

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

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12 https://github.com/MarschmiLab/Pendleton_2025_Absolute_Unifrac_Paper, in addition to a
13 reproducible renv environment. All packages used for analysis are listed in Table S1.

14

15 **Abstract**

16 β -diversity is central to microbial ecology, yet commonly used metrics overlook changes in
17 microbial load, limiting their ability to detect ecologically meaningful shifts. Popular for
18 incorporating phylogenetic relationships, UniFrac distances currently default to relative
19 abundance and therefore omit important variation in microbial abundances. As quantifying
20 absolute abundance becomes more accessible, integrating this information into β -diversity
21 analyses is essential. Here, we introduce *Absolute UniFrac* (U^A), a variant of Weighted UniFrac
22 that incorporates absolute abundances. Using simulations and a reanalysis of four 16S rRNA
23 metabarcoding datasets (from a nuclear reactor cooling tank, the mouse gut, a freshwater lake,
24 and the peanut rhizosphere), we demonstrate that Absolute UniFrac captures microbial load,
25 composition, and phylogenetic relationships. While this can improve statistical power to detect
26 ecological shifts, we also find Absolute UniFrac can be strongly correlated to differences in cell
27 abundances alone. To balance these effects, we also incorporate absolute abundance into the
28 generalized extension (GU^A) that has a tunable α parameter to adjust the influence of abundance
29 and composition. Finally, we benchmark GU^A and show that although computationally slower
30 than conventional alternatives, GU^A is comparably insensitive to realistic noise in load estimates
31 compared to conventional alternatives like Bray-Curtis dissimilarities, particularly at lower α . By
32 coupling phylogeny, composition, and microbial load, Absolute UniFrac integrates three
33 dimensions of ecological change, better equipping microbial ecologists to quantitatively compare
34 microbial communities.

35 **Main Text**

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

43 Sequencing-based microbiome studies therefore rely on relative abundance even when
44 the hypotheses of interest implicitly concern absolute changes in biomass. This creates a
45 mismatch between ecological framing (growth, bloom magnitude, pathogen proliferation,
46 disturbance recovery, or colonization pressure) and the information the β -diversity metric
47 encodes [4]. As a result, β -diversity is often treated as if it includes biomass, even when absolute
48 abundance is either not measured at all or is measured but excluded from the calculation (as in
49 conventional UniFrac). Many studies therefore test biomass-linked hypotheses using a metric
50 that normalizes biomass away [4]. A conceptual correction is needed in which β -diversity is
51 understood as variation along three axes: composition, phylogeny and absolute abundance.

52 Absolute microbial load measurements are now increasingly obtainable through flow
53 cytometry, qPCR, and genomic spike-ins, allowing biomass to be quantified alongside taxonomic
54 composition [4, 5]. These approaches improve detection of functionally relevant taxa and
55 mitigate the compositional constraints imposed by sequencing [1, 2]. Most studies that
56 incorporate absolute abundance counts currently rely on Bray-Curtis dissimilarity, which can
57 capture load but does not consider phylogenetic similarity [5–7]. Unifrac distances provide the
58 opposite strength, incorporating phylogeny but discarding absolute abundance, as they are
59 restricted to relative abundance data by construction [8]. This leaves no phylogenetically
60 informed β -diversity metric that operates on absolute counts, despite the fact that biomass is
61 central to many ecological hypotheses.

62 To evaluate the implications of incorporating absolute abundance into phylogenetic β -
63 diversity, we use both simulations and reanalysis of four 16S rRNA amplicon datasets. The
64 simulations use a simple four-taxon community with controlled abundance shifts to directly
65 compare both Bray-Curtis and UniFrac in their relative and absolute forms, illustrating how each
66 metric responds when abundance, composition, or evolutionary relatedness differ. We then
67 reanalyze four real-world datasets spanning nuclear reactor cooling water [5], the mouse gut [3],
68 a freshwater lake [9], and rhizosphere soil [10]. These differ widely in richness, biomass range,
69 and ecological context, allowing us to test when absolute abundance changes align with or
70 diverge from phylogenetic turnover. Together, these simulations and reanalyses provide the
71 empirical foundation for interpreting Absolute UniFrac relative to existing β -diversity measures
72 across the three axes of ecological difference: abundance, composition, and phylogeny.

73 We also extend Absolute UniFrac as was proposed by [11] to Generalized Absolute
74 UniFrac that incorporates a tunable ecological dimension, α , and evaluate its impact across
75 simulated and real-world datasets. As α increases, β -diversity is increasingly correlated with
76 differences in absolute abundance, allowing researchers to fine tune the relative weight their
77 analyses place on microbial load versus composition.

78 **Defining Absolute UniFrac**

79 The UniFrac distance was first introduced by Lozupone & Knight (2005) and has since
 80 become enormously popular as a measure of β -diversity within the field of microbial ecology
 81 [12]. A benefit of the UniFrac distance is that it considers phylogenetic information when
 82 estimating the distance between two communities. After first generating a phylogenetic tree
 83 representing species (or amplicon sequence variants, “ASVs”) from all samples, the UniFrac
 84 distance computes the fraction of branch-lengths which is *shared* between communities, relative
 85 to the total branch length represented in the tree. UniFrac can be both unweighted, in which only
 86 the incidence of species is considered, or weighted, wherein a branch’s contribution is weighted
 87 by the proportional abundance of taxa on that branch [8]. The Weighted UniFrac is derived:

$$88 \quad U^R = \frac{\sum_{i=1}^n b_i |p_i^a - p_i^b|}{\sum_{i=1}^n b_i (p_i^a + p_i^b)}$$

89 where the contribution of each branch length, b_i , is weighted by the difference in the relative
 90 abundance of all species (p_i) descended from that branch in sample a or sample b . Here, we
 91 denote this distance as U^R , for “Relative UniFrac”. Popular packages which calculate weighted
 92 UniFrac—including the diversity-lib QIIME plug-in and the R packages phyloseq and
 93 GUniFrac—run this normalization by default [11, 13, 14].

94 Importantly, U^R is most sensitive to changes in abundant lineages, which can sometimes
 95 obscure compositional differences driven by rare to moderately-abundant taxa [11]. To address
 96 this weakness, Chen et al. (2012) introduced the generalized UniFrac distance (GU^R), in which
 97 the impact of abundant lineages can be mitigated by decreasing the parameter α :

$$98 \quad GU^R = \frac{\sum_{i=1}^n b_i (p_i^a + p_i^b)^\alpha \left| \frac{p_i^a - p_i^b}{p_i^a + p_i^b} \right|}{\sum_{i=1}^n b_i (p_i^a + p_i^b)^\alpha}$$

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

$$102 \quad U^A = \frac{\sum_{i=1}^n b_i |c_i^a - c_i^b|}{\sum_{i=1}^n b_i (c_i^a + c_i^b)} \quad GU^A = \frac{\sum_{i=1}^n b_i (c_i^a + c_i^b)^\alpha \left| \frac{c_i^a - c_i^b}{c_i^a + c_i^b} \right|}{\sum_{i=1}^n b_i (c_i^a + c_i^b)^\alpha}$$

103 Where c_i^a and c_i^b denote for the absolute counts of species descending from branch b_i in
 104 communities a and b , respectively. We refer to these distances as *Absolute UniFrac* (U^A) and
 105 *Generalized Absolute UniFrac* (GU^A). Although substituting absolute for relative abundances is
 106 mathematically straightforward, we found no prior work that examines UniFrac in the context of
 107 absolute abundance, either conceptually or in application. Incorporating absolute abundances
 108 introduces a third axis of ecological variation: beyond differences in composition and
 109 phylogenetic similarity, U^A also captures divergence in microbial load. This makes interpretation
 110 of U^A nontrivial, particularly in complex microbiomes.

111 **Demonstrating β -diversity metrics’ behavior with a simple simulation**

112 To clarify how U^A behaves relative to existing β -diversity metrics, we first constructed a
113 simple four-taxon simulated community arranged in a small phylogeny (Fig. 1A). By varying the
114 absolute abundance of each ASV (1, 10, or 100), we generated 81 samples and 3,240 pairwise
115 comparisons. For each pair, we computed four dissimilarity metrics: Bray-Curtis with relative
116 abundance (BC^R), Bray-Curtis with absolute abundance (BC^A), Weighted UniFrac with relative
117 abundance (U^R), and Weighted UniFrac with absolute abundance (U^A). These comparisons help
118 to illustrate how each axis of ecological difference—abundance, composition, and phylogeny—is
119 expressed by the different metrics.

120 U^A does not consistently yield higher or lower distances compared to other metrics, but
121 instead varies depending on how abundance and phylogeny intersect (Fig. 1B). In the improbable
122 scenario that all branch lengths are equal, U^A is always less than or equal to BC^A (Fig. S1).
123 These comparisons emphasize that once branch lengths differ, incorporating phylogeny and
124 absolute abundance alters the structure of the distance space. The direction and magnitude of that
125 change depend on which branches carry the abundance shifts. U^A is also usually smaller than
126 BC^A and is more strongly correlated with BC^A (Pearson $r = 0.82$, $p < 0.0001$) than with BC^R (r
127 = 0.41) and U^R ($r = 0.55$).

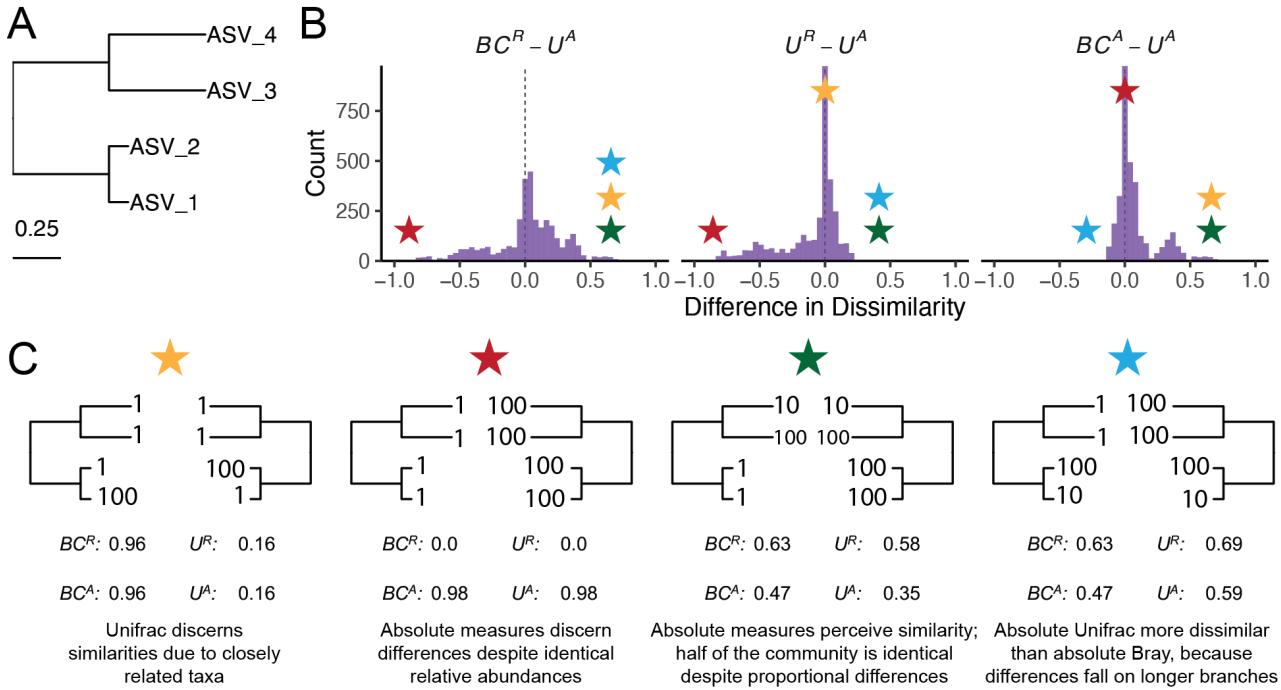
128 To better understand how these metrics diverge, we examined individual sample pairs
129 (Fig. 1C). Scenario 1 (gold star) illustrates the classic advantage of UniFrac: ASV_1 and ASV_2
130 are phylogenetically close, so U^R and U^A discern greater similarity between samples than BC^R
131 and BC^A , which ignore phylogenetic structure. Scenario 2 (red star) highlights a limitation of
132 relative metrics: two samples with identical relative composition but a 100-fold difference in
133 biomass appear identical to BC^R and U^R , but not to their absolute counterparts. In Scenario 3
134 (green star), incorporating absolute abundance decreases dissimilarity. BC^A and U^A are lower
135 than their relative counterparts because half the community is identical in absolute abundance,
136 even though their proportions differ. In contrast, Scenario 4 (blue star) shows that U^A can exceed
137 BC^A when abundance differences occur on long branches, amplifying phylogenetic dissimilarity.

138 These scenarios demonstrate that U^A integrates variation along three ecologically
139 relevant axes: composition, phylogenetic similarity, and microbial load, rather than isolating any
140 single dimension. Because a given U^A value can reflect multiple drivers of community change,
141 interpreting it requires downstream analyses to disentangle the relative contributions of these
142 three axes. To evaluate how this plays out in real systems we next reanalyzed four previously
143 published datasets spanning diverse microbial environments.

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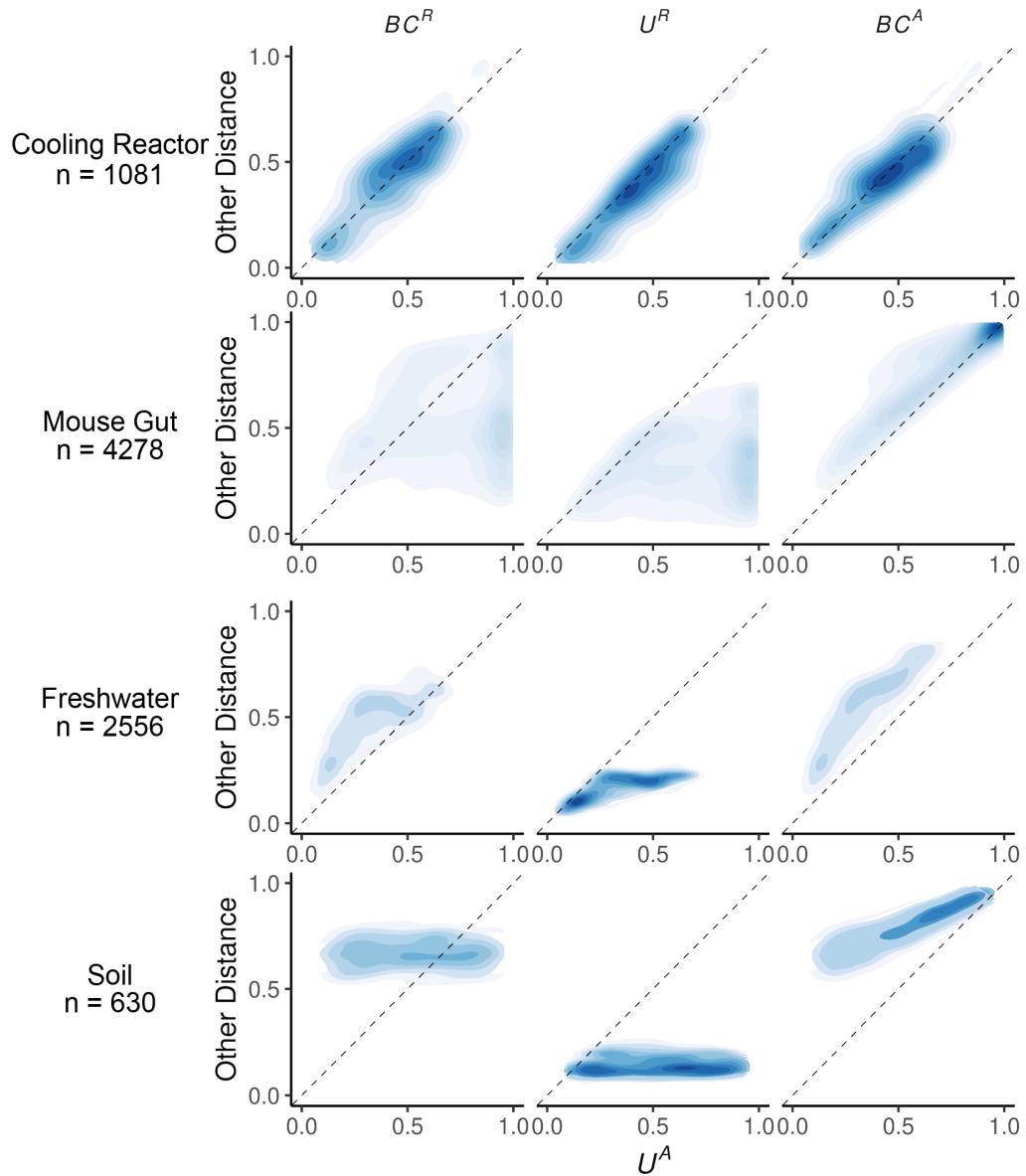
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156 Application of Absolute UniFrac to Four Real-World Microbiome Datasets

157 To illustrate the sensitivity of U^A to both variation in composition and absolute
158 abundance, we re-analyzed four previously published datasets from diverse microbial systems.
159 These datasets included: (i) nuclear reactor cooling water sampled across three reactor cycle
160 phases [5]; (ii) the mouse gut sampled across multiple regions and between two diets [3]; (iii)
161 depth-stratified freshwater communities sampled across two months [9]; and (iv) peanut
162 rhizospheres under two crop rotation schemes (conventional rotation (CR) vs. sod-based rotation
163 (SBR)), plant maturities, and irrigation treatments [10]. Absolute abundance was quantified by
164 flow cytometry for the cooling water and freshwater datasets, droplet digital PCR for the mouse
165 gut, and by qPCR for the rhizosphere dataset. Together these span a wide range of richness—from
166 215 ASVs in the cooling water to 24,000 ASVs in the soil—and microbial load—from as low as
167 4×10^5 cells/ml (cooling water) up to 2×10^{12} 16S rRNA copies/gram (mouse gut). Additional

168 details of the re-analysis workflow, including ASV generation and phylogenetic inference, are
 169 provided in the Supporting Methods.



170 *Figure 2. Absolute UniFrac (U^A) compared with other β -diversity metrics across four real microbial datasets. Each*
 171 *panel shows pairwise sample distances for U^A (x-axis) against another metric (y-axis): Bray-Curtis using relative*
 172 *abundance (BC^R , first column), weighted UniFrac using relative abundances (U^R , second column), and Bray-curtis*
 173 *using absolute abundances (BC^A , third column). Contours indicate the relative density of pairwise comparisons (n*
 174 *shown for each dataset, left), with darker shading corresponding to more observations. The dashed line marks the*
 175 *1:1 relationship. Points above the line indicate cases where U^A is smaller than the comparator metric, while points*
 176 *below the line indicate cases where U^A is larger.*

177 We first calculated four β -diversity metrics for all sample pairs in each dataset and
 178 compared them to U^A (Fig. 2). The degree of concordance between U^A and other metrics was
 179 highly context dependent. In the cooling water dataset, U^A closely tracked all three alternatives,
 180 whereas in the remaining systems it diverged substantially. U^A also spanned a similar or wider

181 range of distances than the other metrics. For example, in the soil dataset BC^R and U^R occupied a
182 narrow range relative to the broad separation observed under U^A .

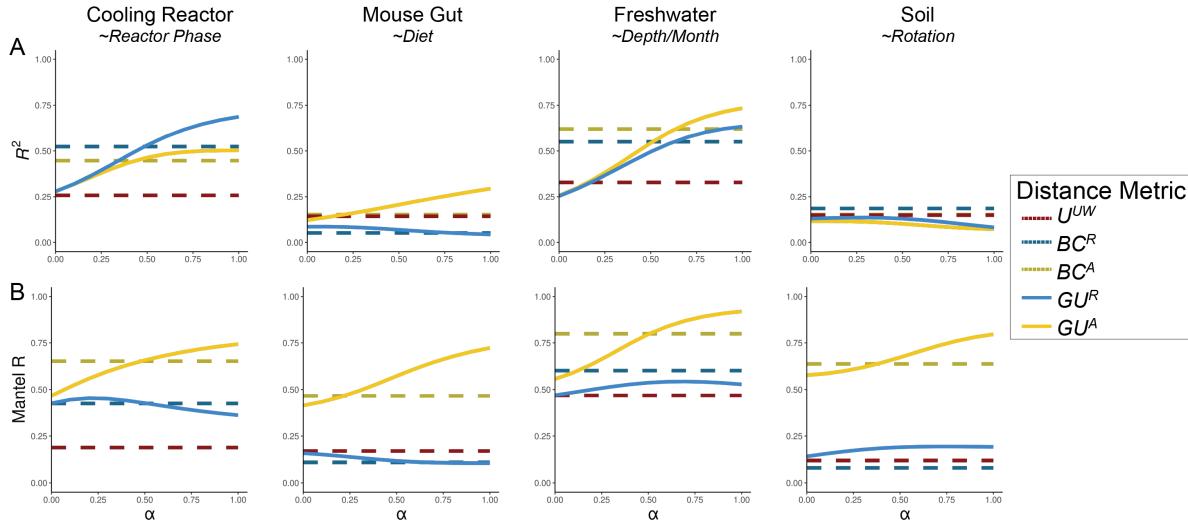
183 U^A generally reported distances that were similar to or greater than U^R , consistent with
184 the simulations shown in Fig. 1B. This reflects the ability of U^A to discern dissimilarity due to
185 differences in microbial load, even when community composition is conserved. In contrast, U^A
186 yielded distances that were similar to or lower than BC^A , again matching the simulated behavior
187 in Fig 1B. In these cases, phylogenetic proximity between abundant, closely-related ASVs leads
188 U^A to register greater similarity than BC^A .

189 Given these differences, we next quantified how well each metric discriminates among
190 categorical sample groups. For each dataset, we ran PERMANOVAs to measure the proportion
191 of variance (R^2) and statistical power (*pseudo-F*, *p*-value) attributable to group structure, using
192 groupings that were determined to be significant in the original publications. To evaluate how
193 strongly absolute abundance contributed to this discrimination, we also calculated GU^A across a
194 range of α values. The resulting R^2 values are displayed in Fig. 3A, with the corresponding
195 *pseudo-F* statistics and *p*-values provided in Fig. S2.

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

202 However, this comes with a major caveat: at high α values, GU^A becomes strongly
203 correlated with total cell count alone (Fig. 3B). Mantel tests confirmed that absolute-abundance
204 metrics are far more sensitive to differences in microbial load than their relative counterparts.
205 This behavior is intuitive, and to some extent desirable, because these metrics are designed to
206 detect changes in microbial load even when composition remains constant. Yet at $\alpha = 1$, U^A
207 approaches a proxy for sample absolute abundance itself. In ordination space (Fig. S3), this can
208 cause Axis 1 to correlate with absolute abundance and in some cases (freshwater and soil)
209 produces strong horseshoe effects [15], potentially distorting ecological interpretation.

210



211 *Figure 3. Discriminatory performance of U^A and related metrics across four microbial systems.* (A) PERMANOVAs
 212 were used to quantify the percent variance (R^2) explained by predefined categorical groups (shown in italics beneath
 213 each dataset name), with 1,000 permutations. PERMANOVA results were evaluated across five metrics and, where
 214 applicable, across eleven α values (0-1 in 0.1 increments). For consistency with the original studies, only samples
 215 from Reactor cycle 1 were used for the cooling-water dataset, only stool samples for the mouse gut dataset, and only
 216 mature rhizosphere samples for the dataset. (B) Mantel correlation (R) between each distance metric and the
 217 pairwise differences in absolute abundance (cell counts or 16S copy number), illustrating the degree to which each
 218 metric is driven by biomass differences.

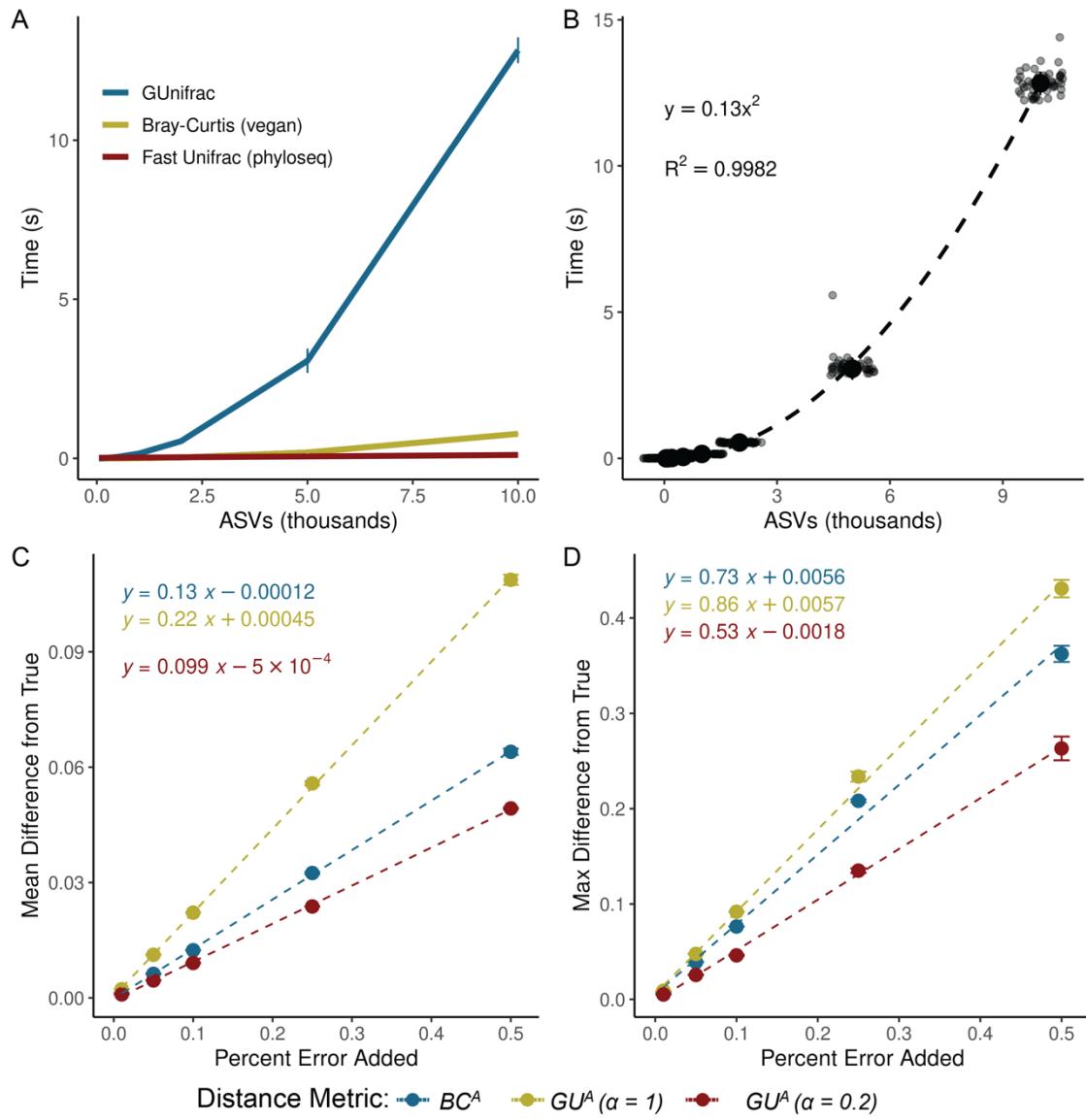
219 We recommend calibrating α based on research goals, modulating this effect by using
 220 GU^A across a range of α rather than relying on U^A ($\alpha = 1$). Researchers should consider how
 221 much emphasis they want their dissimilarity metric to place on microbial load (Box 1). When
 222 biomass differences are central to the hypothesis being tested (for example, detecting
 223 cyanobacterial blooms), high α are recommended. In contrast, if microbial load is irrelevant or
 224 independent of the hypothesis in question, low α (or U^R) may be preferred; for example in the
 225 soil dataset, fine-scale differences in composition may be obscured by random variation in
 226 microbial load.

227 In many systems, microbial biomass is one piece of the story, likely correlated to other
 228 variables being tested. If the importance of microbial load in the system is unknown, one
 229 potential approach is to calculate correlations as demonstrated in Fig. 3B and select an α prior to
 230 any ordinations or statistical testing. Again, correlation to cell count is valuable and is intrinsic to
 231 absolute abundance-aware measures, especially when microbial load is relevant to the
 232 hypotheses being tested. Correlations to cell count in BC^A , an accepted approach in the literature,
 233 ranged from ~0.5 up to ~0.8. As a general recommendation from these analyses, we recommend
 234 α values in an intermediate range from 0.1 up to 0.6, wherein GU^A has similar correlation to cell
 235 count as BC^A .

236 Computational and Methodological Considerations

237 Applying GU^A in practice raises several considerations related to sequencing depth,
 238 richness, and computational cost. Both Bray-Curtis and UniFrac can be sensitive to sequencing
 239 depth because richness varies with read count [16–18]. Methods to address these concerns,
 240 including rarefaction, remain debated and are challenging to benchmark rigorously [19]. We do
 241 not evaluate the sensitivity of U^A or GU^A to different rarefaction strategies here, but we do

242 provide a workflow and code for how we incorporated rarefaction into our own analyses (Box 2
 243 and available code). This approach minimizes sequencing-depth biases while preserving
 244 abundance scaling for downstream β -diversity analysis.



245
 246 *Figure 4.* GU^A requires more computational time but remains resilient to quantification error. (A) Runtime for GU^A
 247 (GUniFrac package), U^R (FastUniFrac in the phyloseq package) and BC^A (vegan package) was benchmarked across
 248 50 iterations on a sub-sampled soil dataset (mature samples only) [10], using increasing ASV richness (50, 100, 200,
 249 500, 1,000, 2,000, 5,000, 10,000), with 10 samples and one α value per run (unweighted UniFrac is also calculated
 250 by default). Error bars represent standard deviation. (B) Quadratic relationship between GU^A computation time and
 251 ASV richness. Large center point represents median across 50 iterations, error bars (standard deviation) are too
 252 small to be seen. (C-D) Sensitivity of GU^A ($\alpha = 1$ and 0.2) and BC^A to measurement error was evaluated by adding
 253 random variation ($\pm 1\%$ to $\pm 50\%$; Supporting Methods) to 16S copy number estimates in stool samples from the
 254 mouse gut dataset [3]. For each error level, 50 replicate matrices were generated and compared to the original
 255 values. Panels reflect the (C) mean difference and (D) max difference between the error-added metrics compared to
 256 the originals. Error bars represent the standard deviation of the average mean and max difference across 50
 257 iterations.

258 GU^A is slower to compute than both BC^A and U^R because it must traverse the phylogenetic
259 tree to calculate branch lengths for each iteration. Computational time of GU^A increases
260 quadratically with ASV number and is 10-20 times slower, though up to 50 times slower, than
261 BC^A (Fig. 4A-B). The number of samples or α values, however, have relatively little effect on
262 runtime (Fig. S4). For a dataset of 24,000 ASVs, computing on a single CPU is still reasonable
263 (1.4 minutes), but repeated rarefaction increases runtime substantially because branch lengths are
264 redundantly calculated with each iteration. Allowing branch-length objects to be cached or
265 incorporated directly into the GUnifrac workflow would considerably improve computational
266 efficiency.

267 We also evaluated the sensitivity of GU^A to measurement error in absolute abundance due
268 to uncertainty arising from the quantification of cell number of 16S copy number. To assess the
269 sensitivity of GU^A and BC^A to measurement error, we added random error to the 16S copy
270 number measurements from the mouse gut dataset, limiting our analyses to the stool samples
271 where copy numbers varied by an order of magnitude. Across 50 iterations, each copy number
272 could randomly vary by a given percentage of error in either direction. We re-calculated β -
273 diversity (BC^A and GU^A at $\alpha = 1$ and $\alpha = 0.2$) and compared these measurements to the original
274 dataset.

275 Introducing random variation into measured 16S copy number altered GU^A values only
276 modestly, and the effect was proportional to the magnitude of the added noise (Fig. 4C-D). At α
277 = 1, each 1% of quantification error introduced an average difference of 0.0022 in GU^A ; at $\alpha =$
278 0.2, GU^A was even less sensitive. Thus, moderate α values provide a balance between
279 interpretability and robustness to noise in absolute quantification. The max deviation from true
280 that added error could inflict on a given metric was also proportional (and always less) than the
281 magnitude of the error itself (Fig. 4D). A more rigorous approach to assessing error propagation
282 within these metrics, including mathematical proofs of the relationships estimated above, is
283 outside the scope of this paper but would be helpful.

284 Ecological Interpretation and conceptual significance

285 Absolute UniFrac reframes the interpretation of β -diversity by making biomass an explicit
286 ecological axis rather than an unmeasured or normalized-away quantity. Conventional UniFrac
287 captures composition and shared evolutionary history, but implicitly invites interpretation as if it
288 also encodes differences on microbial load. By incorporating absolute abundance directly,
289 Absolute UniFrac helps to resolve this mismatch and restores alignment between ecological
290 hypotheses and the quantities represented in the metric. In this view, β -diversity becomes a three-
291 axis ecological measure, incorporating composition, phylogeny and biomass, rather than a two-
292 axis approximation. That said, the additional dimension of microbial load also increases the
293 complexity of applying and interpreting this metric.

294 There are many cases where the incorporation of absolute abundance allows microbial
295 ecologists to assess more realistic, ecologically-relevant differences in microbial communities,
296 especially when microbial load is mechanistically central. Outside of the datasets re-analyzed
297 here [3, 5, 9, 10]; the temporal development of the infant gut microbiome involves both a rise in
298 absolute abundance and compositional changes [6]; bacteriophage predation in wastewater
299 bioreactors can be understood only when microbial load is considered [20]; and antibiotic-driven
300 declines in specific swine gut taxa were missed using relative abundance approaches [21]. As β -

301 diversity metrics (and UniFrac specifically) remain central to microbial ecology, these findings
302 highlight how interpretation changes once biomass is incorporated. Broader adoption of absolute
303 abundance profiling will also depend on data availability. Few studies currently make absolute
304 quantification data publicly accessible, underscoring the need to deposit absolute measurements
305 alongside sequencing reads for reproducibility, ideally as metadata within SRA submissions.

306 While demonstrated here with 16S rRNA data, the approach should extend to other marker
307 genes or (meta)genomic features, provided absolute abundance estimates are available. In this
308 sense, GU^A offers a more ecologically grounded view of lineage turnover by jointly reflecting
309 variation in biomass and phylogenetic structure.

310 No single metric (or α in GU^A), however, is universally “best”. Each β -diversity metric
311 emphasizes a different dimension of community change. Researchers should therefore select
312 metrics based on the ecological quantity that is hypothesized to matter most (Box 1). Here, we
313 demonstrate not that GU^A outperforms other measures, but that it faithfully incorporates the three
314 axes of variation it was designed to incorporate: composition, phylogenetic similarity, and
315 absolute abundance (Fig. 1 and Fig. 2). As with any β -diversity analysis, interpretation requires
316 matching the metric to the ecological question at hand, and exploring sensitivity across different
317 metrics where appropriate [22]. By providing demonstrations and code for the application and
318 interpretation of U^A/GU^A , we hope to encourage the use of these metrics as a tool of microbial
319 ecology.

320 Conclusion

321 By explicitly incorporating absolute abundance, Absolute UniFrac shifts phylogenetic β -
322 diversity from a two-axis approximation to a three-axis ecological measure. This reframing
323 connects the metric to the underlying biological questions that motivate many microbiome
324 studies. As methods for quantifying microbial load continue to expand, the ability to interpret β -
325 diversity in a biomass-aware framework will become increasingly important for distinguishing
326 true ecological turnover from proportional change alone. In this way, Absolute UniFrac is not
327 simply an alternative distance metric but a tool for aligning statistical representation with
328 ecological mechanism.

329

330

331 **Box 1: β -diversity metrics should reflect the hypothesis being tested**

332 Absolute UniFrac is most informative when variation in microbial load is expected to carry
 333 ecological meaning rather than being a nuisance variable. The choice of α determines how
 334 strongly abundance differences influence the metric, and should therefore be selected based on
 335 the hypothesis, not by convention. In settings where biomass is central to the mechanism under
 336 study, higher α values appropriately foreground that signal, whereas in cases where load
 337 variation is incidental or confounding, lower α values maintain interpretability. Framing α as a
 338 hypothesis-driven choice repositions β -diversity from a default normalization step to an explicit
 339 ecological decision.

				Metric to Use	Hypothetical Example
Treat closely related lineages as similar?	Yes	How relevant is microbial load?	Central to hypothesis	$GU^A, \alpha > 0.5$	Cyanobacterial bloom, pathogen proliferation
			Relevant, but associated with other variables of interest	$GU^A, 0.1 < \alpha < 0.5$	Compositional shifts in response to nutrient addition
			Irrelevant	Dominant	$GU^R, \alpha > 0.5$
				Rare	$GU^R, \alpha < 0.5$
			Unknown	GU^A at multiple α	Random variation in microbial load obscured compositional shifts in soil communities
	No	Microbial load relevant?	Yes	BC^A	Strain turnover and proliferation in the infant gut
	No	BC^R	Temporal succession in chemostat		

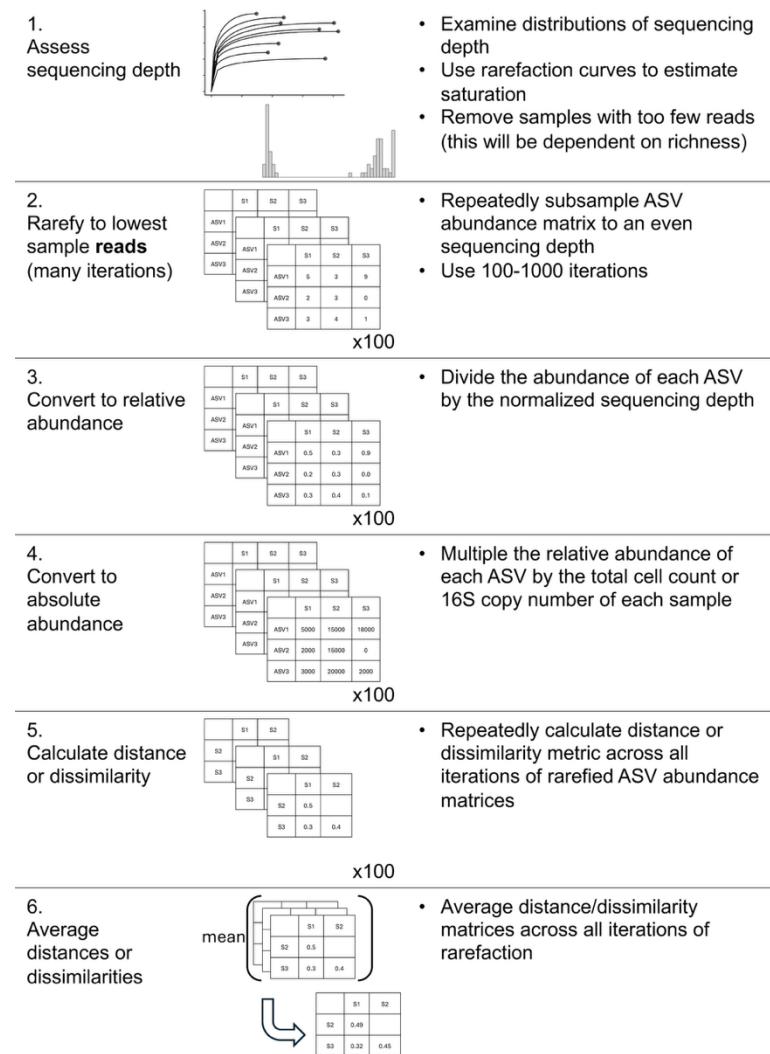
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343 **Box 2: Rarefaction workflow for incorporating absolute abundance**

344 While we refrain from an in-depth
 345 analysis of rarefaction approaches,
 346 here we present our workflow for
 347 incorporating rarefaction alongside
 348 absolute abundance. First, samples
 349 were assessed for anomalously low
 350 read counts and discarded
 351 (sequencing blanks and controls
 352 were also removed). For rarefaction,
 353 each sample in the ASV table was
 354 subsampled to equal *sequencing*
 355 depth (# of reads) across 100
 356 iterations, creating 100 rarefied ASV
 357 tables. These tables were then
 358 converted to relative abundance by
 359 dividing each ASV's count by the
 360 equal sequencing depth (rounding
 361 was not performed). Then, each
 362 ASV's absolute abundance within a
 363 given sample was calculated by
 364 multiplying its relative abundance
 365 by that sample's total cell count or
 366 16S copy number. Methods to
 367 predict genomic 16S copy number
 368 for a given ASV were not used [23].

369 Each distance/dissimilarity metric was then calculated across all 100 absolute-normalized ASV
 370 tables. The final distance/dissimilarity matrix was calculated by averaging all 100 iterations of
 371 each distance/dissimilarity calculation. Of note for future users: pruning the phylogenetic tree
 372 after rarefaction for each iteration is not necessary – ASVs removed from the dataset do not
 373 contribute nor change the calculated of UniFrac distances.



375

376

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