**Interpreting UniFrac with Absolute Abundance: A Conceptual and Practical Guide**

Augustus Pendleton1\* & Marian L. Schmidt1\*

1Department of Microbiology, Cornell University, 123 Wing Dr, Ithaca, NY 14850, USA

**Corresponding Authors:** Augustus Pendleton: arp277@cornell.edu; Marian L. Schmidt: marschmi@cornell.edu

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**Data Availability:** All data and code used to produce the manuscript are available at https://github.com/MarschmiLab/Pendleton\_2025\_Absolute\_Unifrac\_Paper, in addition to a reproducible renv environment. All packages used for analysis are listed in Table S1.

**Abstract**

-diversity is central to microbial ecology, yet commonly used metrics overlook changes in microbial load, limiting their ability to detect ecologically meaningful shifts. Popular for incorporating phylogenetic relationships, UniFrac distances currently default to relative abundance and therefore omit important variation in microbial abundances. As methods for quantifying absolute abundance become more accessible, integrating this information into -diversity analyses becomes increasingly important. Here, we introduce *Absolute UniFrac* (), a variant of Weighted UniFrac that operates on absolute abundances. Using simulations and reanalysis of four 16S rRNA datasets (nuclear reactor cooling tank, the mouse gut, a freshwater lake, and the peanut rhizospere), we demonstrate that Absolute UniFrac jointly captures microbial load differences and phylogenetic turnover in comparison to other abundance-aware -diversity metrics. While this can improve statistical power to detect ecological shifts, Absolute Unifrac can also be strongly correlated to differences in cell abundances alone, especially when changes occur along long branches. To balance these effects, we present a generalized extension () with a tunable parameter to balance sensitivity and interpretability. We recommend evaluating conclusions across multiple parameters and complementary -diversity metrics. Finally, we benchmark *GUA* and show that although computationally slower than conventional alternatives, *GUA* is comparably insensitive to realistic noise in load estimates compared to conventional alternatives (i.e. Bray-Curtis dissimilarities), particularly at lower By coupling phylogeny with microbial load, Absolute UniFrac reframes -diversity as an eco-evolutionary measure of how communities reorganize in both membership and magnitude.

**Main Text**

Microbial ecologists routinely compare communities using -diversity metrics derived from relative abundances. Yet this approach overlooks a critical ecological dimension: microbial load. High-throughput sequencing produces compositional data, in which each taxon’s abundance is constrained by all others [1]. However, quantitative profiling studies show that cell abundance, not only composition, can drive major community differences [2]. In low-biomass samples, relying on relative abundance can allow contaminants to appear biologically meaningful despite absolute counts too low for concern [3].

To overcome compositional constraints, researchers increasingly use flow cytometry, qPCR, and genomic spike-ins to quantify microbial load [4, 5]. These tools improve detection of functionally relevant taxa and mitigate the compositional constraints imposed by sequencing [1, 2]. Most studies that incorporate absolute data have rely on Bray-Curtis dissimilarity, which operates directly on counts rather than normalized proportions [4, 6]. But in the field of microbial ecology, UniFrac distances remain popular when working with relative abundance data. With UniFrac spreading into fields where absolute abundances are already standard, its continued use as a relative-only metric risks obscuring the ecological and evolutionary signals carried by changes in load. Here, we present *Absolute UniFrac*, a direct extension of Weighted UniFrac that incorporates total abundance and evaluate its impact across simulated and real-world datasets.

The UniFrac distance was first introduced by Lozupone & Knight (2005) and has since become enormously popular as a measure of -diversity within the field of microbial ecology [7]. A benefit of the UniFrac distance is that is considers phylogenetic information when estimating the distance between two communities. After first generating a phylogenetic tree representing species (or amplicon sequence variants, “ASVs”) from all samples, the UniFrac distance computes the fraction of branch-lengths which is *shared* between communities, relative to the total branch length represented in the tree. UniFrac can be both “unweighted”, in which only the incidence of species is considered, or “weighted”, wherein a branch’s contribution is weighted by the proportional abundance of taxa on that branch [8]. The Weighted UniFrac is derived:

where the length of each branch is weighted, , by the difference in the relative abundance of all species () descended from that branch in sample or sample . Here, we denote this distance as *UR*, for “Relative UniFrac”. Popular packages which calculate weighted Unifrac, including the diversity-lib QIIME plug-in and the R packages phyloseq and GUniFrac, run this normalization by default.

Importantly, is most sensitive to changes in abundant lineages, which can sometimes obscure compositional differences driven by rare to moderately-abundant taxa [9]. To address this weakness, Chen et al. (2012) introduced the generalized UniFrac distance (), in which the impact of abundant lineages can be mitigated by decreasing the parameter :

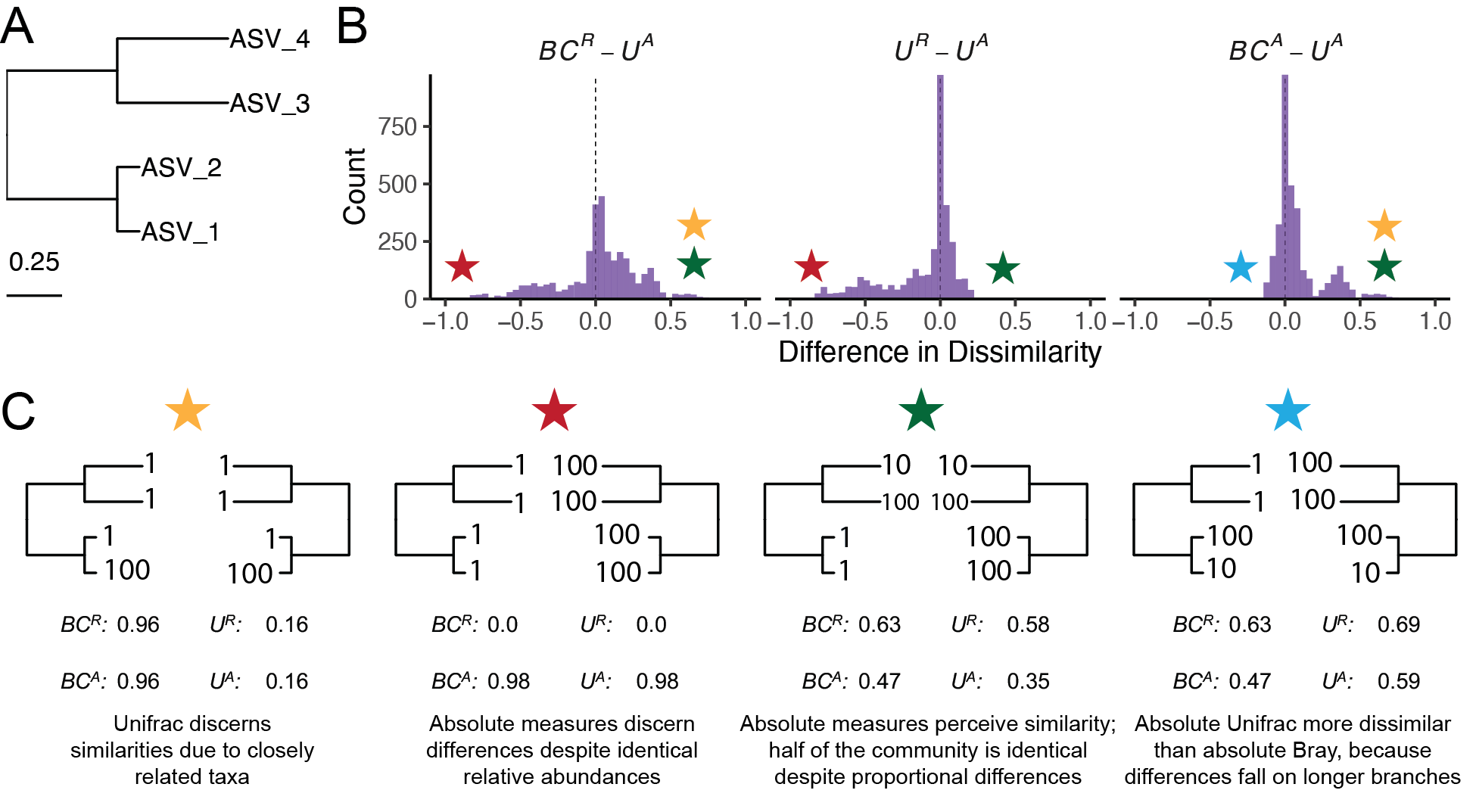
where ranges from 0 (close to Unweighted UniFrac) up to 1 (identical to , above). However, if one wishes to use absolute abundances, both and can be derived without normalizing to proportions:

Where and stands for the absolute counts of species descended from branch in community and , respectively. We refer to these distances as *Absolute Unifrac* (

Consequently, interpreting the behavior of in comparison to other -diversity metrics becomes nontrivial, especially in complex microbiomes with many samples. To illustrate how compares to other metrics, we first constructed a simple example: a simulated community of four ASVs arranged in a simple phylogeny (Fig. 1A). By varying the absolute abundance of each ASV (1, 10, or 100), we generated 81 samples and 3,240 pairwise comparisons. For each pair, we computed four dissimilarity metrics: Bray-Curtis with relative abundance (), Bray-Curtis with absolute abundance (), Weighted UniFrac with relative abundance (), and Weighted UniFrac with absolute abundance ().

does not consistently yield higher or lower distances but instead varies depending on how abundance and phylogeny intersect (Fig. 1B). In the improbable scenario that all branch lengths are equal, is always less than or equal to (Fig. S1). These comparisons emphasize that once branch lengths differ, incorporating phylogeny and absolute abundance alters the structure of the distance space. The direction and magnitude of that change depend on which branches carry the abundance shifts.

To better understand how these metrics diverge, we examined individual sample pairs (Fig. 1C). Scenario 1 (gold star) illustrates the classic advantage of UniFrac: ASV\_1 and ASV\_2 are phylogenetically close, so and discern greater similarity between samples than and , which ignore phylogenetic structure. Scenario 2 (red star) highlights a limitation of relative metrics: two samples with identical relative composition but a 100-fold difference in biomass appear identical to and , but not to their absolute counterparts. In Scenario 3 (green star), incorporating absolute abundance decreases dissimilarity. and are lower than their relative counterparts because half the community is identical in absolute abundance, even though their proportions differ. In contrast, Scenario 4 (blue star) shows that can exceed when abundance differences fall on long branches, magnifying evolutionary distance and amplifying phylogenetic dissimilarity.

*Figure 1. Simulated communities reveal how absolute abundance affects phylogenetic and non-phylogenetic β-diversity measures.* (A) We constructed a simple four-ASV community with a known phylogeny and generated all permutations of each ASV having an absolute abundance of 1, 10, or 100, resulting in 81 unique communities and 3,240 pairwise comparisons. (B) Distributions of pairwise differences between weighted UniFrac using absolute abundance () and three other metrics: Bray-Curtis using relative abundance (), weighted UniFrac using relative abundance (), and Bray-Curtis using absolute abundance (). (C) Illustrative sample pairs demonstrate how absolute abundances and phylogenetic structure interact to increase or decrease dissimilarity across metrics. Colored stars indicate where each scenario falls within the distributions shown in panel B. Actual values for each metric are displayed beneath each scenario.

Across all 3,240 pairwise comparisons, is usually smaller than *BCA* and is more strongly correlated with (Pearsons = 0.82, < 0.0001) than with ( = 0.41) and ( = 0.55), reflecting the effect illustrated in Scenario 1. However, exceptions like Scenario 4 show that can also exceed when abundance differences occur on long branches. These scenarios demonstrate that integrates variation along three ecologically relevant axes: composition, phylogenetic relatedness, and microbial load, rather than isolating any single dimension. Because a given value can reflect multiple drivers of community change, interpreting it requires downstream analyses to disentangle the relative contributions abundance, phylogeny, and composition. To evaluate how this plays out in real systems, we next re-analyzed A graph of different types of data

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*Figure 2. Absolute UniFrac (UA) compared with other β-diversity metrics across four real microbial datasets.* Each panel shows pairwise sample distances for *UA* (x-axis) against another metric (y-axis): Bray-Curtis using relative abundance (*BCR*, first column), weighted UniFrac using relative abundances (*UR*, second column), and Bray-curtis using absolute abundances (*BCA*, third column). Contours indicate the density of pairwise comparisons (n shown for each dataset, left), with darker shading corresponding to more observations. The dashed line marks the 1:1 relationship. Points above the line indicate cases where *UA* is smaller than the comparator metric, while points below the line indicate cases where *UA* is larger.

To illustrate the sensitivity of to both variation in composition and absolute abundance, we re-analyzed four previously published datasets from diverse microbial systems. These included: (i) nuclear reactor cooling water sampled across three reactor cycle phases [4]; (ii) the mouse intestinal track sampled across gut regions and under two diets [3]; (iii) depth-stratified freshwater communities sampled across two months [10]; and (iv) peanut rhizosphere microbiomes under two crop rotation regimes (conventional rotation (CR) vs. sod-based rotation (SBR)), plant maturities, and irrigation treatments [11]. Absolute abundance was quantified by flow cytometry for the cooling water and freshwater datasets, droplet digital PCR for the mouse gut, and by qPCR for the rhizosphere dataset. Together these span a wide range of richness–from 215 ASVs in the cooling water to 24,000 ASVs in the soil–and microbial load–from as low as 3.99x105 cells/ml (cooling water) up to 2x1012 16S rRNA copies/gram (mouse gut). Additional details of the re-analysis workflow, including ASV generation and phylogenetic inference, are provided in the Supporting Methods. Collectively, these re-analyses highlight that integrates ecological realism by incorporating differences in both lineage identity and total biomass.

We first calculated four β-diversity metrics for all sample pairs in each dataset and compared them to *UA* (Fig. 2). The degree of concordance between *UA* and other metrics was highly context dependent*.* In the cooling water dataset, *UA* closely tracked all three alternatives, whereas in the remaining systems it diverged substantially. *UA* also spanned a similar or wider range of distances than the other metrics. For example, in the soil dataset *BCR* and *UR* occupied a narrow range relative to the broad separation observed under *UA.*

*UA* generally reported distances that were similar to or greater than *UR,* consistent with the simulations shown in Fig. 1B. This reflects the ability of *UA* to discern dissimilarity due to differences in microbial load, even when community composition is conserved. In contrast, *UA* yielded distances that were similar to or lower than *BCA,* again matching the simulated behavior in Fig 1B. In these cases, phylogenetic proximity between abundant, closely-related ASVs leads *UA* to register greater similarity than *BCA.*

Given these differences, we next quantified how well each metric discriminates among categorical sample groups. For each dataset, we ran PERMANOVAs to measure the proportion of variance (*R2*) and statistical power (*pseudo-F, p-*value) attributable to group structure, using groupings that were determined to be significant in the original publications. To evaluate how strongly absolute abundance contributed to this discrimination, we also calculated *GUA* across a range of values. The resulting *R2* values are displayed in Fig. 3A, with the corresponding *pseudo-F* statistics and *p*-values provided in Fig. S2.

As in Fig. 2, the performance of each metric was strongly context dependent (Fig. 3A). In the mouse gut and freshwater datasets, absolute-abundance-aware metrics (*BCA* and *GUA*) explained the greatest proportion of variance (*R2*), and *R2* generally increasing as increased. In contrast, relative metrics captured more variation in the cooling water dataset (again at higher , and all metrics explained comparably little variance in the soil dataset. Taken at face value, these trends might suggest that higher values typically improve group differentiation.

However, this comes with a major caveat:

*Figure 3. Discriminatory performance of UA and related metrics across four microbial systems.* (A) PERMANOVAs were used to quantify the percent variance (*R2*) explained by predefined categorical groups (shown in italics beneath each dataset name), with 1,000 permutations. Metrics were evaluated across five metrics and, where applicable, across eleven values (0-1 in 0.1 increments). For consistency with the original studies, only samples from Reactor cycle 1 were used for the cooling-water dataset, only stool samples for the mouse gut dataset, and only mature rhizosphere samples for the dataset. (B) Mantel correlation (R) between each distance metric and the pairwise differences in absolute abundance (cell counts or 16S copy number), illustrating the degree to which each metric is driven by biomass differences.

We recommend calibrating based on research goals, mitigating this effect by using across a range of values rather than relying on or a single value. Researchers should decide how strongly they want their dissimilarity metric to place on microbial load. One potential approach is to calculate correlations as demonstrated in Fig. 3B and select an prior to any ordinations or statistical testing. Again, absolute metrics are expected to correlate to differences in cell count, as we hope absolute abundance measures incorporate differences in cell counts, especially when microbial load is relevant to the hypotheses being tested. Correlations to cell count in *BCA,* an accepted approach in the literature, ranged from ~0.5 up to ~0.8. As a general recommendation from these analyses, we recommend values in an intermediate range from 0.25 up to 0.75.

**Practical Concerns:**

Finally, we sought to quantify some of the practical concerns researchers may have when applying *GUA* to their own data. Both Bray-Curtis and Unifrac measures can be sensitive to differences in richness as a result of uneven sequencing depth [13–15]. Methods to address these concerns, including rarefaction, are highly debated in the literature, and the simulations to test the impact of rarefaction decisions are complex (e.g. [16]). We do not explore the specific sensitivity of *UA/GUA* to rarefaction decisions here, but do provide a practical framework and code for how we incorporated rarefaction into our own analyses (Fig. S4 and available code). Put simply, we rarefy our ASV tables to an even sequencing depth across many iterations (here, 100); transform each ASV to relative abundance; normalize the abundance of each ASV in each sample by multiple the relative abundance by the absolute abundance of that sample; and average the distance measure across all iterations. Future work which assesses the sensitivity of *UA* to A collage of graphs and diagrams

AI-generated content may be incorrect.richness and sequencing depth, compared to *UR* and *BCA,* would be useful to the field.

*Figure 4. Performance of GUA in terms of computational time and resilience to quantification error.* (A) The computational time to calculate *GUA* (from the GUnifrac package), *UR* (implemented as FastUnifrac in the phyloseq package) and *BCA* (from the vegan package) was measured across 50 iterations on the sub-sampled soil dataset (only mature samples) [11] across a range of ASV numbers (50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000), holding a constant of 10 samples and 1 requested value (though unweighted Unifrac is also calculated by default). (B) Linear relationships between *GUA* computation time and ASV number. (C-D) Using only the stool samples from the mouse gut dataset [3], random error (ranging from ±1% variation up to ±50% variation) was added to the 16S copy number of each sample (additional details in Supporting Methods). Across 50 iterations, the difference between *BCA, GUA* ( =1) and *GUA* ( =0.2) on the error-added dataset was compared to the original calculation of that metric for each sample comparison. Points reflect the (C) mean difference and (D) max difference between the error-added metrics compared to the original calculation. Error bars represent the standard deviation of the average mean and max difference across 50 iterations.

*GUA* is slower to compute than other metrics, including *BCA* and *UR* (Fig. 4A). On average, *GUA* is two-four orders of magnitude slower than these metrics. The computation time of *GUA* is driven primarily, and linearly, by the number of ASVs in the dataset, as traversing the tree to calculate branch length is computationally expensive. In contrast, the number of samples or number of values to calculate weakly influences the computation time (Fig. S5). While computation time on a 10,000 ASV dataset with a single CPU is still reasonable (~6 minutes), when calculating *GUA* over many iterations of rarefaction the total computation time can become inconvenient, as branch lengths are redundantly calculated each time. If rarefaction could be incorporated into the GUnifrac package, or a branch-lengths object could be saved and re-used such that branch lengths are only calculated once, this would greatly improve computational efficiency.

It is also expected that estimation of -diversity metrics which rely on absolute abundance will be sensitive to errors or uncertainty arising from the quantification of cell number of 16S copy number. To assess the sensitivity of *GUA* and *BCA* to measurement error, we added random error to the 16S copy number measurements from the mouse gut dataset, limiting our analyses to the stool samples where copy numbers varied by an order of magnitude. Across 50 iterations, each copy number could randomly vary by a given percentage of error in either direction. We re-calculated -diversity (*BCA* and *GUA* at = 1 and = 0.2) and compared these measurements to the original dataset.

All three measurements were resilient to added error (Fig. 4C). At an = 1, for every 1% of random variation we added to absolute quantification *GUA* differed from the original measurement by only 0.0022; even at ±50% added noise, *GUA* only differed (on average) by 0.1. And while *GUA* ( = 1) was more sensitive to error than *BCA,* at = 0.2 it was *less* sensitive, again highlighting the potential benefits of moderate values. The max deviation from true that added error could inflict on a given metric was also proportional (and always less) than the magnitude of the error itself (Fig. 4D). Put simply, if one adds 10% of random error to a measurement, they can expect *GUA* to change, at max, by 0.1; in general the deviation from true will be much less. A more rigorous approach to assessing error propagation within these metrics, including mathematical proofs of the relationships estimated above, is outside the scope of this paper but would be helpful.

**INSERT HEADER:**

There are many cases where the incorporation of absolute abundance allows microbial ecologists to assess more realistic, ecologically-relevant differences in microbial communities, especially in contexts where microbial load matters. Outside of the datasets re-analyzed here [3, 4, 10, 11], the temporal development of the infant gut microbiome involves both a rise in absolute abundance and compositional changes [6]; bacteriophage predation in wastewater bioreactors can be understood only when microbial load is considered [17]; and antibiotic-driven declines in specific swine gut taxa were missed using relative abundance approaches [18]. As -diversity metrics (and UniFrac specifically) remain central to microbial ecology, we encourage researchers to adopt when applicable. In addition, our re-analysis efforts were limited by ßœfew papers sharing the absolute quantification data. The open sharing of absolute quantification data, ideally as metadata within SRA submissions, is just as essential for reproducibility as the sequencing files themselves.

While demonstrated here with 16S rRNA data, the approach should be generalizable to other marker genes or (meta)genomic features, provided absolute abundance estimates are available. In doing so, offers not only a more grounded picture of lineage differences but also sensitivity to both biomass variation and phylogenetic depth.

In reality, no one metric (or in *GUA*) is “better” than another – all metrics shown here calculate distance or dissimilarity along valid, but different, axes of variation in microbial communities. Researchers should select β-diversity measures to address their specific hypotheses on how two microbiomes may differ, and interpret the results of a β-diversity measure in terms of what information that measure incorporates. Here, we demonstrate **not** that *GUA* outperforms other measures, but that it does incorporate the axes of variation (composition, phylogenetic similarity, and absolute abundance) it was designed to incorporate (Fig. 1 and Fig. 2). As with any -diversity study, researchers should interpret results critically, explore sensitivity of results across different metrics, and justify their choice of metrics (and when applicable [19]). By providing demonstrations and code for the application and interpretation of *UA/GUA,* we hope to encourage the use of these metrics as a tool of microbial ecology.

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