CONCEPTS & SYNTHESIS
EMPHASIZING NEW IDEAS TO STIMULATE RESEARCH IN ECOLOGY

Ecology, 99(3), 2018, pp. 550–556

Toward more robust plant-soil feedback research

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Abstract. Understanding if and how plant-soil biota feedbacks (PSFs) shape plant communities has become a major research priority. In this paper, we draw on a recent, high-profile PSF study to illustrate that certain widely used experimental methods cannot reliably determine if PSFs occur. One problem involves gathering soil samples adjacent to multiple conditioning plants, mixing the samples and then growing phytometers in the mixtures to test for PSFs. This mixed soil approach does not establish that the conditioning plant being present *caused* the soil biota to be present, the first step of a PSF. Also, soil mixing approximates replacing raw data with averages prior to analysis, a move certain to generate falsely precise statistical estimates. False precision also results from sample sizes being artificially inflated when phytometers are misinterpreted as experimental units. Plant biomass ratios become another source of false precision when individual plant values contribute to multiple ratio observations. Any one of these common missteps can cause still living null hypotheses to be pronounced dead, and risks of this increase with numbers of missteps. If soil organisms truly structure plant communities, then null hypotheses indicating otherwise will not survive proper testing. We discuss conceptual, experimental and analytical refinements to facilitate accurate testing.

Key words: biodiversity; data quality; experimental design; log response ratio; nutrient acquisition strategy; phytometer; plant traits; plant-soil feedback; pseudoreplication; soil biota; type 1 error.

Introduction

Researchers are increasingly investigating the role of soil biota in regulating plant range expansions/invasions (Engelkes et al. 2008, Reinhart et al. 2010b), species diversity (Klironomos 2002, Mangan et al. 2010, Liu et al. 2012, Bennett et al. 2017) and diversity-productivity relationships (Schnitzer et al. 2010, Maron et al. 2011). Plant-soil feedbacks (PSF) are the main means by which soil organisms are hypothesized to impact plants. PSFs occur when plants foster buildups of organisms in their soil surroundings that facilitate or inhibit recruitment of their own or other species (Bever 1994). PSFs are often termed positive or negative depending on whether plants perform (i.e., grow, survive) better in their own or other species soil, respectively.

In order to more mechanistically understand PSFs and better predict when they will be negative, neutral and positive, recent studies have investigated relationships between PSFs and plant traits (e.g., Baxendale et al. 2014, Cortois et al. 2016, FitzPatrick et al. 2017). In this vein, it has recently been theorized that nutrient acquisition strategy (NAS) is a plant trait explaining variation in PSFs (Laliberte et al. 2015, Laliberte 2017). A study recently published in *Science* (Teste et al. 2017), and featured in a popular-press article in that same journal (van der Putten 2017), purports to provide evidence for this theory. In this paper, we illustrate that the experimental and analytical methods of Teste et al. can neither confirm that PSFs occur nor that they vary by NAS. One reason for pointing this out is to prevent this high-profile study from misdirecting understanding of soil biota effects on plants. More importantly, however, except for a problem with missing terms in their statistical model, none of the issues we discuss are at all unique to Teste et al. By explaining the problems, we hope to steer researchers toward more reliable methods and more accurate interpretation of studies.

We begin by explaining experimental approaches and findings of Teste et al. We then illustrate that some of their key methods, though widely used, cannot be considered reliable. Next, we reanalyze their data to show how experimental issues are compounded by unmet statistical assumptions. We close by highlighting some conceptual, methodological and analytical refinements to improve PSF research.

Experiment of Teste et al

Methods

In Australian shrublands, Teste et al. (2017) gathered soil cores within 1.0 m of 7 individuals of 26 shrub species. Each species had one of five NASs: arbuscular mycorrhizal, ectomycorrhizal, ericoid mycorrhizal, N-fixing, or nonmycorrhizal, cluster-rooted. Soil samples were combined by NAS to create five soil mixtures (Appendix S1: Fig. S1). The

Manuscript received 19 July 2017; revised 22 November 2017; accepted 20 December 2017. Corresponding Editor: Joseph B. Yavitt.
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arbuscular mycorrhizal mixture, for example, contained soil gathered near 49 individual shrubs = (seven individuals per arbuscular mycorrhizal shrub species) × (seven arbuscular mycorrhizal shrub species). To assess feedbacks, seedlings (i.e., phytometers) of 16 of the 26 species were grown in glasshouse pots containing the NAS mixtures. Except for ericoid mycorrhizal phytometers, phytometers representing each NAS were grown. When phytometers were grown in soil from their own NAS, conspecific soil was excluded from the NAS mixture. In addition to NAS mixtures, one live and one sterile conspecific soil mixture was formed for each of the 16 species by combining soil from the seven individuals per species, and the 16 species were grown in their conspecific soil mixtures. Each combination of phytometer species and soil mixture was replicated 10 times: 70 pots per phytometer species = 10 replications × (one live conspecific mix + one sterile conspecific mix + five NAS mixes). Survival and biomass of phytometers were measured following nine months of growth.

Key findings

Important results are reproduced in Figs. 1a, b and 2a–d. In short, Teste et al. concluded that PSFs occur in their system and that the direction (positive vs. negative) of PSFs varies with the NAS of the phytometer and the NAS of the soil mixture.

Methodological issues.—PSFs occur in two steps: (1) plants alter soil microbe compositions, and (2) the alterations in turn impact plant performance (Bever 1994, Ehrenfeld et al. 2005). Combining soil samples by NAS and species prevented Teste et al. from confirming step 1. Soil microbe compositions can vary widely across small spatial scales (Monard et al. 2016, Zhang et al. 2016, Ping et al. 2017, Seuradge et al. 2017, Wang et al. 2017), so some sampled microbial species may have occurred near just one or a few field plants. Even microbes occurring near a single plant would presumably be distributed to 20 conspecific glasshouse pots and ≥150 NAS mixture pots (Appendix S1: Fig. S1). Particular microbes could occur near a few plants just by chance, not because particular plant species *caused* the microbes to be present, so the Teste et al. data do not confirm the first step of a PSF (Bever 1994). Other studies following similar methods suffer similar inconclusiveness (e.g., van der Putten et al. 1993, Callaway et al. 2013, Valliere and Allen 2016). A related problem is that more soil cores were used to construct heterospecific than conspecific soil mixtures (i.e., 49 vs. seven cores), so heterospecific mixtures had greater probabilities of supporting beneficial and harmful microbes purely by chance. These issues can be avoided by subjecting each phytometer to soil gathered near one unique plant. If such phytometers show consistent evidence of associating with/responding to microbes, this provides at least some assurance the plant species may have cultured the microbes.

Instead of different plants growing with different microbial species, another possibility is every member of a plant

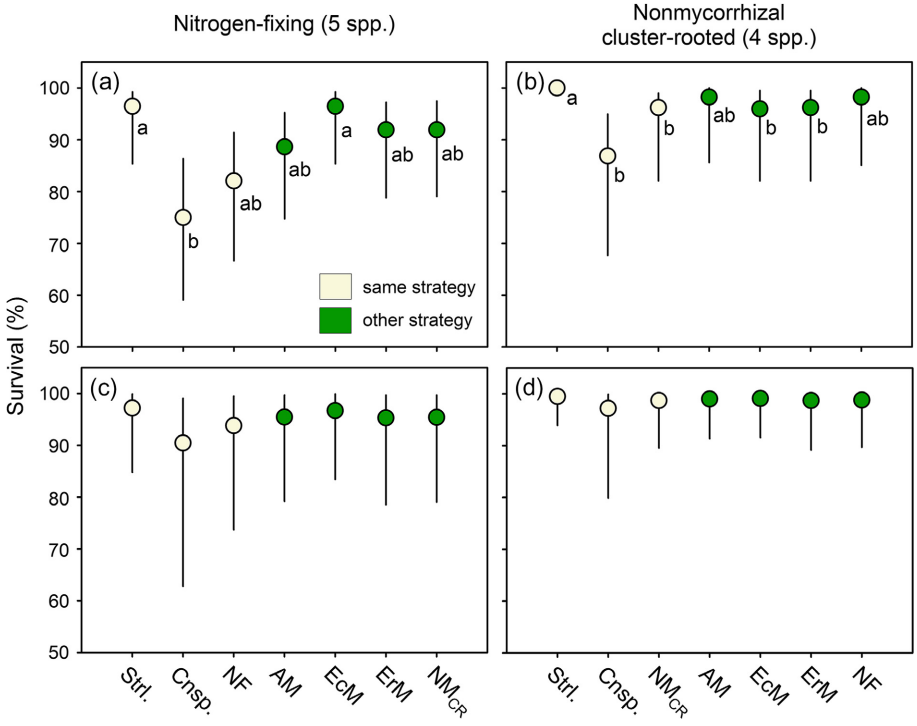

FIG. 1. Point estimates (dots) and 95% confidence intervals (bars) estimating average survival rates across five nitrogen-fixing species and four nonmycorrhizal species grown in soil mixtures. Panels a and b depict estimates from Teste et al., and panels c and d depict estimates from a reanalysis that corrected unmet statistical assumptions. Sterilized (Strl.) and not sterilized (Cnsp.) conspecific soil mixtures were formed by combining soil gathered near conspecific individuals. Heterospecific soil mixtures were formed by combining soil gathered near nitrogen-fixing (NF), arbuscular mycorrhizal (AM), ectomycorrhizal (EcM), ericoid mycorrhizal (ErM), and nonmycorrhizal cluster rooted (NM_{CR}) plants. Bars with different letters are significantly different ($P \leq 0.05$).

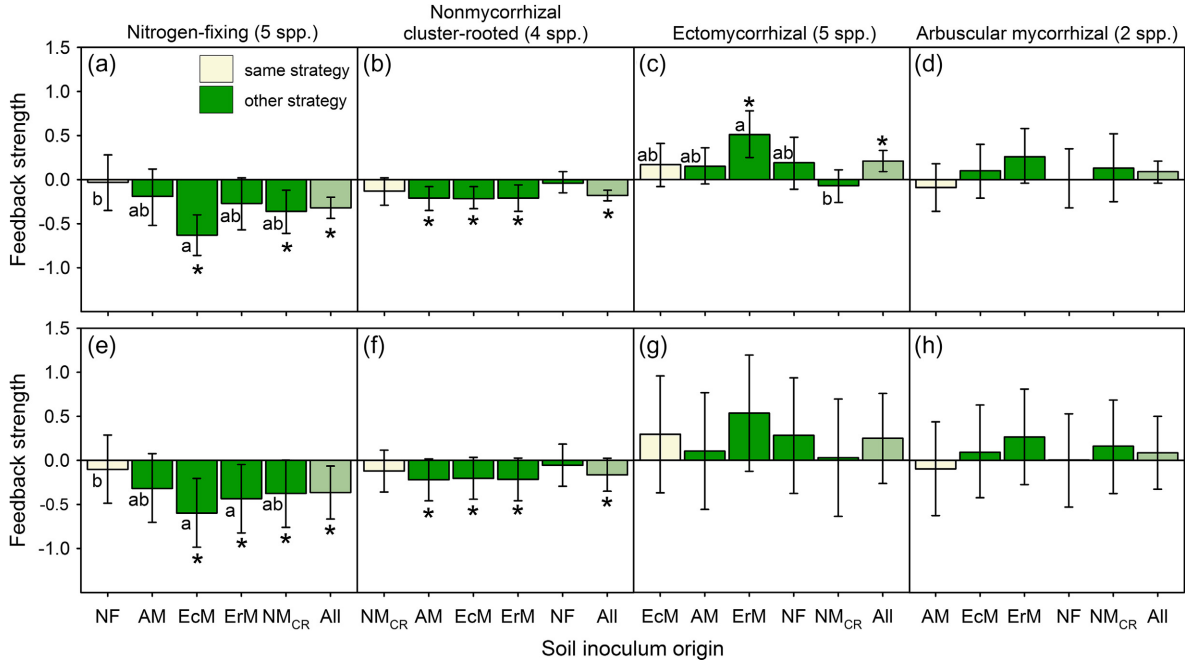

FIG. 2. Point estimates (dots) and 95% confidence intervals (bars) estimating plant soil feedbacks. Panels a–d depict estimates from Teste et al., and panels e–h depict estimates from a reanalysis that corrected for correlated residuals in the Teste et al. analysis. Soil mixtures were formed by combining soil gathered near nitrogen-fixing (NF), arbuscular mycorrhizal (AM), ectomycorrhizal (EcM), ericoid mycorrhizal (ErM), and nonmycorrhizal cluster rooted (NM_{CR}) plants. "All" values estimate feedbacks averaged over heterospecific soil inocula. The number of phytometer species "spp." subjected to soil mixtures varied by NAS. Bars with different letters are significantly different, and asterisks denote significant differences from zero ($P \leq 0.05$).

species grows with the same beneficial and/or harmful microbes, and variation in microbe densities causes variation in phytometer performance (e.g., Reinhart et al. 2010a). It has been asserted that in this case mixed soil sample approaches can reliably identify PSFs (Cahill et al. 2017, Gundale et al. 2017), but this is untrue (Rinella and Reinhart 2017, Smith-Ramesh and Reynolds 2017). We have recently shown that mixing soils across experimental units in the manner of Teste et al. and many other studies (e.g., Felker-Quinn et al. 2011, Hilbig and Allen 2015) creates a mismatch between the data generating process and whatever statistical model is used to analyze the data (Rinella and Reinhart 2017). Growing phytometers in mixtures of soil from multiple experimental units approximates the unusual approach of growing phytometers in soil from individual experimental units (e.g., plots, sites, field shrubs) and then replacing the raw phytometer data with corresponding treatment (e.g., fertilizer, region, shrub NAS) means before analysis, an approach guaranteed to generate falsely precise inferences (Rinella and Reinhart 2017).

Statistical analysis of Teste et al

Due to the experimental issues raised above, any analysis of the Teste et al. data will give suspect conclusions. However, we nevertheless reanalyzed the Teste et al. data to explore two issues with their analysis. One issue involves terms missing from their model, an issue appearing somewhat unique to Teste et al. The other issue involves the use of a biomass ratio that elevates Type I error rates, an issue compromising other studies (Brinkman et al. 2010).

Survival analysis

Teste et al. reported phytometer survival rates varied by soil mixture (Fig. 1a, b). However, because their survival model excluded soil mixture × phytometer species interactions, they assumed species sharing the same NAS shared the same survival rate when exposed to the same soil mixture, and this assumption was unmet. For example, survival of species with the N-fixing NAS ranged from 20 to 100% in conspecific soil mixtures. Like Teste et al., we fit a binomial mixed effects regression model to data on the two phytometer types Teste et al. identified as exhibiting survival differences (N-fixing and nonmycorrhizal). In addition to terms of the Teste et al. model (i.e., species, soil mixture), our model included a term for the evident soil mixture × phytometer species interaction (Appendix S2).

Our reanalysis provided no evidence that survival rates varied by soil mixture (Fig. 1c, d). As such, important conclusions of Teste et al. are incorrect. Unfounded conclusions include "Plant survival. . .(was) strongly influenced by the origin of the soil inoculum, and the effects varied among nutrient-acquisition strategies" and "Survival of N-fixing and nonmycorrhizal cluster-rooted plants declined when inoculated with conspecific soil, suggesting a response to soil-borne pathogens or other antagonists."

Feedback analysis

The Teste et al. feedback response was $\log_{10}\frac{y_{i1k}}{y_{ijk}}$, where $i = 1, 2, \ldots, 16$ and $k = 1, 2, \ldots, 10$ index species and replications, respectively, y_{i1k} is biomass growth in unsterilized

conspecific soil and y_{ijk} is growth in the five, $j = 2, 3, 4, 5, 6$, heterospecific soil mixtures (Fig. 2a–d). Accordingly, each phytometer grown in conspecific soil formed the numerator of five observations. Such observation groups are known to exhibit residual correlation (Kulmatiski et al. 2008), so unsurprisingly, we found *t*-tests of Teste et al. violated the independence assumption ($P < 0.01$). Our reanalysis used $\log_{10} y_{ijk}$ as the response instead of $\log_{10} \frac{y_{i1k}}{y_{ijk}}$ (Appendix S2). With this response, regression coefficients retain units of Teste et al. $\left(\text{i.e. } \log_{10} \frac{y_{i1k}}{y_{ijk}}\right)$, and the residual correlation is eliminated. For N-fixing ($P = 0.05$) and ectomycorrhizal ($P = 0.01$) phytometer species, there was also residual correlation owing to soil mixture × species interactions, so we included this interaction in our model (Appendix S2).

For nitrogen-fixing and nonmycorrhizal cluster rooted phytometers, our reanalysis largely agrees with Teste et al. (Fig. 2a, b, e, f), but for other plant groups, our results do not agree with theirs' (Fig. 2c, d, g, h). Their conclusion that "growth of ectomycorrhizal plants was enhanced in conspecific soil" is not supported (Fig. 2). Their claims that "N-fixing plants grew best in ectomycorrhizal soil" and "nonmycorrhizal plants grew best in soil from all three mycorrhizal types" are supported neither by their own analysis nor our reanalysis (Fig. 2). Also, although there were only two to five species per NAS, Teste et al. identified no cases where all species of a given NAS exhibited positive or negative PSFs in a soil mixture (Fig. S7 of Teste et al.), and our reanalysis confirms this finding (data not shown). Concerningly, the Teste et al. analysis and our reanalysis revealed cases in which different species with the same NAS exhibited *opposite* PSFs (some negative, some positive) in the same soil mixture (Fig. S7 of Teste et al.). This removes all confidence that "feedback between plants and their associated soil biota critically depends on nutrient-acquisition strategy."

Recommendations for Future Research

Correctly identify experimental units and treatments

In part, the mistake of combining soils from multiple experimental units stems from confusion over what the experimental units exactly are in PSF studies, confusion not unique to this line of research (Jenkins 2002, Lazic 2010, Prosser 2010). Some studies, like that of Teste et al., subject test plants to soil/biota sampled directly from the field, and other studies first "condition" field soil/biota by growing plants in it. When soils/biota are not conditioned, the experimental units are necessarily dictated by the field soil sampling design. Accordingly, the experimental units of Teste et al. were shrub microenvironments; i.e., 200 [diam.] × 20-cm soil cylinders surrounding shrubs where soil samples were taken. Teste et al. interpreted plants grown in the soil samples as their experimental units, but these plants were simply phytometers used to measure traits of the experimental units. Moreover, Teste et al. interpreted soil mixtures as their "soil inoculum origin treatments," but the treatments were actually the species and NAS of the shrubs inhabiting the microenvironments. More precisely, Teste et al. was a part natural, part experimental study: The shrub species and NAS were natural treatments "applied" to the experimental units, and soil sterilization was an experimental treatment applied to (a sample from) the experimental units. Like Teste et al., Valliere and Allen (2016) and Lozano et al. (2017) also identified mixtures of inocula from multiple experimental units as their treatments, and their experimental units were also actually shrub microenvironments.

PSF studies that begin with a conditioning phase are also prone to misidentifying experimental units. This is most easily understood with studies that condition a soil by growing plants in pots containing the soil. After being conditioned, the soil in each pot becomes an experimental unit. Yet, studies sometimes mix soil from multiple pots and interpret phytometers grown in the mixture as experimental units (e.g., Callaway et al. 2013, Cortois et al. 2016, FitzPatrick et al. 2017, Stanescu and Maherali 2017). When used correctly, each phytometer bioassays a single experimental unit.

Misidentifying experimental units can lead researchers to artificially inflate sample sizes. To quantify how species grew in their own soil, Teste et al. studied 10 phytometers per species. They interpreted the 10 phytometer observations per species as independent data points, even though there were only seven experimental units per species. It is again illuminating to consider the correct approach of subjecting each phytometer to soil from one experimental unit. With the correct approach, having seven experimental units and 10 phytometers would imply three experimental units were double-sampled. Therefore, had Teste et al. not combined soils, three of every 10 observations would have been pseudoreplicates (Hurlbert 1984), and combining soils does not change the underlying pseudoreplication issue. Like mixing soils, pseudoreplication leads to falsely precise statistical estimates. Other studies have also artificially inflated sample sizes as a consequence of interpreting phytometers as experimental units (e.g., Li et al. 2009, 2014, Mangan et al. 2010).

Ecological research articles usually do not explicitly identify experimental units and treatments, likely because these items are often implicitly clear. This is not, however, the case with PSF research, and we believe clearly stating experimental units and treatments could alleviate confusion and lead to better PSF study designs.

Ensure analyses of ratio data meet statistical assumptions, or avoid analyzing ratios

Plant biomass ratios regularly serve as response variables in PSF research (e.g., Perkins and Nowak 2013, Baxendale et al. 2014, Johnson et al. 2016, FitzPatrick et al. 2017, Stanescu and Maherali 2017). A common ratio response is the natural log of one plant weight, y_i, divided by another plant weight, $y_{\neq i}$, $\log \frac{y_i}{y_{\neq i}}$. Whereas correct analysis of this and other ratios is certainly possible, ratios may contribute to confusion over experimental units, because they create atypical situations where individual data points, $\log \frac{y_i}{y_{\neq i}}$, describe observations on multiple experimental units, y_i and $y_{\neq i}$. For this and other reasons, we prefer $\log y_i$ to $\log \frac{y_i}{y_{\neq i}}$ as the response. Model coefficients can be made to retain the $\log \frac{y_i}{y_{\neq i}}$ interpretation when $\log y_i$ is the response. To demonstrate this, we consider the hypothetical experiment of Brinkman et al. (2010) in which $i = 1, 2 \ldots, 10$ plants are grown in soil conditioned by their "own" species and 10

plants $i = 11, 12\ldots, 20$ are grown in "foreign" soil. A common log-ratio analysis would pair treated and control plants, either arbitrarily or by a blocking factor, and then model the $j = 1, 2\ldots, 10$ pairs as

$$\log\frac{y_{\text{own},j}}{y_{\text{foreign},j}} \sim N(\mu, \sigma), \quad (1)$$

where $N(\mu, \text{sigma})$ is the normal distribution with mean μ, standard deviation sigma. Once Eq. 1 is assumed, the null hypothesis $\log\frac{y_{\text{own},j}}{y_{\text{foreign},j}} = 0$ is amenable to a one-sample t-test. Assuming no blocking for simplicity, these same data can be analyzed without pairing using the linear regression

$$\log y_i \sim N(\beta_0 + \beta_1 x_i, \tau), \quad (2)$$

where x_i equals 0 or 1 for plants grown in foreign and own soil, respectively. Both μ and β_1 have the same log-ratio units: $\beta_1 = \log\frac{e^{\beta_0+\beta_1}}{e^{\beta_0}} = \log\frac{y_{\text{own}}}{y_{\text{foreign}}}$. The desired summaries (i.e., standard error or confidence interval for $\log\frac{y_{\text{own},j}}{y_{\text{foreign},j}}$ and test of $\log\frac{y_{\text{own},j}}{y_{\text{foreign},j}} = 0$) are standard output from all major statistical packages: These summaries are those provided automatically for the regression coefficient β_1. Moreover, if $\log\frac{y_i}{y_{\neq i}}$ is normally distributed, then $\log y_i$ and $\log y_{\neq i}$ are normally distributed, so Eqs. 1 and 2 make the same distributional assumptions. While Eqs. 1 and 2 give similar results, Eq. 2 is more easily adapted to complex study designs (e.g., multiple species and soil treatments, blocked designs) where it becomes more difficult to meet statistical assumptions involving ratios (e.g., James et al. 2011).

Another ratio sometimes used is $\frac{y_{\text{own},j}}{\bar{y}_{\text{foreign}}}$ where $\bar{y}_{\text{foreign}}$ is mean plant weight in foreign soil (e.g. Troelstra et al. 2001, Brinkman et al. 2005), and a simulation by Brinkman et al. (2010) indicated this ratio severely elevates Type I errors. We repeated the Brinkman et al. (2010) simulation with two other published ratios, $\frac{y_{\text{own},j}-\bar{y}_{\text{foreign}}}{\bar{y}_{\text{foreign}}}$ (e.g. Bezemer et al. 2006, Kardol et al. 2007) and $\frac{y_{\text{conditioned},j}-\bar{y}_{\text{unconditioned}}}{y_{\text{conditioned},j}+\bar{y}_{\text{unconditioned}}}$ (Perkins et al. 2016), and found these ratios also elevate Type I errors (i.e. to 15% and 40%, respectively, from the nominal rate of 5%). These ratios, like the Teste et al. ratio and other recently published PSF ratios (Heinze et al. 2016, Gomez-Aparicio et al. 2017), lead to residual correlation because individual plants contribute to multiple data points. In our reanalysis of Teste et al., there were advantages to changing the response from biomass ratios to biomass per plant beyond helping maintain intended Type I error rates. In particular, it avoided having to arbitrarily pair numerator and denominator plants and having to discard the many replications where numerator plants died.

Clarity, parsimony and consistency provide added motivation for moving away from ratios. It is often impossible to determine how PSF ratios were calculated based on provided descriptions (e.g., formula and stated bounds for ratio do not match [Gomez-Aparicio et al. 2017, Mehrabi et al. 2015], unspecified if ratio of means or mean of ratios [Stanescu and Maherali 2017], not indicated which if any terms are means [Perkins and Nowak 2013]). Also, biomass ratios encourage redundant analyses. Researchers often fit one model to biomass data and a second model to biomass ratios when all the desired estimates could be obtained from the biomass (or log-biomass) model alone (e.g., Johnson et al. 2016, Schittko et al. 2016, Gomez-Aparicio et al. 2017). Finally, ratios encourage conflicting analytical assumptions. Researchers sometimes simultaneously assume biomass and biomass ratios are normally distributed (te Beest et al. 2009, Baxendale et al. 2014, Perkins et al. 2016), which is logically inconsistent because ratios (and log ratios) of normally distributed random variables are not normally distributed.

Design studies to rule out as many hypotheses as possible

In order to conclude that phytometers grown in unconditioned field soil measure PSFs occurring in nature, it must be assumed that field plants causally determine which soil biota occur where. This is not a trivial assumption: Mounting evidence indicates soil biota compositions vary widely across small spatial scales due to dispersal processes, soil physicochemical properties, soil water content, and other factors (Monard et al. 2016, Zhang et al. 2016, Ping et al. 2017, Seuradge et al. 2017, Wang et al. 2017). Insofar as stochastic or abiotic factors determine which soil biota occur where, phytometers will be misleading indicators of PSFs. For example, if abiotic features determine which microbes occur at which microsites, and plant species preferentially establish at microsites supporting their beneficial microbes, phytometers will incorrectly suggest positive PSFs when grown in their own species soil. Likewise, if plant species and their deleterious microbes co-occur because they both happen to be most fit under the same abiotic microsite conditions, then phytometers will erroneously suggest negative feedbacks. Also, if two plant species are most fit when associated with the same microbes, and the most competitive species preempts microsites occupied by those microbes, the weak competitor will be relegated to areas lacking those microbes, and phytometers of the weak competitor will incorrectly suggest negative feedbacks. These are but a few of the ways in which phytometers grown in unconditioned field soil could incorrectly suggest PSFs.

In short, even when problematic methods we outlined are avoided, data on phytometers grown in unconditioned soil are compatible with several theories, not exclusively PSF theories under test. This is not necessarily to imply the underdetermination of theory by data is so severe the research is meritless, but it does highlight the importance of designing studies to test theories of interest while ruling out as many alternative theories as possible. Given that PSF research is innately predisposed to false positives, methods like those we've critiqued that unnecessarily elevate false positive risks must be avoided. Studies could be made more conclusive without being made larger. Teste et al. could address their research questions with a similarly sized incomplete factorial experiment that subjects each phytometer to soil from one experimental unit. The paradigmatic biodiversity experiments provide a useful analog. These studies replicated species compositions little (Hector et al. 1998) or not at all (Tilman et al. 2001) but extensively replicated species numbers (i.e., species per plot) using random draws from a species pool. With no increase in experiment size, Teste et al. could use a similar approach to replicate

soil treatments extensively at the NAS level while replicating less at the species level. It will be appropriately harder to reject null hypotheses using more reliable designs, but if soil biota truly drive plant community dynamics, null hypotheses indicating otherwise will not survive accurate tests.

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