Electronic supplementary material for

Armitage, D.W. Time-variant species pools shape competitive dynamics and biodiversity-ecosystem function relationships.

Supplementary material and methods

Sample collection & strain isolation

At the start of the growing season in 2014, I identified and tagged 5 emerging pitcher leaves of equivalent developmental stage, taking care to select leaves off different individual plants (separated by at least 25 m). The exact date of each pitcher's opening was recorded, allowing me to return to tagged leaves throughout their lifespan and know the precise age of their associated communities. I visited this cohort of leaves every 11 days to collect samples of pitcher fluid. To remove pitcher fluid without damaging the leaf, I used a vacuum aspirator to gently agitate the pitcher contents and then siphon 0.5 mL pitcher fluid into a sterile centrifuge tube. I performed this on all 5 pitchers, sterilizing the tubing and rubber stopper with 70% ethanol and a 4% bleach-tween solution between samples. When pitcher leaves did not contain enough fluid to be aspirated, I added 2 mL of sterile phosphate buffer to the leaf, waited 5 minutes, and then collected a 0.5 mL sample. The centrifuge tubes were placed on ice and immediately returned to the lab. I replaced the fluid collected from each pitcher with an equal volume of sterile phosphate buffer to minimize damage to the leaf tissue and its associated aquatic food web. Because of the high bacterial abundances in open pitchers (10^7 – 10^9 cells mL^{-1}), disturbance to the food web was likely minimal and was unlikely to influence pitcher community composition more than the marked temporal variation previously documented in undisturbed pitchers [1].

I spread dilutions of each leaf's contents onto individual R2A agar plates and incubated them at 25° C. After 48 hours, I isolated all visually distinct colony phenotypes onto their own plates based on colony morphology. I verified the genetic basis for these

isolates' morphologies by serially re-plating them three times and observing no phenotypic changes. I selected bacterial strains to be used in the experiment based on their unique colony morphologies/pigmentations and cell morphologies (viewed under 1000× phase contrast microscopy). From this remaining pool of bacterial isolates, I selected the 10 most abundant strains to represent the pool of isolates for a given pitcher age (table S1). I repeated this process sampling from the same five leaves every 11 days until day 88, and then returned to the population in June 2015 to resample the senescing leaves for a 365-day time point. Although 16s ribosomal RNA identification of colony isolates would verify the unique identities of each strain, such information would not provide significant additional insight. Even if ribosomal RNA sequences suggested two isolates belonged to the same operational taxonomic unit (defined as 97% similarity in 16s rRNA), there is no guarantee that they share similar ecological niches, particularly given their divergence in phenotypic characters [2]. I collected samples from the same five leaves every 11 days until day 88, and then returned to the population in June 2015 to resample the senescing leaves for a 365-day time point.

When assembling artificial communities, pooling isolates from pitchers of a given age was necessary, as there were never 10 distinct cultivable phenotypes fitting my selection criteria within a single leaf, although I attempted to minimize this bias by preferentially selecting strains originating from the same pitcher leaves. Despite this caveat, strain overlap among pitchers appeared high based on colony morphology, and both plating and rRNA amplicon surveys of pitchers revealed their communities to strongly cluster by age [1,3]. In addition, the majority of sequenced isolates from a previous experiment matched the most abundant strains from culture-independent surveys [1,3]. Supplemental figure S1 provides a graphical walkthrough of the experimental procedure.

Microcosm experimental protocols

The bacterial strains used to seed the experimental communities were grown in shaken R2A broth to mid-log phase and diluted using PBS to an OD_{600} of 0.25. Aliquots of these isolates were then centrifuged at $10,000 \times G$ for 5 minutes at $4°$ C. I removed the supernatant broth from these samples and washed the pellet of R2A medium by adding 1 mL sterile PBS, re-suspending the pellet, and centrifuging it again. This step was repeated twice and the pellets were left to starve for 2 hours at room temperature to consume any residual medium. Pellets were then resuspended and seeded into 1.2 mL 96-well plates containing 700 μL of autoclaved medium comprised of M9 salt solution (NH_4Cl 1 g L^{-1}, Na_2HPO_4 6 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl 0.5 g L^{-1}, pH 6.0) and 3 g L^{-1} of powder from ground, freeze-dried crickets. 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 approximately 10^4 colony forming units). Once assembled, plates were sealed using a sterile, perforated rubber gasket. I placed over this gasket an inverted 96-well plate containing cresol red dye (12.5 μg mL^{-1}), KCl (150 mM) and $NaHCO_3$ (2.5 mM) set in 1% purified agar. CO_2 respired by the microbial communities passes through the perforated gasket where it reacts with the agar following the equation $CO_2 + H_2O + HCO_3^- \rightarrow 2\ CO_3^- + 3\ H^+$. This redox reaction induces a colorimetric change in the dye that can be read on a 96-well spectrophotometer [4].

All experimental communities from a source pitcher age were run simultaneously on at $25°$ C for three days. I estimated percentage of CO_2-C in each agar well by measuring its absorbance at 570 nm and comparing these values to a calibration curve. Next, I estimated the rate of carbon respired by each community using the ideal gas formula

$$\mu g\ CO_2 - C\ mL^{-1}\ d^{-1} = \left\{ \frac{\frac{\%CO_2}{100} \cdot H \cdot \frac{44}{22.4} \cdot \frac{12}{44} \cdot \frac{273}{273+T}}{V} \right\} \cdot \frac{1}{t} \qquad (1)$$

where H is the headspace volume of the culture well (400 μL), T is the temperature (25° C), V is the volume of medium (800 μL), and t is the incubation time (3 days). After three days, I removed the colonies and plated the 10-strain communities onto agar in order to assess whether extinctions had taken place. This procedure was repeated every 11 days using different pools of isolates collected from the same pitchers.
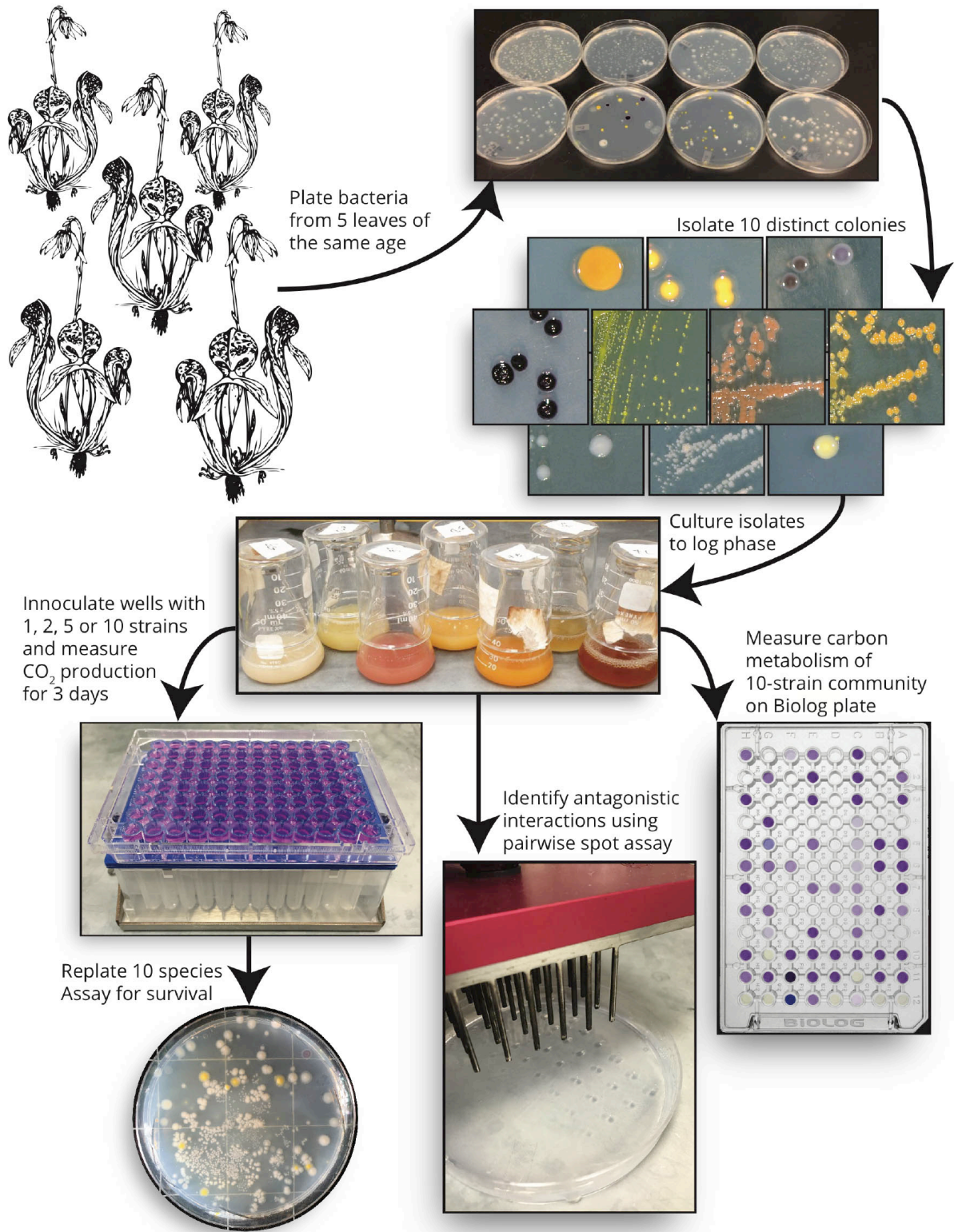
Community metabolic fingerprinting

I measured the carbon metabolic profiles of each 10-strain community using the GN2 microplate assay (Biolog, Inc.). I inoculated each dilute, starved, 10-strain mixture onto the 96-well GN2 plate, which consisted of 95 unique carbon compounds and a blank. The inoculum contained dye that turns violet when reduced. The optical absorbance at 570 nm of these wells is proportional to the productivity of the community on the particular substrate. The pattern of metabolite use provides a unique metabolic "fingerprint" that can serve as a basis for the comparison of different communities. I performed this assay on each 10-strain community in triplicate. After three days of incubation at 25° C I visually scored each well. If a violet color had developed in the same well across the three replicates, I scored that substrate as an electron donor for at least one strain in the community. With these data, I constructed a logical matrix from which I calculated the pairwise Jaccard distances between each community's carbon metabolic profiles and ordinated these distances using Principal Coordinates Analysis (PCoA).

Supplementary references

1. Armitage, D. W. 2016 Microbes in time: incorporating bacteria into ecosystem development theory. Ph.D thesis. UC Berkeley.

2. Shapiro, B. J., Friedman, J., Cordero, O. X., Preheim, S. P., Timberlake, S. C., Szabó, G., Polz, M. F. & Alm, E. J. 2012 Population Genomics of Early Events in the Ecological Differentiation of Bacteria. *Science* **336**, 48–51. (doi:10.1126/science.1218198)

3. Petrosky, A. 2015 Local adaptation of bacteria and bacteriophage in California pitcher plants (*Darlingtonia californica*). Honor's Thesis, UC Berkeley.

4. Campbell, C. D., Chapman, S. J., Cameron, C. M., Davidson, M. S. & Potts, J. M. 2003 A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil. *Appl. Environ. Microbiol.* **69**, 3593–3599. (doi:10.1128/AEM.69.6.3593-3599.2003)

Plate bacteria from 5 leaves of the same age

Isolate 10 distinct colonies

Culture isolates to log phase

Innoculate wells with 1, 2, 5 or 10 strains and measure CO_2 production for 3 days

Measure carbon metabolism of 10-strain community on Biolog plate

Identify antagonistic interactions using pairwise spot assay

Replate 10 species Assay for survival

Supplementary figure S1. Graphical flowchart of the experimental procedure. This process was repeated every 11 days until day 88 using the same pitcher leaves. Five additional year-old leaves were also sampled.
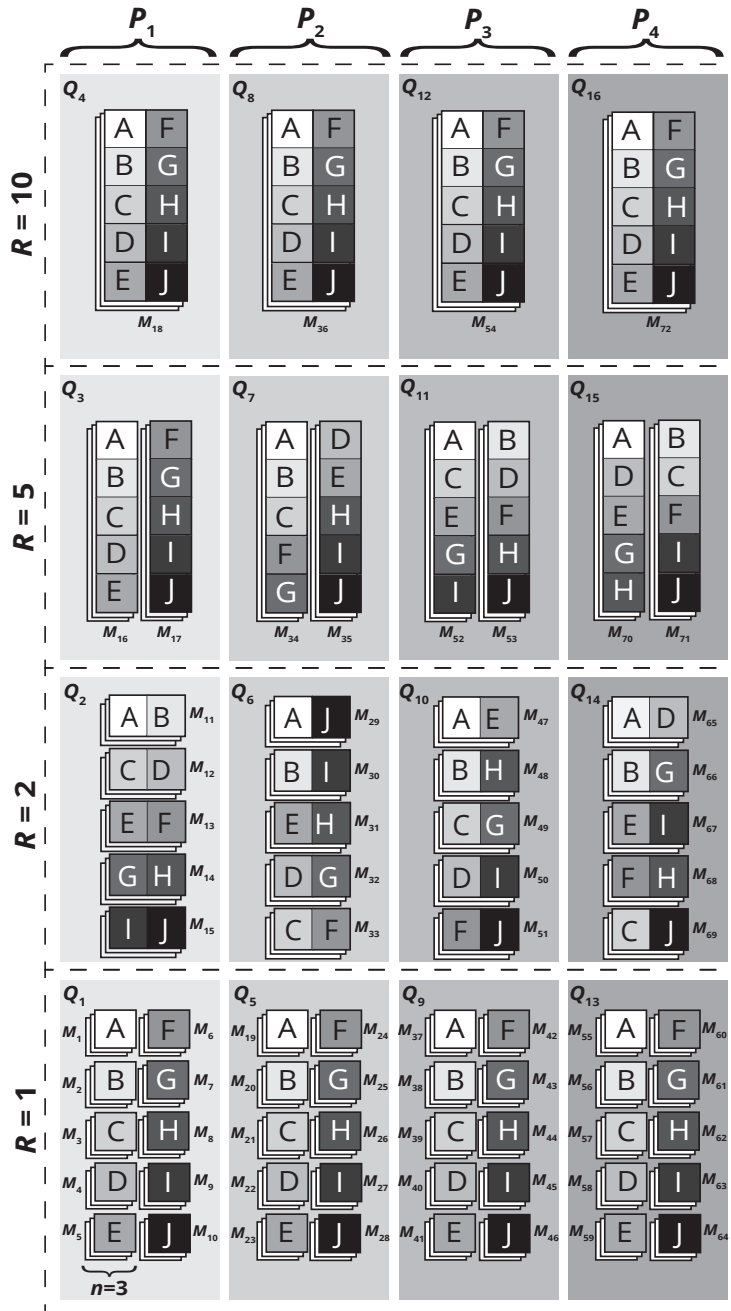
Supplementary figure S2. Diversity-ecosystem function experimental design. Each lettered box represents one of 10 individual bacterial strains isolated from a source pitcher leaves of a particular age. Each partition *P* represents a unique assignment of species to communities, *M*, where a species is chosen without replacement. These communities span 4 levels of species richness and are replicated three times per species pool *Q*. This design was used for each set of bacterial isolates from each source community age.
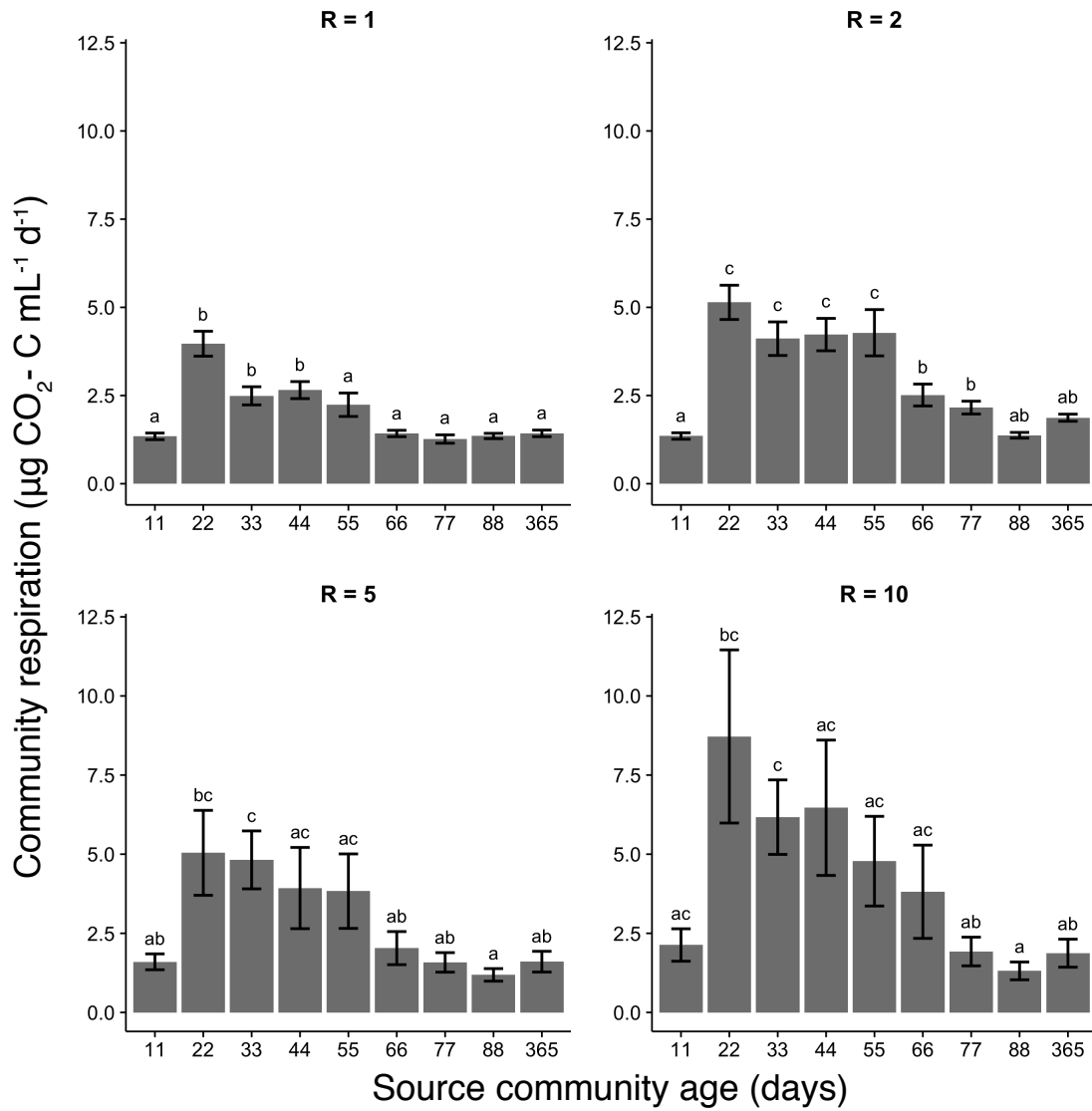
Supplementary figure S3. Mean rates of carbon mineralization for synthetic bacterial communities peaked in mid-successional communities. Different letters within each richness level indicate significant differences between ages according to Tukey's range test ($p < 0.05$). Bars denote standard errors.
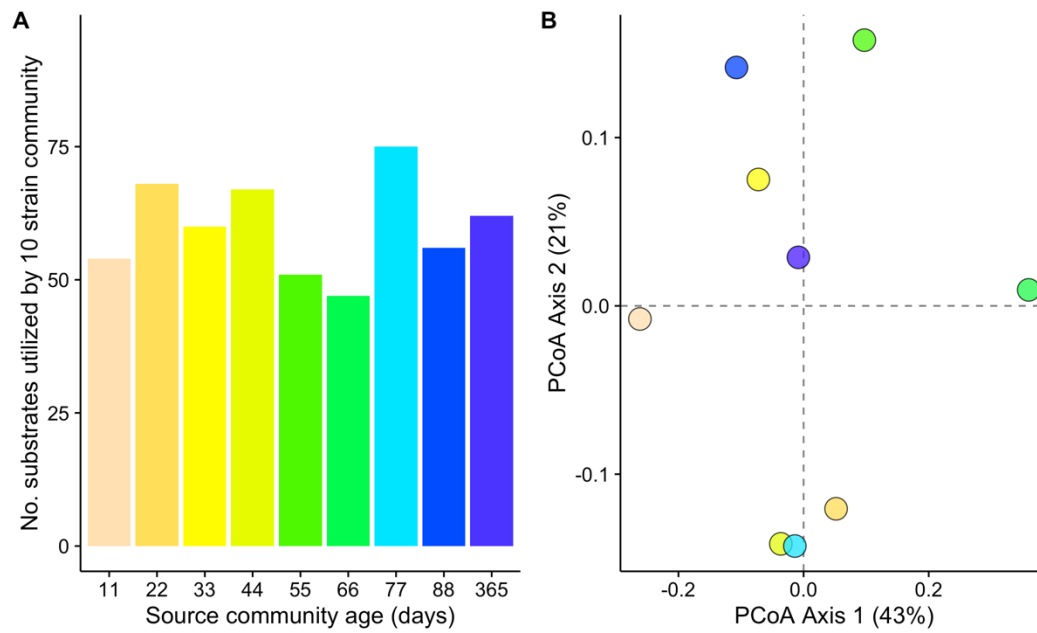
Supplementary figure S4. (a) The number of carbon substrates metabolized by each 10-species community on the Biolog GN2 microplate. (b) Jaccard distances between each community's carbon utilization profile plotted on first two principal coordinates. Colors represent source community ages as in (a).