mean values were significantly greater than zero for all richness treatments ($\beta_{0,R2} = 1.04 \pm 0.26$, $\beta_{0,R5} = 7.25 \pm 0.48$, $\beta_{0,R10} = 16.05 \pm 0.62$, $p < 0.0001$ for all cases). The magnitude of this inhibitory effect increased with strain richness (figure 4 and table 1). I detected only 12 antagonistic interactions between eight pairs of strains (out of 405 total). These interactions occurred only in 11- and 44-day source pools. Furthermore, there was no detectable temporal trend among source pool ages in either the total number of carbon substrates used (electronic supplementary material, figure S4) or their multivariate Jaccard similarities (electronic supplementary material, figure S4).

4. Discussion

(a) Dynamic species pools impact biodiversity–ecosystem function relationships

I encountered a mid-successional peak in the rates of carbon mineralization, independent of taxonomic richness. This implies that when placed into identical environments, bacterial strains isolated from leaves of intermediate ages

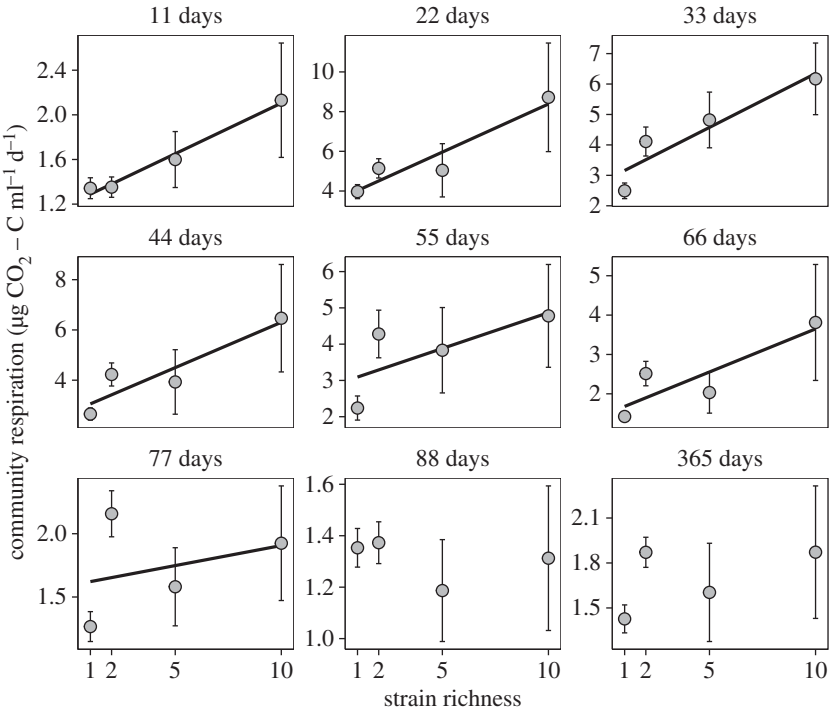

Figure 2. Relationships between strain richness and community respiration for synthetic bacterial communities assembled from pitchers of different ages. Black lines denote significant linear richness fits for individual communities within each age group ($p < 0.05$). Mean values for the response variables are presented for clarity. Bars denote standard error measurements.

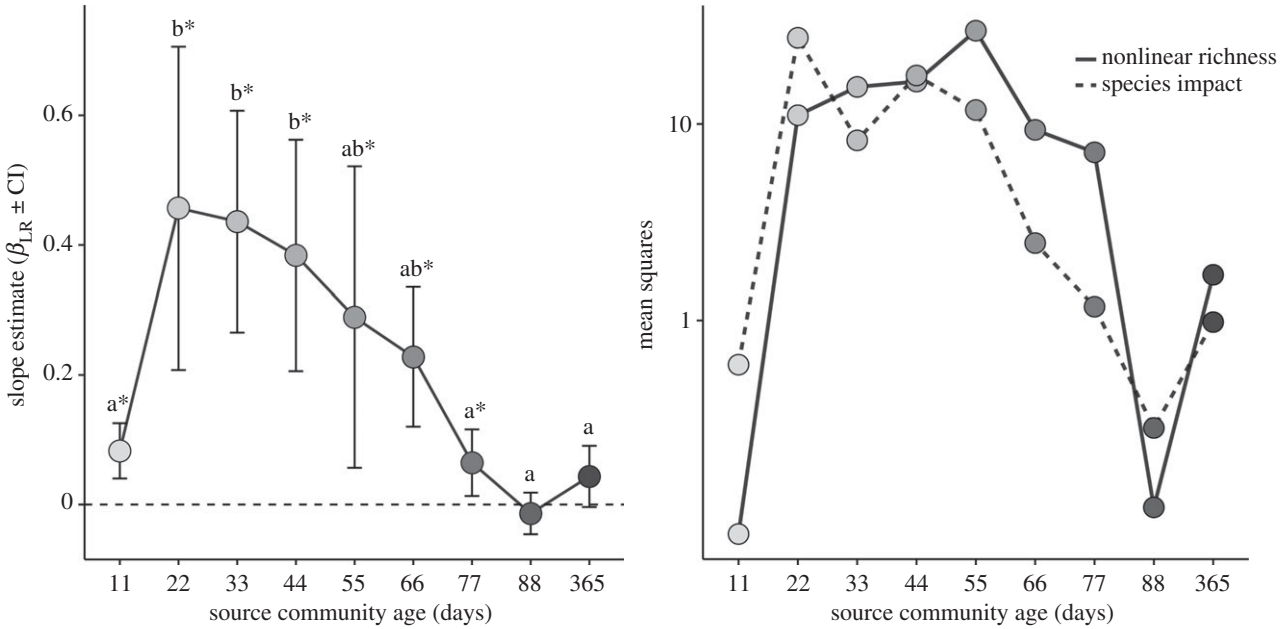

Figure 3. (*a*) Linear richness (β_{LR}) regression coefficients as a function of source community age. Bars denote 95% confidence intervals and shared letters between ages signify an overlap between the two estimates. Asterisks denote coefficients found to be significantly greater than zero (*F*-test, $p < 0.05$). (*b*) Log mean-square estimates for the species impact (β_i) and nonlinear richness (β_{NLR}) parameters. These values represent the relative contributions of species-specific effects and species interactions, respectively, on respiration rates. None of these coefficients were significantly greater than zero.

(22–55 days old) were better able to mineralize carbon in the growth medium. This result could not be explained by differences in the taxon pools' carbon metabolic profiles. Rather, the increase in strains' average respiration rates during this period coincided with the greatest rates of prey capture by the pitcher leaf [32,46]. It is possible that the relatively low respiration rates of late-stage bacterial communities reflect an adaptive strategy for living in nutrient-poor pitcher environments. This is supported by the observation of lower average ribosomal RNA copy numbers—a trait correlated with growth rate—as succession proceeds [32,47]. However, information on all strains' relative performances across different nutrient concentrations would be required to experimentally verify this hypothesis. A recent study found that both BEF effects and competitive interactions decreased in bacterial microcosms over time, as highly productive taxa were outcompeted by specialists capable of efficient use of recalcitrant resources [42]. This observation mirrors and lends support to

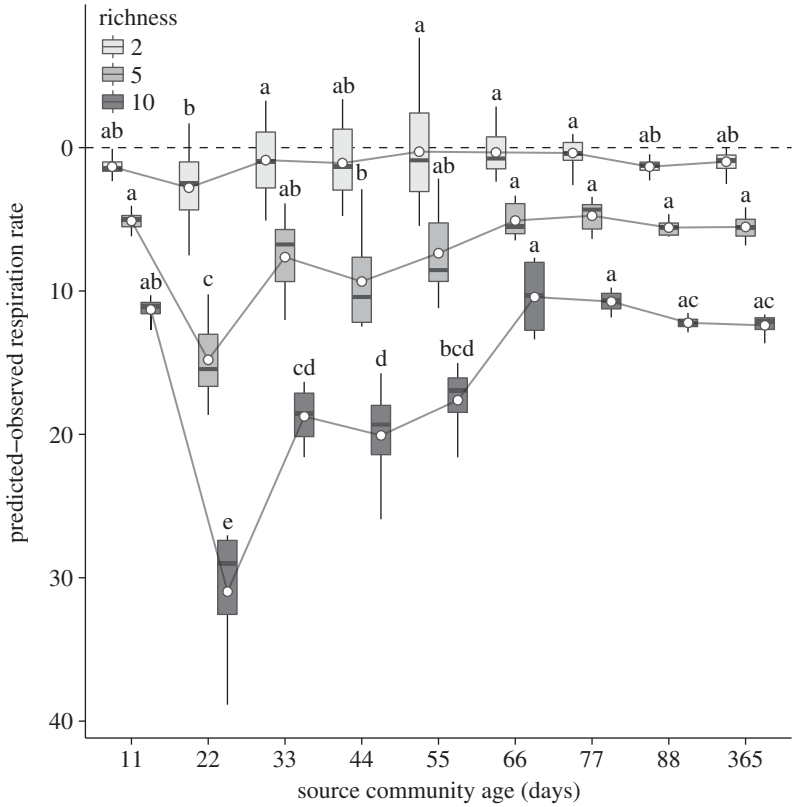

Figure 4. Relative inhibition of bacterial respiration in polycultures measured as the difference between additive predicted and observed rates. Values of zero indicate that the sum of community members' respirations in monoculture equalled the community's performance in polyculture. Values greater than zero indicate that observed rates were less than predicted rates and provide evidence for interspecific competitive or antagonistic inhibition. The *y*-axis has been reversed to more clearly illustrate this inhibition. Letters shared by points within a richness group indicate that their means (white points) do not significantly differ from one another (Tukey's range test, $p < 0.05$). Shading denotes richness treatments.

my results, and suggests that the community dynamics observed in closed microcosms may approximate those from more natural systems.

The effects of a community's richness on respiration rates were generally positive, but varied over time such that the slope estimates peaked in pitcher leaves of intermediate age. These positive BEF relationships appeared to be driven by linear, additive contributions of taxa, as evidenced by strong positive linear richness terms, but weak nonlinear richness and species impact terms. This observation implies that, on average, community members had similar relative respiration rates and low levels of niche overlap. This interpretation is supported by the lack of dominance by any one or more strains in 10-strain communities, which would be predicted to lead to significant species impact terms. Inhibition of a community's potential additive respiration rates was common in all polycultures and peaked in communities assembled from intermediate-aged pitcher leaves. This observation, combined with an absence of direct antagonistic interactions, provides evidence for diffuse competition limiting a strain's potential respiration in polyculture. Although I failed to detect significant negative nonlinear richness terms indicative of strong competition, I did encounter an increase in the effect of nonlinear richness coinciding with the periods of highest respiration inhibition. This general pattern of diffuse competition in polycultures is commonly found in bacterial microcosm experiments [40,48] but may not be typical of bacteria within pitcher plants owing to my isolation procedure. By using a single medium to isolate bacteria, it is likely that the strains I sampled were more phenotypically similar to one another than to a random sample of all bacteria in a pitcher leaf. Thus, the strains used in this study should be considered members sampled from a guild of aerobic, heterotrophic bacteria and are expected to compete with one another for resources and express similar rates of carbon respiration. However, this is no different from most plant and microbial BEF studies, which commonly draw inference at the guild level. A useful follow-up to this experiment would investigate the effects of increasing the phenotypic diversity of the taxon pool by adding strains obtained using a broader range of media.

Competition among isolates is predicted to decrease in bacterial communities over time owing to divergent evolution and can lead to changes in ecosystem functioning [49–51]. The relatively low levels of competitive inhibition among strains from late-stage pitcher leaves may represent indirect evidence of divergence. This scenario is plausible, given the rapid generation times and population sizes of the isolates. A recent study by Fiegna *et al*. [48] showed that the experimental evolution of bacterial isolates over five weeks can alter the BEF relationship via a relaxation of competition. Although such an effect is possible in natural systems, its demonstration would require tracking individual bacterial lineages over time and regularly assaying their competitive interactions. Miller & Kneitel [52] attempted this by measuring the degree of competitive inhibition of four bacterial colony morphotypes isolated from the same pitcher leaves 7 and 42 days after opening. The authors found that the competitive abilities (relative to a common bacterial competitor) of two of the four strains decreased with pitcher age while two did not appear to change [52]. These results match my observation of increased competitive inhibition of potential respiration on a similar timescale (11- and 44-day leaves).

(b) Potential drivers of biodiversity–ecosystem function relationships

To date, few studies have directly estimated the impacts of natural successional dynamics in the context of BEF [26,53,54]. Using 15 years of observational data from regenerating tropical forest plots, Lasky *et al*. [26] documented a decreasing effect of species richness on rates of above-ground biomass production in mid- and late-successional tropical forest plots. These results matched both theoretical predictions [14] and experimental studies in which diversity effects were tracked over time within individual microcosms without immigration [18,22]. My results conform to those of other BEF time-series experiments, despite marked differences in design. In concert, these findings challenge the common observation that the effects of richness on productivity become more positive over time [21], though further investigation is necessary to uncover the mechanisms leading to these contrasting outcomes.

One mechanism for generating non-positive BEF relationships is the negative selection effect [7,27]. This phenomenon occurs when the competitively dominant taxa in a community are those that contribute least to the measured ecosystem function. Three lines of evidence from my experiments suggest that the negative selection effect does not occur in late-stage source communities. First, I did not detect any trends towards increasing rates of competitive exclusions in late-stage source communities. Second, these communities had some of the smallest nonlinear richness (i.e. species interaction) terms and extents of inhibition. These lines of evidence signify a low contribution of negative species interactions to the diminished respiration in late-stage pitchers [39]. Further study, however, is needed to determine: (i) whether observed successional decreases in competition result from decreasing niche overlap within late-stage communities; and (ii) the relative influence of competition versus habitat filtering during different stages of ecosystem development and how these factors, acting historically, contribute to contemporary community structure.

5. Conclusion

All previous experimental studies measuring the BEF relationship over time do so using communities with finite resources and no immigration. Consequently, the closed nature of these systems may have influenced the resulting community dynamics and ecosystem processes. My study, however, measured individual 'snapshots' of communities assembled from a temporal gradient of natural, open source pools. Furthermore, my microcosms were assembled with equal starting concentrations of bacterial strains and resources, which may have prevented communities from becoming resource limited prior to measuring their respirations. Despite these differences, however, decreases in microbial BEF relationships of both static species pools over time and dynamic species pools at a single time point suggest that similar ecological processes may govern these patterns in microbial communities.

In leaves of the pitcher plant *D. californica*, bacterial degradation of organic matter is a process critical for the uptake of prey-derived nitrogen and phosphorous in the nutrient-poor habitats to which these plants are adapted. Using bacterial strains isolated from pitcher leaves at regular intervals over a 1 year period, I determined the magnitude of the BEF relationship to peak in mid-successional communities. This positive richness effect on respiration was driven primarily by strains' relatively equivalent contributions to ecosystem function. At the same time, respiration was constrained by diffuse competition among strains in polyculture. This study represents an initial attempt to integrate BEF effects over successional time and concludes that the functional consequences of diversity loss on a host or ecosystem may vary along a successional gradient. Future studies on BEF relationships are encouraged to adopt a dynamic species pool framework to improve the generalizability of their results.

Data accessibility. The datasets supporting this article have been uploaded as a part of the electronic supplementary material.

Authors' contributions. D.W.A. conceived of the study, collected all data, performed the analyses and drafted the manuscript.

Competing interests. I declare I have no competing interests.

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