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The Leinster and Cobbold indices improve inferences about microbial diversity

Stavros D. VERESOGLOU[a,b,*], Jeff R. POWELL[c], John DAVISON[d], Ylva LEKBERG[e], Matthias C. RILLIG[a,b]

[a]Freie Universität Berlin, Institut für Biologie, Berlin, Germany
[b]Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), D-14195 Berlin, Germany
[c]Hawkesbury Institute for the Environment, University of Western Sydney, Australia
[d]Department of Botany, Institute of Ecology and Earth Sciences, University of Tartu, Tartu, Estonia
[e]MPG Ranch and College of Forestry and Conservation, University of Montana, Missoula, MT, USA

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ABSTRACT

An increasing number of ecological studies compare the diversity of microbial taxa along environmental gradients or between imposed treatments. Estimates are often based on analysis-of-variance of taxon-richness inferred from pyrosequencing data. We conducted a reanalysis of three 454-pyrosequencing studies on arbuscular-mycorrhizal-fungal diversity to evaluate the suitability of using the Leinster and Cobbold diversity-indices (LCdis) to assess diversity. We expected that the potential of LCdis to consider phylogenic relationships could resolve problems arising from ambiguous species-delineation in microbial-systems. Our reanalysis showed that comparisons between studies differing considerably in sequencing depth may be risky. Moreover, we show that LCdis not only reproduce the results of analyses of variance but can also resolve issues connected to variation in sequence read number, while additionally representing a less conservative metric of diversity than analysis-of-variance of taxa-richness. Based on these results we advocate the use of inclusive diversity indices in ecological studies targeting microbial communities.

Introduction

Recent decades have increasingly seen ecological theory being tested using microbial systems (Poisot et al., 2013). There are several reasons for this, the most obvious being that shifts in community composition and structure can be observed over small temporal and spatial scales (Jessup et al., 2004). The development of pyrosequencing technologies have greatly contributed to the accumulation of such studies, and will continue to do so, due to their cost-efficiency (per sequence; Rothberg and Leamon, 2008) and their ability to detect rare individuals that cannot be adequately sampled using earlier approaches (Öpik et al., 2009). Although it is common for such studies to incorporate microbial community information in analyses of α- and β- diversity after pooling data, in many studies the unit for analyses of diversity is the replicated sample.

The most common way to summarize diversity information is to apply analysis of variance (ANOVA) to estimates of species richness (e.g. Öpik et al., 2009; Lekberg et al., 2011), which is the only diversity metric robust to potential PCR-related biases in quantitative-community matrices.

* *Corresponding author*. Freie Universität Berlin, Institut für Biologie, Berlin, Germany. Tel.: +49 3083853172.
E-mail addresses: sveresoglou@zedat.fu-berlin.de, seby31@zeroone.net (S.D. Veresoglou).
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Community richness represents one extreme in diversity assessment in which rare species are assigned the same weight as abundant species. The other extreme is the inverse of the Berger and Parker index (Berger and Parker, 1970), in which only abundant species are considered. Both of these extreme approaches for assessing community diversity may be problematic. The former may be misleading since it does not consider shifts in evenness among taxa, a characteristic of communities that has been demonstrated to predict functional resilience (Wittebolle et al., 2009). The latter ignores rare or elusive taxa that may become important under certain environmental conditions, such as in the lead-up to toxic algal blooms (Burkholder et al., 1992; Hooper et al., 2005). Therefore, a robust representation of species abundance is necessary in order to accurately represent diversity in ecological studies of microbes.

Leinster and Cobbold (2012) proposed the use of diversity profiles (a series of multiple diversity estimates that differ in the relative weighting of abundant vs. rare taxa), as opposed to point estimates of diversity (e.g. richness and Shannon diversity index), to visually represent the importance of rare taxa in making comparisons among sample groups. They also used an extension of Hill numbers (Hill, 1973) to account for gradients in similarity among species; diversity profiles may then compare naive diversity profiles to profiles that explicitly account for genetic or functional variation among species within communities. A major novelty of these indices is their ability to consider phylogenetic relationships between taxa; according to the non-naive version of the indices a site with a given number of species that belong to a single genus is typically less diverse than another site with an equal number of species that belong to different genera. This attribute may be of particularly high applied value in microbial ecology, where considerable uncertainty surrounds approaches to species delineation (e.g. Stockinger et al., 2010). Here we use the term "inclusive diversity indices" to describe metrics that address diversity on the basis of diversity profiles, i.e., considering the entire range of weighting applied to rare (richness) and abundant (evenness) species. The result is a generalized comparison of diversity between communities that requires no *a priori* assumptions about the importance of species abundance.

Here, we use the approach of Leinster and Cobbold (2012) to reanalyze data from published studies that have applied next-generation sequencing. We had three objectives. First, we highlight the importance of standardizing sequencing depth in pyrosequencing studies before conducting assessments of diversity. While the sample size dependency of diversity metrics such as species richness has been known for a long time (e.g. Smith and van Belle, 1984) this is a component of bioinformatics that has not been sufficiently stressed even within the latest pyrosequencing user guides (e.g. Lindahl et al., 2013). Then we assess whether the results of recent next-generation sequencing studies are comparable to earlier studies that were based on lower numbers of sequences and which consequently detected a much smaller fraction of each community. This objective is important as it will allow researchers to determine whether the inferences made from Sanger sequencing studies are comparable to those made from pyrosequencing studies. Finally, we assess whether the conclusions that had been reached through implementation of traditional comparison techniques such as ANOVAs would be robust to consideration of alternative diversity metrics. Specifically we consider the diversity profiles that are obtained from inclusive diversity indices when rare species are not weighted equally to abundance species, i.e., we ask whether attributing variable importance to abundant species might change our view of diversity responses in some cases.

Materials and methods

We focus here on ecological studies of arbuscular mycorrhizal fungi (AMF), ubiquitous obligate symbionts associating with the roots of most terrestrial plant species (Smith and Read, 2008). We chose AMF as a model for our purposes because this system provides a clear sampling criterion delineating a community of interacting individuals (within the root system of a single host plant). This criterion is difficult to satisfy for many ecological studies of microbes, where issues of scale always exist and are dependent on the quantity and properties of the substratum sampled (e.g. Kang and Mills, 2006), which can lead to confused definitions of α- and β- diversity (Whittaker et al., 2001). In addition, a recurrent problem in ecological studies of microbes is the recognition of 'species' and 'individuals' and the delineation of clusters of DNA sequences into these groups (Smith et al., 1992; Powell, 2012). This problem has been shown to have consequences for our understanding of the ecological characteristics of AMF (Krüger et al., 2011; Powell et al., 2011) and other microorganisms (Koeppel et al., 2008). Finally, AMF represent a group of ecologically important microbes that has been studied extensively in the past (Öpik et al., 2010). We did not consider multiple microbial groups as we wanted our datasets to be sufficiently homogenous to be comparable. However, we acknowledge that this limits our ability to generalize our results to other microbial groups.

We reanalyzed two studies that utilized next-generation sequencing to assay AMF communities from plant roots (Lekberg et al., 2011; Becklin et al., 2012). To expand our dataset we further used a study that assayed AMF communities from soil (Davison et al., 2012); at the time when the project was initiated these were the only published next-generation sequencing studies on AMF that both reported abundances and included replicated designs. In all three studies phylogenetic information had been partitioned into operational taxonomic units – OTUs; in our reanalysis we adopted these OTU definitions as a means of delineating species – a surrogate of a species in plants and animals.

Lekberg et al. (2011) used eleven replicates per treatment of plants (*Plantago lanceolata*): (i) subjected to limited disturbance; (ii) disturbed with recolonization from the surrounding AMF community prevented; (iii) disturbed with recolonization from the surrounding AMF community possible; and (iv) mycorrhizal plants adjacent to these units; the native *P. lanceolata* treatment was dropped to generate a balanced dataset (Table 1).

Becklin et al. (2012) included 4–8 replicates per treatment (*Taraxacum ceratophorum*, *T. officinale* and *P. viscosum* harvested from open meadow plants and willow understory habitats) (Table 1).

Table 1 – Comparative statistics for the three studies that were reanalyzed

Study	Locus	Primers	Mean sequencing depth/ sample	Sequence dissimilarity cut-off	Number of samples	Number of treatments	Number of OTUs retrieved	Mean richness	Accessions
Becklin et al., 2012	SSU rRNA	nu-SSU-0817, nu-SSU-1536	12	97 %	36	2 × 3	44	4	SRA023882.1
Davison et al., 2012	SSU rRNA	NS31, AML2	378	97 %	27	4	36	17*	HE659710-HE659982**
Lekberg et al., 2011	LSU rRNA	Glo454, NDL22	1841	97 %	48	11 × (4–5)	32	11	SRA029261

*Aggregates of 4–9 replicates; **some of the sequences listed are not related to the publication.

Davison et al. (2012) included soil samples from different plots in a forest reserve along a 4 month temporal gradient (4–9 replicates per group; we only considered replicated data for plot A, which was the only plot that was replicated in time). Replicated information per treatment was used to retrieve more accurate estimates of α-diversity through separately calculating the diversity metrics for each of the samples and then averaging the result across the samples that belonged to the specific treatment (diversity metrics were normally distributed) (Table 1).

Our analysis was subject to the following assumptions. First, the number of DNA sequence reads represents a surrogate of the relative abundance of AMF taxa within each sample; we are aware that this assumption is controversial due to potential biases in DNA amplification efficiency and variation between taxa in investment to storage structures inside roots (e.g. Kiers et al., 2011). Second, with the exception of the downsampling procedure (details follow), we assumed that sequencing depth across the different samples was sufficient to accurately characterize abundance, which is generally highly dependent on sample size (e.g. Smith and van Belle, 1984).

To estimate the effect of sequencing depth, a proxy for comparisons between current next-generation studies and earlier studies with lower sampling depth, we downsampled the datasets using a bootstrapping procedure (Efron, 1979). We subsampled with replacement each dataset 1 000 times yielding each time the desired number of sequences, (16, 32, 64, 128, and 256 sequences; except for Becklin et al., from which we subsampled 4, 8, 16, 32, and 64 sequences due to the low number of available reads), and then averaged the richness of these 1 000 subsamples. Subsequently, we evaluated whether the ranking of the means of within-study treatments had been modified as a consequence of the downsampling, or even more importantly if the significance of a comparison was altered. Although we believe that making inferences simply based on the ranking of the treatment means is not statistically robust, we based our assessments on considerations of the entire sequencing depth profile in a way consistent with the approach used to assess inclusive diversity indices, i.e., two samples were considered different when the sequencing-depth profiles did not intersect. Moreover, in molecular biology it is relatively common for authors to base their conclusions on the rankings of treatment means (e.g. Singh et al., 2008). We anticipated that consideration of the full datasets (i.e., no downsampling – only standardization) would produce the most reliable results; downsampling was simply carried out to assess whether the original authors might have arrived at different conclusions if the number of sequences per treatment was lower. Standardizing to a relatively high number of sequences is a procedure that resolves bias in comparisons of taxon richness in samples that differ in size (Smith and van Belle, 1984). We repeated the downsampling procedure for a range of targeted sequences per sample to assess the extent to which sequencing effort may affect richness estimates. By using sequence numbers representative of early sequencing attempts and modern 454 sequencing analyses, our goal was to determine the comparability of these types of study. Approaches to standardization of pyrosequencing datasets may differ considerably from those examined here – in part because sequencing depth is typically higher – and have been the subject of a number of specialized studies (e.g. Aguirre de Cárcer et al. 2011).

To assess the performance of inclusive diversity indices, we generated diversity profiles by implementing the methodology proposed by Leinster and Cobbold (2012). When assessing inclusive diversity indices, the number of replicates is important for retrieving an improved estimate of the mean (as in the case of ANOVA estimates) but is unimportant with regards to determining whether differences in diversity exist. This is determined based on whether diversity profiles of different treatments intersect or not (Leinster and Cobbold, 2012). The Leinster and Cobbold (2012) is formulated as follows:

$$ {}^{q}D^{Z}(n) = \left(\sum p_n (Zp)_n^{q-1} \right)^{\frac{1}{1-q}} \quad (1) $$

where ${}^{q}D^{Z}(n)$ is the diversity profile for sample n, p is an $n \cdot s$ table describing the relative abundance of each species in each sample with n samples containing a total of s species, Z is an optional $s \cdot s$ table describing the similarity level among the s species (zero no similarity, one maximum similarity). The statistic is influenced by a parameter q that determines the relative importance of rare species; $q = 0$ represents a scenario in which rare species have a strong influence on diversity estimation (e.g. species richness), while rare species contribute less as q increases. In each diversity profile q represents a continuous variable that takes values from zero to infinity. An important point to note is that q cannot take the value one. To address this issue we used an approximation of q when it approaches one ($q = 0.99$). The statistic has two formulations: the naive form where different species are considered equally dissimilar (the similarity table Z is replaced by an identity matrix), and the non-naive form where information on

similarity across different species, usually inferred following phylogenetic analysis, is included in the calculation. Diversity groups or treatments that did not intersect in the diversity profiles were considered genuinely different in diversity.

For each of the three studies we retrieved sequence information for representatives of the recovered OTUs (operational taxonomic units) provided by the authors. We represented the phylogenetic structure of AMF taxa using a maximum clade credibility tree that was generated from a Bayesian phylogenetic analysis (using Beast v1.7.2; Drummond and Rambaut, 2007). The chain consisted of 5×10^6 generations, sampling every 1 000 trees; the first 500 trees were discarded as the burn-in, leaving 4 500 trees for estimating topology and node heights. Phylogenetic similarity was assessed as the difference of a rescaled cophenetic distance of any two taxa in the range of zero to one (achieved through dividing with the maximum cophenetic distance in the dataset) subtracted from one.

Results

Standardizing the sampling procedure altered the ranking of the four treatments in Lekberg et al. (2011) with regards to AMF taxa richness (Fig 1A: 256 sequences vs. all). Downsampling further to 32 sequences only altered the ranking of the three less diverse (with regards to AMF richness) treatments (Fig 1A). In their original paper, Lekberg et al. (2011) stated that there were no significant differences in OTU richness across treatments and, indeed, following standardization there was no treatment effect. However, after downsampling to 16 sequences (a sequencing depth representative of Sanger-sequencing based studies) paired *t*-tests between the minimal disturbance treatment, which appeared to be richer than the other treatments (the control treatment), and any other treatment were often significant ($0.028 < P < 0.037$ for disturbance with recolonization; $0.021 < P < 0.028$ for disturbance without recolonization; $0.045 < P < 0.059$ for surrounding plants – *P* values obtained in a series of 20 bootstraps). Diversity metrics based on the Leinster and Cobbold naive index for $q \neq 0$ effectively reproduced the AMF richness ranking after downsampling to fewer than 32 sequences (Fig 2A). The non-naive version of the index gave comparable results. The minimal disturbance treatment was more diverse than the disturbance treatment without re-colonization (Fig 2A,D).

Despite the fact that Becklin et al. (2012) recovered a low number of reads, averaging 12.2 AMF-sequence-reads per sample, the standardization procedure altered the ranking of richness estimates for two of the treatments in the open meadow (Fig 1B). Further downsampling had little effect on the relative ranking of the treatments. In our reanalysis we adopted a slightly different approach to Becklin et al. (2012) and included in the dataset the samples that were reported to have been successfully sequenced but yielded no Glomeromycota. Our ANOVA did not reveal significance of either habitat type or plant species but did reveal a significant interaction. When habitat was not considered in the ANOVA, any plant species differences were non-significant (Supplementary material). The Leinster and Cobbold approach confirms the results of our ANOVA: AMF diversity associated with *T. ceratophorum* was lower than *T. officinale* in the open meadow but higher under willow understory; thus the interaction has to be of importance (Fig 2B, E).

Standardization of the number of reads across treatments altered the ranking of richness estimates for two of the treatments in Davison et al. (2012). However, the ranking of these two treatments reverted again with further downsampling. In Davison et al. (2012) analysis of variance did not reveal any significant effect of seasonality on AMF richness. The Leinster and Cobbold approach revealed that diversity declined from May to Jun. (Fig 2C, F). However, the non-naive version of the index revealed an additional interesting pattern (Fig 2F). For high *q* values, the most diverse treatment was the harvest of Sep.; however, this treatment was the least diverse when rare species were given equal weight to common ones (low *q*). This was due to a shift of the AMF community from Glomeraceae group A phylotypes towards representatives of

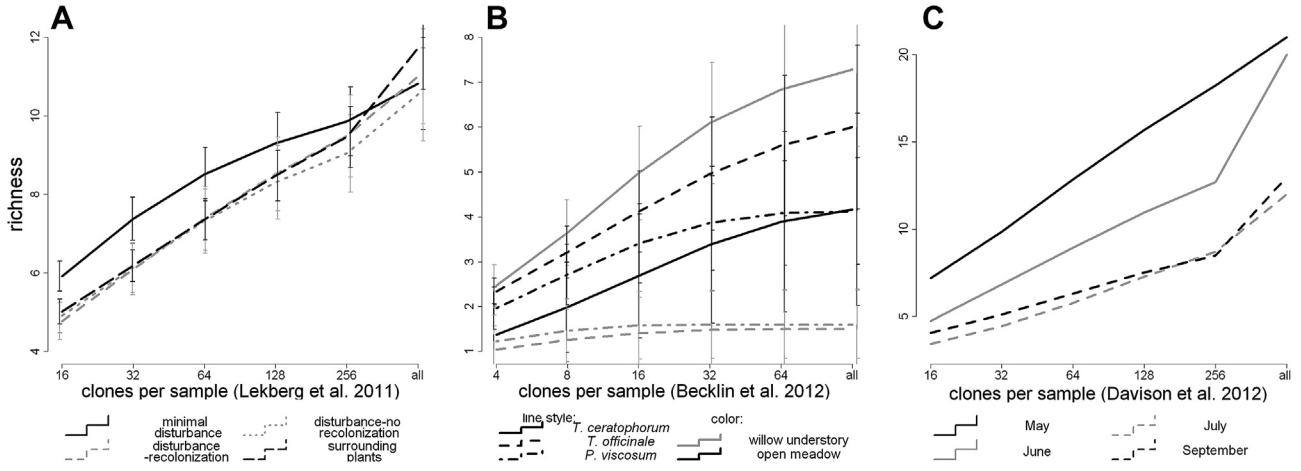

Fig 1 – Impact of standardization of AMF taxa sequence reads through a bootstrapping procedure to a lower number of sequences (*x*-axes) in the three studies (A, Lekberg et al., 2011; B, Becklin et al., 2012; C, Davison et al., 2012) compared to the original estimate of taxon richness (tagged as '*all*'). Error bars represent standard errors of the means; no error bars could be generated for Davison et al. (2012) as only cumulative data per treatment were presented.

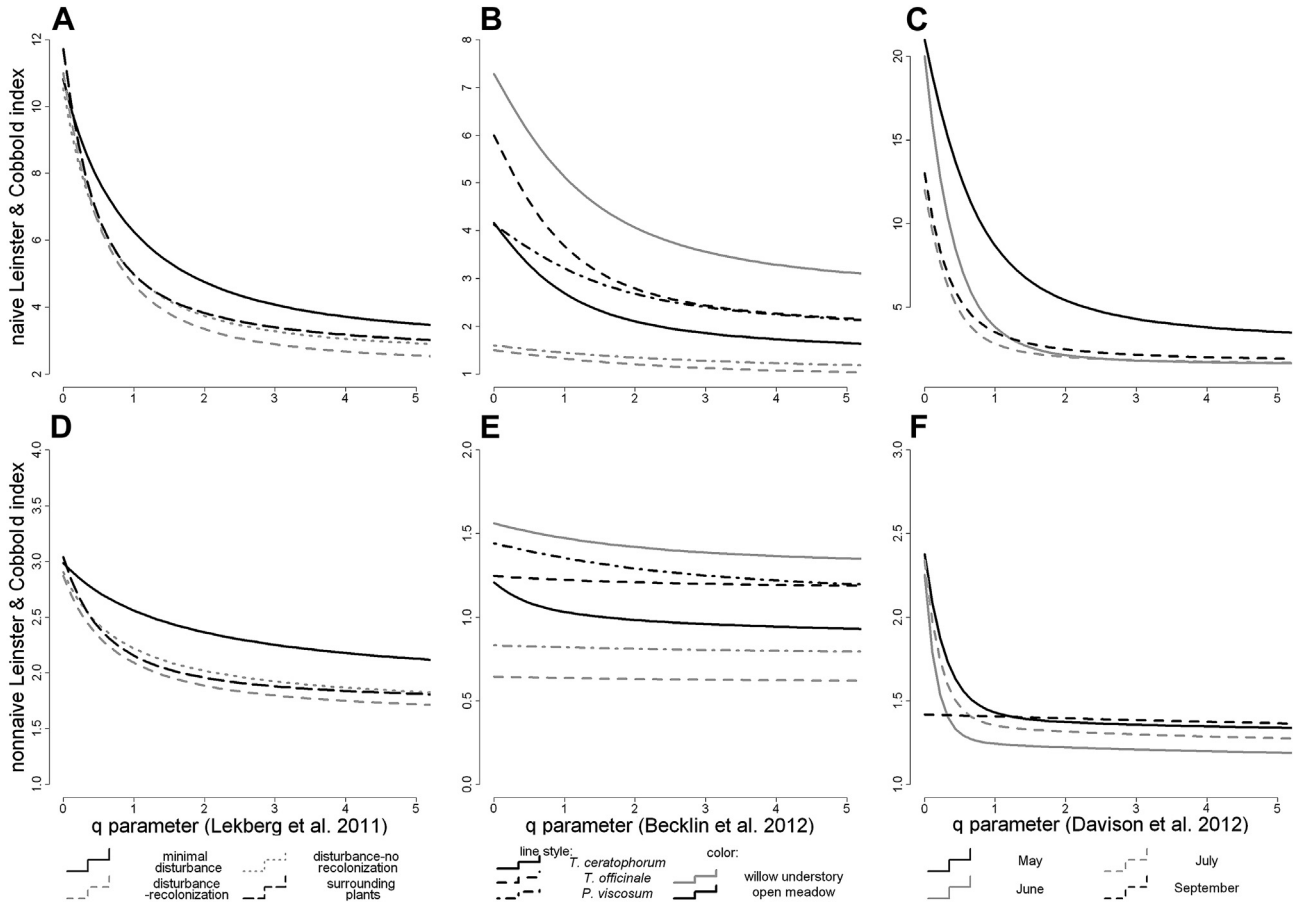

Fig 2 – Diversity profiles according to Leinster and Cobbold (2012) for the three studies included in the reanalysis. The *x*-axes represent the *q* parameter (determines the relative influence of rare species) whereas the *y*-axes highlight the estimated diversity with either the naive (species similarities ignored; A, B, C) or the non-naive (species similarities included in the statistic; D, E, F) form of the index. The six panels show data from A, D: Lekberg et al. (2011); B, E: Becklin et al. (2012); and C, F: Davison et al. (2012). Differences in diversity between two samples are indicated by intersections in their diversity profiles (naive or non-naive; Leinster and Cobbold, 2012). Analyses were carried out on the unstandardized sequence tables that were published with the original manuscripts.

the *Glomus* group B. This is a qualitative change in the AMF community that could only be detected when AMF phylogeny is considered.

In all three studies the use of the naive Leinster and Cobbold approach with $q > 0$ ranked the treatments in a way that resembled the ranking after standardization: in Lekberg et al. (2011) diversity was highest in the minimal disturbance treatment followed by the surrounding plants treatment and the disturbance without recolonization treatment; in Becklin et al. (2012) the open meadow *T. ceratophorum* treatment was less diverse than the respective *P. viscosum* treatment; and in Davison et al. (2012) for intermediate *q* values (around one) the diversity ranking was May, Jun., Sep., Jul..

Discussion

We found that the combined use of a naive and non-naive version of the Leinster and Cobbold index, despite not explicitly considering replicates, could largely replicate the results of ANOVA on AMF richness, and was more likely to uncover subtle treatment effects. ANOVA is often a conservative way to assess treatment effects on estimates of richness. Despite the fact that multifold differences are often observed in AMF richness, to the best of our knowledge, there have been few instances where a significant effect was reported following analysis of sequencing data with ANOVA (e.g. Öpik et al., 2009; Wang et al., 2011). Consequently, the less conservative nature of the Leinster and Cobbold approach is a potentially desirable property. Our reanalysis highlighted the importance of standardizing the number of sequence reads per sample before making any inferences about diversity. While this is well established in the ecological literature (e.g. Hellmann and Fowler, 1999), molecular microbial ecologists are often unaware of these complications (but see Gilbert et al., 2009). Although the degree of downsampling (we consider the extremes in the downsampling gradient to address objective one) had less of an impact on richness estimates than the standardization of sampling depth across samples, we recorded instances where the ranking of richness changed after downsampling.

This result suggests that the conclusions of previous Sanger sequencing studies, in which sequencing depth was much reduced, are not directly comparable with the outcomes of studies using deep sequencing with next-generation technologies (assuming sampling depths are standardized across samples). Additionally, we highlighted the importance of considering a range of diversity indices (the Leinster and Cobbold indices for different q values correspond to distinct diversity indices) before making generalizations about treatment effects on diversity. An increasing number of ecologists have expressed their support for the use of inclusive sets of diversity indices in drawing ecological inferences (e.g. Chao et al., 2012). The approach is of particular importance for microbial ecologists because it has the advantages that: (i) it strongly limits the effect of chimeras or other artifacts that may appear as rare taxa in a dataset; (ii) the indices can be robust to the issue of false negatives (accounting for rare taxa that may have been present in the community but were not included in the fingerprint); and (iii) when phylogenetic information is integrated in the form of a similarity index, many complications arising from species delineation are largely resolved (in the non-naive version of the Leinster and Cobbold index, closely-related taxa are considered less independent than those distantly related – e.g. Fig 2F).

Demonstrating the absence of diversity differences across treatments in any given situation, especially in complex designs with high replication, should not necessarily be treated as a negative result; it could become a starting point for further exploration. For instance, as was shown in the case of Davison et al. (2012), a qualitative shift in the AMF community accounted for the absence of diversity differences according to the non-naive Leinster and Cobbold index between the late September harvest and all other treatments. Highlighting the reasons why two diversity profiles do not differ may be just as informative as reporting actual differences. An important point that needs to be reiterated, however, is that community assembly analysis alone can potentially be misleading in cases when there are huge differences in abundance; complementary use of other quantitative methods such as RT-PCR is always advisable. Our research demonstrated the suitability of inclusive diversity indices in the study of AMF. While the fundamental challenges faced by microbial ecologists are comparable irrespective of their study organisms (e.g. Prosser et al., 2007), and organism identity should consequently be of minor importance when it comes to the analysis of microbial data, future research should address the extent to which these findings apply to other taxa.

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Supplementary material

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