

1 **Title:** To rarefy or not to rarefy: Enhancing diversity analysis of microbial communities through
2 next-generation sequencing and rarefying repeatedly

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11

12 **Abstract**

13 Amplicon sequencing has revolutionized our ability to study DNA collected from environmental
14 samples by providing a rapid and sensitive technique for microbial community analysis that
15 eliminates the challenges associated with lab cultivation and taxonomic identification through
16 microscopy. In water resources management, it can be especially useful to evaluate ecosystem
17 shifts in response to natural and anthropogenic landscape disturbances to signal potential water
18 quality concerns, such as the detection of toxic cyanobacteria or pathogenic bacteria. Amplicon
19 sequencing data consist of discrete counts of sequence reads, the sum of which is the library size.
20 Groups of samples typically have different library sizes that are not representative of biological
21 variation; library size normalization is required to meaningfully compare diversity between them.
22 Rarefaction is a widely used normalization technique that involves the random subsampling of
23 sequences from the initial sample library to a selected normalized library size. Rarefying is often
24 dismissed as statistically invalid because subsampling effectively discards a portion of the
25 observed sequences. Nonetheless, it remains prevalent in practice. Notably, the superiority of
26 rarefying relative to many other normalization approaches has been argued in diversity analysis.
27 Here, repeated rarefying is proposed as a tool for diversity analyses to normalize library sizes.
28 This enables (i) proportionate representation of all observed sequences and (ii) characterization
29 of the random variation introduced to diversity analyses by rarefying to a smaller library size
30 shared by all samples. While many deterministic data transformations are not tailored to produce
31 equal library sizes, repeatedly rarefying reflects the probabilistic process by which amplicon
32 sequencing data are obtained as a representation of the source microbial community.
33 Specifically, it evaluates which data might have been obtained if a particular sample's library

34 size had been smaller and allows graphical representation of the effects of this library size
35 normalization process upon diversity analysis results.

36 **Keywords (Maximum 6 keywords)**

37 Library size normalization, amplicon sequencing, alpha diversity, beta diversity, Shannon index,
38 Bray-Curtis dissimilarity

39 **1. Introduction**

40 Next-generation sequencing (NGS) has revolutionized the understanding of environmental
41 systems through the characterization of microbial communities and their function by examining
42 DNA collected from samples that contain mixed assemblages of organisms (Bartram et al., 2011;
43 Hugerth and Andersson, 2017; Shokralla et al., 2012). It is well known that fewer than 1% of
44 species in the environment can be isolated and cultured, limiting the ability to identify rare and
45 difficult-to-cultivate members of the community (Bodor et al., 2020; Cho and Giovannoni, 2004;
46 Ferguson et al., 1984). In addition to the limitations of culturing, microscopic evaluation of
47 environmental samples remains of limited utility because of challenges in high-resolution
48 taxonomic identification and the inability to infer function from morphology (Hugerth and
49 Andersson, 2017). Metagenomic evaluations employ NGS technology to analyze large quantities
50 of diverse environmental DNA (Thomas et al., 2012) and have largely eliminated challenges
51 associated with culturing and microscopic identification (McMurdie and Holmes, 2014).

52 Metagenomics encompasses a conglomerate of different sequencing experimental designs,
53 including amplicon sequencing (sequencing of amplified genes of interest) and shotgun
54 sequencing (sequencing of fragments of present genetic material). While shotgun sequencing
55 allows characterization of the entire community, including both taxonomic composition and
56 functional gene profiles, it is not widely accessible due to high sequencing costs and

57 computational requirements for analysis (Bartram et al., 2011; Clooney et al., 2016; Langille et
58 al., 2013). In contrast, the relatively low cost of amplicon sequencing has made it an increasingly
59 popular technique (Clooney et al., 2016; Langille et al., 2013). The amplification and sequencing
60 of specific genes (e.g., taxonomic marker genes) enables characterization of microbial
61 community composition (Hodkinson and Grice, 2015); as a result, it has been successfully
62 applied in many areas of environmental and water research. For example, amplicon sequencing
63 has been used to characterize and predict cyanobacteria blooms (Tromas et al., 2017), describe
64 microbial communities found in aquatic ecosystems (Zhang et al., 2020), and evaluate
65 groundwater vulnerability to pathogen intrusion (Chik et al., 2020). It has also been applied to
66 water quality and treatment performance monitoring in diverse settings (Vierheilig et al., 2015),
67 including drinking water distribution systems (Perrin et al., 2019; Shaw et al., 2015), drinking
68 water biofilters (Kirisits et al., 2019), anaerobic digesters (Lam et al., 2020), and cooling towers
69 (Paranjape et al., 2020).

70 Processing and analysis of amplicon sequencing data are statistically complicated for a
71 number of reasons (Weiss et al., 2017). In particular, library sizes (i.e., the total number of
72 sequencing reads within a sample) can vary widely among different samples, even within a
73 single sequencing run, and the disparity in library sizes between samples may not represent
74 actual differences in microbial communities (McMurdie and Holmes, 2014). Amplicon
75 sequencing libraries cannot be compared directly for this reason. For example, two replicate
76 samples with 5,000 and 20,000 sequence reads, respectively, are likely to have different read
77 counts for specific sequence variants simply due to the difference in library size. While
78 parametric tools such as generalized linear modelling (e.g., McMurdie and Holmes, 2014) can
79 provide a statistically sound framework for differential abundance analysis, drawing biologically

80 meaningful diversity analysis conclusions from amplicon sequencing data typically requires
81 normalization of library sizes to account for the additional variation in counts that is attributable
82 to differences in library sizes between samples (McKnight et al., 2019). For example, larger
83 samples may appear more diverse than smaller samples (Hughes and Hellmann, 2005). Notably,
84 a variety of normalization techniques that may affect the analysis and interpretation of results
85 have been suggested, including rarefaction (i.e., the process of rarefying libraries to a common
86 size).

87 Rarefaction is a normalization tool initially developed for ecological diversity analyses to
88 allow for sample comparison without associated bias from differences in sample size (Sanders,
89 1968). Rarefaction normalizes samples of differing sample size by subsampling each to a shared
90 threshold. Although initially developed for use in ecological studies, rarefaction is a commonly
91 used library size normalization technique for amplicon sequencing data. As a result, it is the
92 subject of considerable debate and statistical criticism (Gloor et al., 2017; McMurdie and
93 Holmes, 2014). Rarefying is typically conducted in a single iteration that only provides a
94 snapshot of the community that might have been observed at the smaller normalized library size.
95 This omits a random subset of observed sequences and potentially also samples with small
96 library sizes and introduces artificial variation to the data (McMurdie and Holmes, 2014).

97 Repeatedly rarefying, on the other hand, has the potential to address the statistical concerns
98 associated with omission of data and could provide a more statistically acceptable technique than
99 performing a single iteration of rarefying for diversity analyses. It characterizes what data might
100 have been obtained if a particular sample's library size had been smaller, revealing what can be
101 inferred about community diversity in the source from samples of equal library size. Rarefying
102 repeatedly has received only trivial consideration in the literature (e.g., McMurdie and Holmes,

103 2014; Navas-Molina et al., 2013). Diversity analysis approaches grounded in statistical inference
104 about source microbial diversity (that address the random probabilistic processes through which
105 NGS yields libraries of sequence reads) could conceptually be superior to rarefying (Willis,
106 2019), but they are not yet fully developed or readily available for routine diversity analysis to
107 support study of environmental microbial communities.

108 Here, we investigate the application of repeatedly rarefying as a library size normalization
109 technique specifically for diversity analyses. This paper graphically evaluates the impact of
110 subsampling with or without replacement and normalized library size selection on diversity
111 analyses such as the Shannon index and Bray-Curtis dissimilarity ordinations, specifically.
112 Rather than representing diversity as a single numerical value or point in an ordination plot
113 (often following transformation that may not be designed to compensate for differing library
114 sizes), rarefying repeatedly yields bands of values or patches of points that characterize how
115 diversity may vary among or between samples at a particular library size.

116 **2. Theory**

117 *2.1 Amplicon Sequencing and Diversity Analysis for Microbial Communities in Water – An* 118 *Overview*

119 Due to the inevitable interdisciplinarity of environmental water quality research and the
120 complexity and novelty of next generation sequencing relative to traditional microbiological
121 methods used in water quality analyses, further detail on amplicon sequencing is provided.
122 Amplification and sequencing of taxonomic marker genes has been used extensively to examine
123 phylogeny, evolution, and taxonomic classification of numerous groups across the three domains
124 of life (Quast et al., 2013; Weisburg et al., 1991; Woese et al., 1990). Taxonomic marker genes
125 include the 16S rRNA gene in mitochondria, chloroplasts, bacteria and archaea (Case et al.,

126 2007; Tsukuda et al., 2017; Weisburg et al., 1991; Yang et al., 2016), or the 18S rRNA gene
127 within the nucleus of eukaryotes (Field et al., 1988). Widely used reference databases have been
128 developed containing marker gene sequences across numerous phyla (Hugerth and Andersson,
129 2017).

130 The 16S rRNA gene consists of nine highly conserved regions separated by nine
131 hypervariable regions (V1-V9; Gray et al., 1984) and is approximately 1,540 base pairs in length
132 (Kim et al., 2011; Schloss and Handelsman, 2004). While sequencing of the full 16S rRNA gene
133 provides the highest taxonomic resolution (Johnson et al., 2019), many studies only utilize partial
134 sequences due to limitations in read length of NGS platforms (Kim et al., 2011). Next-generation
135 sequencing on Illumina platforms (Illumina Inc., San Diego, California) produces reads that are
136 up to 350 base pairs in length, requiring selection of an appropriate region of the 16S rRNA gene
137 to amplify and sequence for optimal taxonomic resolution (Bukin et al., 2019; Kim et al., 2011).
138 Sequencing the more conservative regions of the 16S rRNA gene may be limited to resolution of
139 higher levels of taxonomy, while more variable regions can provide higher resolution for the
140 classification of sequences to the genus and species levels in bacteria and archaea (Bukin et al.,
141 2019; Kim et al., 2011; Yang et al., 2016).

142 Different variable regions of the 16S rRNA gene may be biased towards different taxa
143 (Johnson et al., 2019) and be preferred for different ecosystems (Escapa et al., 2020). For
144 example, the V4 region has been shown to strongly differentiate taxa from the phyla
145 Cyanobacteria, Firmicutes, Fusobacteria, Plantomycetes, and Tenericutes but the V3 region best
146 differentiates taxa from the phyla Proteobacteria (e.g., *Escherichia coli*, *Salmonella* spp.,
147 *Campylobacter* spp.), Acidobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes,
148 Nitrospirae, and Spirochaetae (Zhang et al., 2018). The V4 region of the 16S rRNA gene is

149 frequently targeted using specific primers designed to minimize amplification bias while
150 accounting for common aquatic bacteria (Walters et al., 2015) and is frequently used in aquatic
151 studies (Zhang et al., 2018). It is important to consider suitability of a 16S rRNA region for the
152 habitat (Escapa et al., 2020) and the taxa present in the microbial community due to potential
153 bias of analyzing differing subregions of the 16S rRNA gene (Johnson et al., 2019; Zhang et al.,
154 2018).

155 The use of amplicon sequencing of partial sequences of the 16S rRNA gene allows
156 examination of microbial community composition and the exploration of shifts in community
157 structure in response to environmental conditions (Hodkinson and Grice, 2015), and
158 identification of differentially abundant taxa between samples (Hugerth and Andersson, 2017).
159 Amplicon sequencing datasets can be analyzed using a variety of bioinformatics pipelines for
160 sequence analysis (e.g., sequence denoising, taxonomic classification, diversity analysis)
161 including *mothur* (Schloss et al., 2009) and *QIIME2* (Bolyen et al., 2019). Previously,
162 sequencing analysis involved the creation of dataset-dependent operational taxonomic units
163 (OTUs) by clustering sequences into groups that met a certain similarity threshold, resulting in a
164 loss of representation of variation in sequences and precluding cross-study comparison (Callahan
165 et al., 2017). Advances in computational power have allowed a shift from use of OTUs to
166 amplicon sequence variants (ASVs) representative of each unique sequence in a sample, which
167 allows for the comparison of sequence variants generated in different studies and retains the full
168 observed biological variation (Callahan et al., 2017). The implementation of tools included
169 bioinformatics pipelines, such as *DADA2* (Callahan et al., 2016) or *Deblur* (Amir et al., 2017),
170 allows quality control of sequencing through the removal of sequencing errors and for the
171 creation of ASVs.

172 Quality controlled sequencing data for a particular run is then organized into large matrices
173 where columns represent experimental samples and rows contain counts for different ASVs
174 (Weiss et al., 2017). Amplicon sequencing samples have a total number of sequencing reads
175 known as the library size (McMurdie and Holmes, 2014), but do not provide information on the
176 absolute abundance of sequence variants (Gloor et al., 2016, 2017). This data can be used for
177 studies on taxonomic composition, differential abundance analysis and diversity analyses (Figure
178 1). Taxonomic classification of 16S rRNA sequences using rRNA databases including SILVA
179 (Quast et al., 2013), the Ribosomal Database Project (Cole et al., 2014) and GreenGenes
180 (DeSantis et al., 2006) allows for construction of taxonomic community profiles (Bartram et al.,
181 2011). Taxonomic composition analysis allows for characterization of microbial communities by
182 classifying sequence variants based on similarities to sequences in online databases. The creation
183 of taxonomic composition graphs frequently expresses community composition in proportions.
184 Differential abundance analysis is utilized to explore whether specific sequence variants are
185 found in significantly different proportions between samples (Weiss et al., 2017) to identify
186 potential biological drivers for these differences. This application is outside the scope of this
187 work and is frequently performed using programs initially designed for transcriptomics, such as
188 *DESeq2* (Love et al., 2014) and *edgeR* (Robinson et al., 2009), or programs designed to account
189 for the compositional structure of sequence data *ALDeX2* (Fernandes et al., 2014). The final
190 potential application of this data is diversity analyses, which can be evaluated on varying scales
191 from within sample (alpha) to between samples (beta; Sepkoski, 1988) but is associated with the
192 challenge of the true diversity of environmental sources largely remaining unknown (Hughes et
193 al., 2001).

194 Alpha diversity serves to identify richness (e.g., number of observed sequence variants) and
195 evenness (e.g., allocation of read counts across observed sequence variants) within a sample
196 (Willis, 2019). Comparison of alpha diversity among samples of differing library sizes may
197 result in inherent biases, with samples having larger library sizes appearing more diverse due to
198 the potential presence of more sequence variants in samples with larger libraries (Hughes and
199 Hellmann, 2005; Willis, 2019). This has commonly required samples to have equal library sizes
200 before comparison to prevent bias fabricated only from differences in initial library size.
201 Diversity indices used to characterize the alpha diversity of samples include but are not limited
202 to the Shannon index (Shannon, 1948), Chao1 index (Chao and Bunge, 2002), and Simpson
203 index (Simpson, 1949), but unique details of such indices should be understood for correct
204 usage. For example, Chao1 relies on the observation of singletons in data to estimate diversity
205 (Chao and Bunge, 2002), but denoising processes for sequencing data may remove singleton
206 reads making the Chao1 estimator invalid for accurate analysis. The Shannon index, used in this
207 study, is affected by differing library sizes because the contribution of rare sequences to total
208 diversity is progressively lost with smaller library sizes.

209 Similar to alpha diversity, samples with differing library sizes in beta diversity analyses may
210 produce erroneous results due to the potential for samples with larger library sizes to have more
211 unique sequences simply due to the presence of more sequence variants (Weiss et al., 2017). A
212 variety of beta diversity metrics can be used to compare sequence variant composition between
213 samples including Bray-Curtis (Bray and Curtis, 1957) or Unifrac (Lozupone and Knight, 2007)
214 distances, which can then be visualized using ordination techniques (e.g., PCA, PCoA, NMDS).
215 Bray-Curtis dissimilarity, used in this study, includes pairwise comparison of the numbers for

216 each ASV between two samples, which are expected to be quite dissimilar (even if the
217 communities they represent are not) if library sizes vary substantially.

218 *2.2 Limitations of Library Size Normalization Techniques*

219 Diversity analysis, as it is presently applied, usually requires library size normalization to
220 account for bias introduced through varying read counts in samples. For example, samples with
221 larger library sizes may appear more diverse simply due to the presence of more sequences.
222 Normalization techniques that feature various statistical transformations have been proposed for
223 use in place of rarefying or proportions (McKnight et al., 2019), including upper-quartile log fold
224 change (e.g., Robinson et al., 2009), centered log-ratio transformations (e.g., Gloor et al., 2017),
225 geometric mean pairwise ratios (e.g., Chen et al., 2018), variance stabilizing transformations
226 (e.g., Love et al., 2014) or relative log expressions (e.g., Badri et al., 2018). McKnight et al.
227 (2019) noted that the failure of most normalization techniques to transform data to equal library
228 sizes for diversity analysis “is discouraging, as standardizing read depths are the initial impetus
229 for normalizing the data (i.e., if all samples had equal read depths after sequencing, there would
230 be no need to normalize”.

231 These proposed alternatives to rarefying are also often compromised by the presence of large
232 proportions of zero count data in tabulated amplicon sequencing read counts. Zero counts
233 represent a lack of information (Silverman et al., 2018) and may arise from true absence of the
234 sequence variant in the sample or a loss resulting in it not being detected when it was actually
235 present (Tsilimigras and Fodor, 2016; Wang and LêCao, 2019). Nonetheless, many
236 normalization procedures for amplicon sequencing datasets require zero counts to be omitted or
237 modified, especially when applying transformations that utilize logarithms (e.g., centered log-
238 ratio, relative log expressions, geometric mean pairwise ratios). Methods that utilize logarithms

239 involve fabricating count values (pseudocounts) for the many zeros of which amplicon
240 sequencing datasets are comprised and selecting a pseudocount value is an additional challenge
241 (Weiss et al., 2017) that may be accomplished using probabilistic arguments (Gloor et al., 2016;
242 2017). Zeros are a natural occurrence in discrete, count-based data such as the counting of
243 microorganisms or amplicon sequences and adjusting or omitting them can introduce substantial
244 bias into microbial analyses (Chik et al., 2018).

245 McMurdie and Holmes (2014) noted that use of proportions is problematic due to
246 heteroscedasticity: for example, one sequence read in a library size of 100 is a far less precise
247 representation of source composition than 100 sequence reads in a library size of 10,000, even
248 though both comprise 1% of the observed sequences. McKnight et al. (2019) favour use of
249 proportions in diversity analysis without noting how precision of proportions, and the degree to
250 which alpha diversity in the source is reflected (Willis, 2019), varies with library size. Willis
251 (2019) also points towards a conceptually better approach to diversity analysis that accounts for
252 measurement error and the difference between the sample data and the population
253 (environmental source) of which the sample data are only a partial representation. Diversity
254 analysis in general does not do this, as it applies a set of calculations to sample data (or some
255 transformation thereof) to obtain one value of alpha diversity or one point on an ordination plot.
256 Pending further development of such approaches, this study revisits rarefying because of the
257 practical simplicity of comparing diversity among samples of equal library size.

258 McMurdie and Holmes (2014) propose that rarefying is not a statistically valid normalization
259 technique due to the omission of valid data, which may be resolved for the purposes of diversity
260 analysis by rarefying repeatedly to represent all sequences in the proportions with which they
261 were observed and compare sample-level microbial community diversity at a particular library

262 size. In addition, McMurdie and Holmes (2014) dismissed repeatedly rarefying as a
263 normalization technique, in part because repeatedly rarefying an artificial library consisting of a
264 50:50 ratio of two sequence variants does not yield a 50:50 ratio at the rarefied library size and
265 this added noise could affect downstream analyses. However, such error is inherent to
266 subsampling, whether from a population or from a larger sequence library and has thus already
267 affected samples with smaller library sizes; it is the reason why simple proportions are less
268 precise in samples with smaller library sizes.

269 McMurdie and Holmes (2014), also cited the investigation of Navas-Molina et al. (2013) as
270 an example of repeatedly rarefying to normalize library sizes and used it to support their
271 dismissal of this technique due to the omission of valid data and added variability. However, it is
272 critical to note that the work in Navas-Molina et al. (2013) reported using jackknife resampling
273 of sequences, which cannot be equated to repeatedly rarefying (random resampling with or
274 without replacement). Hence, it is necessary to build upon preliminary analysis of repeatedly
275 rarefying as a normalization technique and to explore the impact of subsampling approach and
276 normalized library size on diversity analysis results.

277 **3. Methods**

278 *3.1 Example Data – DNA Extraction and Amplicon Sequencing*

279 Samples used in this study are part of a larger study at Turkey Lakes Watershed (North Part,
280 ON), but only an illustrative subset of samples is considered for the purpose of evaluating
281 rarefaction rather than for ecological interpretation. This allows evaluation of repeated rarefying
282 as a normalization technique without utilizing simulated data. DNA extracts isolated from
283 environmental samples were submitted for amplicon sequencing using the Illumina MiSeq
284 platform (Illumina Inc., San Diego, California) at the commercial laboratory Metagenom Bio

285 Inc. (Waterloo, Ontario). Primers designed to target the 16S rRNA gene V4 region [515FB
286 (GTGYCAGCMGCCGCGGTAA) and 806RB (GGACTACNVGGGTWTCTAAT; Walters et
287 al., 2015)] were used for PCR amplification.

288 *3.2 Sequence Processing and Library Size Normalization*

289 The program *QIIME2* (v. 2019.10; Bolyen et al., 2019) was used for bioinformatic processing of
290 sequence reads. Demultiplexed paired-end sequences were trimmed and denoised, including the
291 removal of chimeric sequences and singleton sequence variants to avoid sequences that may not
292 be representative of real organisms, using *DADA2* (Callahan et al., 2016) to construct the ASV
293 table. Zeroing all singleton sequences could erroneously remove legitimate sequences,
294 particularly if the sequence in question is detected in large numbers in other similar samples;
295 however, the potential effect of such error upon diversity analysis is beyond the scope of this
296 work. Output files from *QIIME2* were imported into R (v. 4.0.1; R Core Team, 2020) for
297 community analyses using *qiime2R* (v. 0.99.23; Bisanz, 2018). Initial sequence libraries were
298 further filtered using *phyloseq* (v. 1.32.0; McMurdie and Holmes, 2013) to exclude amplicon
299 sequence variants that were taxonomically classified as mitochondria or chloroplast sequences.
300 We developed a package called *mirlyn* (Multiple Iterations of Rarefaction for Library
301 Normalization; Cameron and Tremblay, 2020) that facilitates implementation of techniques used
302 in this study built from existing R packages (Table S1). Using the output from *phyloseq*, *mirlyn*
303 was used to (1) generate rarefaction curves, (2) repeatedly rarefy libraries to account for
304 variation in library sizes among samples, and (3) plot diversity metrics given repeated
305 rarefaction.

306 *3.3 Community Diversity Analyses on Normalized Libraries*

307 The impact of normalized library size on the Shannon index (Shannon, 1948), an alpha diversity
308 metric, was evaluated. Normalized libraries were also used for beta diversity analysis. A
309 Hellinger transformation was applied to normalized libraries to account for the arch effect
310 regularly observed in ecological count data and Hellinger-transformed data were then used to
311 calculate Bray-Curtis distances (Bray and Curtis, 1957). Principal component analysis (PCA)
312 was conducted on the Bray-Curtis distance matrices.

313 *3.4 Study Approach*

314 Typically, rarefaction has only been conducted a single time in microbial community analyses,
315 and this omits a random subset of observed sequences, introducing a possible source of error. To
316 examine this error, samples were repeatedly rarefied 1000 times. This repetition provides a
317 representative suite of rarefied samples capturing the randomness in sequence variant
318 composition imposed by rarefying. The sections below address the various decisions that must be
319 made by the analyst and factors affecting reliability of results when rarefaction is used.

320 *3.4.1 The Effects of Subsampling Approach – With or Without Replacement*

321 Rarefying library sizes may be performed with or without replacement. To evaluate the effects of
322 subsampling replacement approaches, we repeatedly rarefied filtered sequence libraries with and
323 without replacement. Results of the two approaches were contrasted in diversity analyses to
324 evaluate the impact of subsampling approach on interpretation of results.

325 *3.4.2 The Effects of Normalized Library Size Selection*

326 Rarefying involves the selection of an appropriate sampling depth to be shared by each sample.
327 To evaluate the effects of different rarefied library sizes, filtered sequence libraries were rarefied

328 repeatedly to varying depths. Results for various sampling depths were contrasted in diversity
329 analyses to evaluate the impact of normalized library size selection on interpretation of results.

330 **4. Results and Discussion**

331 *4.1 Use of Rarefaction Curves to Explore Suitable Normalized Library Sizes*

332 Rarefying requires the selection of a potentially arbitrary normalized library size, which
333 can impact subsequent community diversity analyses and therefore presents users with the
334 challenge of making an appropriate decision of what size to select (McMurdie and Holmes,
335 2014). Suitable sampling depths for groups of samples can be determined through the
336 examination of rarefaction curves (Figure 2). By selecting a library size that encompasses the
337 flattening portion of the curve for each sample, it is generally assumed that the normalized
338 library size will adequately capture the diversity within the samples despite the exclusion of
339 sequence reads during the rarefying process (i.e., there are progressively diminishing returns in
340 including more of the observed sequence variants as the rarefaction curve flattens).

341 Suggestions have previously been made encouraging selection of a normalized library
342 size that is encompassing of most samples (e.g., 10,000 sequences) and advocacy against
343 rarefying below certain depths (e.g., 1,000 sequences) due to decreases in data quality (Navas-
344 Molina et al., 2013). However, generic criteria may not be applicable to all datasets and
345 exploratory data analysis is often required to make informed and appropriate decisions on the
346 selection of a normalized library size. Although previous research advises against rarefying
347 below certain thresholds, users may be presented with the dilemma of selecting a sampling depth
348 that either does not capture the full diversity of a sample depicted in the rarefaction curve (Figure
349 2 – I) or would require the omission of entire samples with smaller library sizes (Figure 2 – III).
350 The implementation of multiple iterations of rarefying library sizes will aid in alleviating this

351 dilemma by capturing the potential losses in community diversity for samples that are rarefied to
352 lower than ideal depth. Doing so with two or more normalized library sizes may reveal
353 differences in diversity attributable to relatively rare variants that could be suppressed by
354 normalizing to too small of a library size.

355 *4.2 The Effects of Subsampling Approach and Normalized Library Size Selection on Alpha* 356 *Diversity Analyses*

357 The differences in how rarefying samples may be carried out requires users to be diligent
358 in the selection of appropriate tools and commands for their analysis. The R package *phyloseq*, a
359 popular tool for microbiome analyses, has default settings for rarefying including sampling with
360 replacement to optimize computational run time and memory usage (McMurdie and Holmes,
361 2013). Sampling without replacement, however, is more appropriate statistically because it draws
362 a subset from the observed set of sequences (as though the sample had yielded only the specified
363 library size), whereas sampling with replacement fabricates a set of sequences in similar
364 proportions to the observed set of sequences (Figure 3). Sampling with replacement can
365 potentially cause a rare sequence variant to appear more frequently in the rarefied sample than it
366 was in the original library.

367 Rarefying libraries with or without replacement was not found to substantially impact the
368 Shannon index in the scenarios considered in this study (Figure 4-A), but users should still be
369 aware of potential implications of sampling with or without replacement when rarefying
370 libraries. Libraries rarefied with replacement are observed to have a slightly reduced Shannon
371 index relative to libraries rarefied without replacement at many library sizes because rare
372 sequences are excluded more often when sampling with replacement.

373 The conservation of larger normalized library sizes allows detection of more diversity
374 with minimal variation observed between the iterations of rarefaction (Figure 4-A). The largest
375 considered normalized library size (the sample with the smallest library size has 11,213
376 sequences) captured the highest Shannon index values, while the Shannon index diminishes for
377 all samples at lower normalized library sizes. The use of repeated iterations of rarefying allows
378 variation introduced through subsampling to be represented in the diversity metric, which is
379 small at larger library sizes. While there was only slight disparity in the Shannon index values
380 between the largest library size and unnormalized data, this may not always be the case and is
381 dependent on the sequence variant composition of the samples. Samples dominated by a large
382 number of low-abundance sequence variants are more likely to have a substantially reduced
383 Shannon index value at a larger normalized library size. Alternatively, samples dominated by
384 only a few highly abundant sequence variants will be comparatively robust to rarefying. A plot
385 of the Shannon index as a function of rarefied library size (Figure 4-B) demonstrates the overall
386 robustness of the Shannon index of these samples for larger library sizes (e.g., > 5,000
387 sequences) and the increased variation and diminishing values when proceeding to smaller
388 rarefied library sizes. When the normalized library size was decreased to 5,000, the Shannon
389 index is still only slightly reduced by the rarefaction but there is greater variability introduced
390 from rarefying.

391 The consistency of the diversity metric when rarefying repeatedly is extremely degraded
392 when libraries were rarefied to the smallest considered library size of 500 sequences. It illustrates
393 the potential to reach incorrect conclusions if rarefying is completed only once. When rarefying
394 repeatedly to a small library size, however, diversity index values that are both highly
395 inconsistent and suppressed relative to the diversity of the unrarefied data may lead to

396 inappropriate claims of identical diversity values between samples (e.g., samples A, B, and C
397 become indistinguishable). The extreme reduction and introduced variation of the Shannon index
398 suggests that the selection of smaller rarefied library sizes should be approached with caution
399 when using alpha diversity metrics, while larger normalized library sizes prevent loss of
400 precision and reduction of the Shannon index value. However, as previously noted, the reduction
401 in the value of the Shannon index will be dependent on the sequence variant composition of the
402 samples.

403 Previous research evaluating normalization techniques has focused on beta diversity
404 analysis and differential abundance analysis (Gloor et al., 2017; McMurdie and Holmes, 2014;
405 Weiss et al., 2017), but the appropriateness of library size normalization techniques for alpha
406 diversity metrics must be evaluated due to the prerequisite of having equal library sizes for
407 accurate calculation. Utilization of unnormalized library sizes with alpha diversity metrics may
408 generate bias due to the potential for samples with larger library sizes to inherently reflect more
409 of the diversity in the source than a sample with a small library size. The repeated iterations of
410 rarefying library sizes allow characterization of the variability introduced to sample diversity by
411 rarefying at any rarefied library size (Figure 4) but does not allow evaluation of uncertainty
412 about the diversity in the source from which the sample was taken, as is the case for all
413 normalization-based approaches.

414 *4.3 The Effects of Subsampling Approach and Normalized Library Size Selection on Beta* 415 *Diversity Analysis*

416 When samples were repeatedly rarefied to a common normalized library size with and
417 without replacement, similar amounts of variation in the Bray-Curtis PCA ordinations were
418 observed between the sampling approaches (Figure 5). This indicates that although rarefying

419 with replacement seems potentially erroneous due to the fabrication of count values that are not
420 representative of actual data, the impact on the variation introduced into the Bray-Curtis
421 dissimilarity distances is not large and will likely not interfere with the interpretation of results.
422 However, rarefying without replacement should be encouraged because it is more theoretically
423 correct to represent possible data if only the smaller library size had been obtained, and it has not
424 been comprehensively demonstrated that sampling with replacement is a valid approximation for
425 all types of diversity analysis or library compositions.

426 When larger normalized library sizes are maintained through rarefaction, there is less
427 potential variation introduced into beta diversity analyses, including Bray-Curtis dissimilarity
428 PCA ordinations. For example, in the largest normalized library size possible for these data
429 (Figure 5A), a minimal amount of variation was observed within each community, indicating
430 that the preservation of higher sequence counts minimizes the amount of artificial variation
431 introduced into datasets by rarefaction (including no variation for Sample F because it is not
432 actually rarefied in this scenario). For this reason, rarefying to the smallest library size of a set of
433 samples is a sensible guideline. Although, a normalized library size of 5,000 is lower than the
434 flattening portion of the rarefaction curve for samples A, B, and C (Figure 2), the selection of
435 this potentially inappropriate normalized library size (Figure 5C) can still accurately reflect the
436 diversity between samples without excess artificial variation introduced through rarefaction. Due
437 to the variation introduced to the Bray-Curtis dissimilarity ordinations in the smaller rarefied
438 library sizes (Figure 5E/G), it is critical to include computational replicates of rarefied libraries
439 to fully characterize the introduced variation in communities. As discussed above, it has been
440 suggested that repeatedly rarefying is inappropriate due to the introduction of “added noise”.
441 However, as demonstrated, the maintenance of larger rarefied library sizes when repeatedly

442 rarefying does not impact interpretation of beta-diversity analysis results. Without this
443 replication, rarefaction to small, normalized library sizes could result in artificial similarity or
444 dissimilarity identified between samples.

445 Beta diversity analysis of very small, rarefied library sizes (Figure 6A, B, C) can still
446 reflect similar clustering patterns observed in larger library sizes but with a much lower
447 resolution of clusters. Rarefying has previously been shown to be an appropriate normalization
448 tool for samples with low sequence counts (e.g., <1,000 sequences per sample) by Weiss et al.
449 (2017), which is promising for datasets containing samples with small initial library sizes or
450 potentially analyzing subsets of data to explore diversity within specific phyla (e.g.,
451 Cyanobacteria). Caution must be taken to avoid selection of an excessively small, normalized
452 library size due to the introduction of extreme levels of artificial variation that compromises
453 accurate depiction of diversity (Figure 6D) and suppresses the contribution of rare variants to
454 overall diversity. The tradeoff between rarefying to a smaller than advisable library size or
455 excluding entire samples with small library sizes remains and can possibly be resolved by
456 analyzing results with all samples and a small, rarefied library size as well as with some omitted
457 samples and a larger rarefied library size.

458 Although rarefying has the potential to introduce artificial variation into data used in beta
459 diversity analyses, these results suggest that rarefying repeatedly does not become problematic
460 until normalized library sizes are very small (e.g., 500 sequences or less) for the samples
461 considered. While we saw a degradation of the consistency and value of the alpha diversity
462 Shannon index at 500 sequences, beta diversity analyses may be more robust to rarefaction and
463 capable of reflecting qualitative clusters in ordination as previously discussed in Weiss et al.
464 (2017). The artificial variation introduced to beta diversity analyses by rarefaction could lead to

465 erroneous interpretation of results, but the implementation of multiple iterations of rarefying
466 library sizes allows a full representation of this variation to aid in determining if apparent
467 similarity or dissimilarity is a chance result of rarefying.

468 The use of non-normalized data has been shown to be more susceptible to the generation
469 of artificial clusters in ordinations, and rarefying has been demonstrated to be an effective
470 normalization technique for beta diversity analyses (Weiss et al., 2017). However, the use of a
471 single iteration of rarefying does result in the omission of valid data (McMurdie and Holmes,
472 2014). Repeated iterations of rarefying in this study demonstrated that rarefying repeatedly does
473 not substantially impact the output and interpretation of beta diversity analyses unless rarefying
474 to sizes that are inadvisably small to begin with. McMurdie and Holmes (2014) were dismissive
475 of rarefying repeatedly due to the variability it introduces, but such repetition was not evaluated
476 in the context of beta-diversity analysis. In the case of differential abundance analysis, the added
477 variability of rarefying would be statistically inappropriate relative to generalized linear
478 modelling that can account for varying library sizes. Additionally, repeatedly rarefying allows
479 for characterization of variation introduced through subsampling while accounting for
480 discrepancies in library size, supporting the potential utility of the normalization technique for
481 beta diversity analyses. McKnight et al. (2019) preferred use of proportions in diversity analysis
482 over rarefying (arguing that both were superior to other normalization approaches). While
483 proportions normalize the sum of the ASV weights to one for each sample, we note that the
484 approach does not normalize the library size in terms of sequence counts. This is important
485 because sample proportions will provide a more precise reflection of the true proportions of
486 which the set of sequences is believed to be representative in samples with larger libraries than in
487 samples with smaller libraries. In particular, using proportions of unnormalized sequence count

488 libraries in beta diversity analysis overlooks the loss of alpha diversity associated with smaller
489 library sizes when comparing samples with different library sizes.

490 *4.4 Perspectives on Library Size Normalization*

491 The increasing popularity and accessibility of amplicon sequencing has enabled the
492 scientific community to gain access to a wealth of microbial community data that would
493 otherwise not have been accessible. However, despite amplicon sequencing of taxonomic marker
494 genes being the gold standard approach for microbial community analysis, the data handling and
495 statistical analysis is still in the early stages of development. The diversity analyses that the
496 scientific community desires to perform on amplicon sequencing data require library sizes to be
497 normalized across samples, which creates the challenge of determining appropriate
498 normalization techniques. New normalization techniques and tools are constantly being
499 developed and released to the community with claims that the newest technique is the best and
500 only solution that should be utilized for analysis, but they may be associated with data handling
501 limitations, be too specifically tailored to a particular type of analysis or desired property, or not
502 normalize the library sizes that motivated the need for normalization (McKnight et al., 2018).
503 For example, the centered-log ratio transformation (Gloor et al., 2016) cannot be used with zero
504 count data and amplicon sequencing datasets must be augmented with an artificial pseudocount
505 to apply the normalization technique. The limitations of normalization techniques may affect
506 downstream analyses, making it critical to understand the implications of the technique chosen.

507 Further discussion within the scientific community is needed to ensure rigorous interpretation
508 of amplicon sequencing data without unwarranted bias introduced by the normalization
509 technique. Approaches to microbiome data analysis that recognize data as samples from a source
510 population and seek to draw inference about diversity in the source rather than just calculating

511 diversity in the (transformed) sample are desirable. Random errors are inherent to sample
512 collection, handling, processing, amplification, and sequencing and should be reflected in how
513 resulting data are analyzed. Pending further research on such approaches, rarefying remains
514 common in current research requiring library size normalization despite potential limitations,
515 especially for diversity analysis. The implementation of a single iteration of rarefying is
516 problematic due to the omission of valid data and should not be used for library size
517 normalization. Conducting repeated iterations of rarefying, however, does not discard valid
518 sequences and allows for the characterization of variation introduced through random
519 subsampling in diversity analyses.

520 **Conclusions**

- 521 ▪ Repeated rarefying (e.g., 1000 times if computationally feasible) statistically describes
522 possible realizations of the data if the number of sequences read had been limited to the
523 normalized library size, thus allowing diversity analysis using samples of equal library
524 size in a way that accounts for the data loss in rarefying.
- 525 ▪ Rarefying with or without replacement did not substantially impact the interpretation of
526 alpha (Shannon index) or beta (Bray-Curtis dissimilarity) diversity analyses considered in
527 this study, but rarefying without replacement is theoretically more appropriate and will
528 provide more accurate reflection of sample diversity.
- 529 ▪ The use of larger normalized library sizes when rarefying minimizes the amount of
530 artificial variation introduced into diversity analyses but may necessitate omission of
531 samples with small library sizes (or analysis at both inclusive low library sizes and
532 restrictive higher library sizes).

- 533 ▪ Ordination patterns are relatively well preserved down to small, normalized library sizes
534 with increasing variation shown by repeatedly rarefying, whereas the Shannon index is
535 very susceptible to being impacted by small, normalized library sizes both in declining
536 values and variability introduced through rarefaction.
- 537 ▪ Even though repeated rarefaction can characterize the error introduced by excluding some
538 fraction of the sequence variants, rarefying to extremely small sizes (e.g., 100 sequences)
539 is inappropriate because the substantial introduced variation leads to an inability to
540 differentiate between sample clusters and suppresses contribution of rare variants to
541 diversity.
- 542 ▪ Further development of strategies (e.g., data handling, library size normalization for
543 diversity analyses) for ensuring rigorous interpretation of amplicon sequencing data is
544 required.

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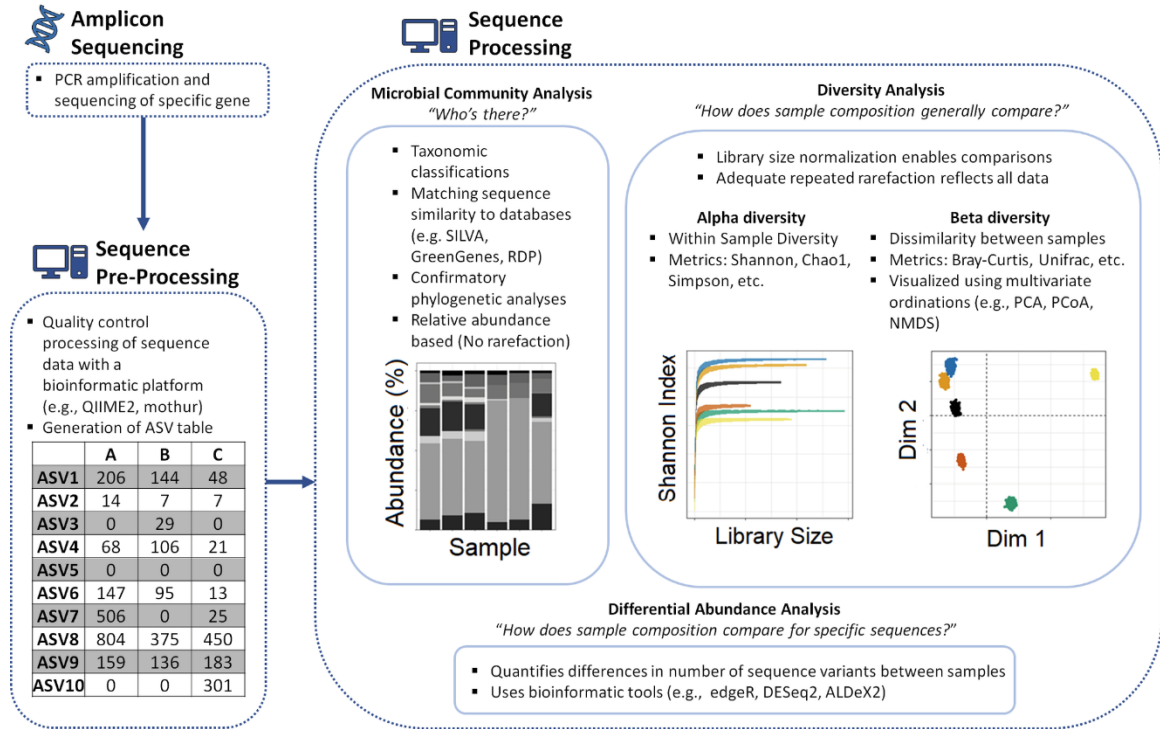


Figure 1 Schematic of general workflow in amplicon sequencing of samples.

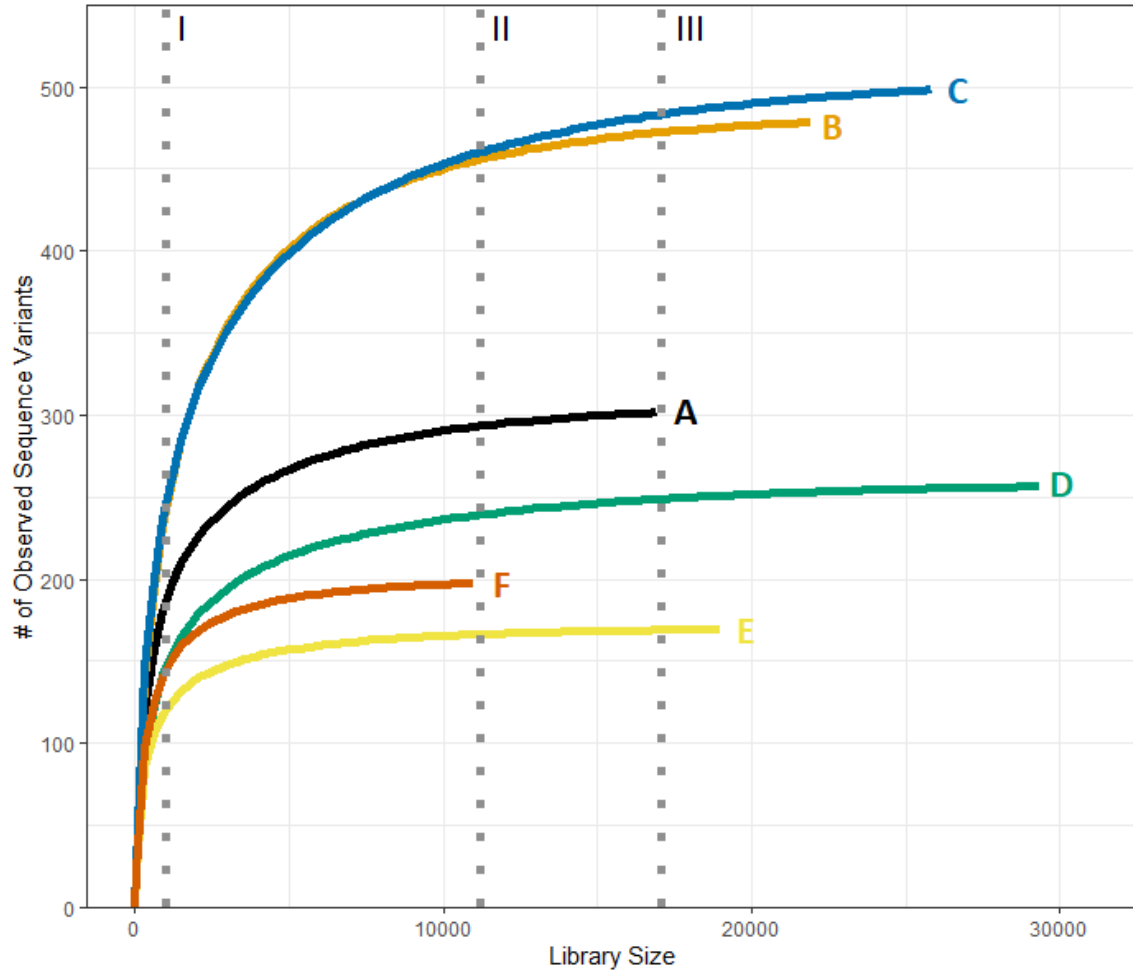
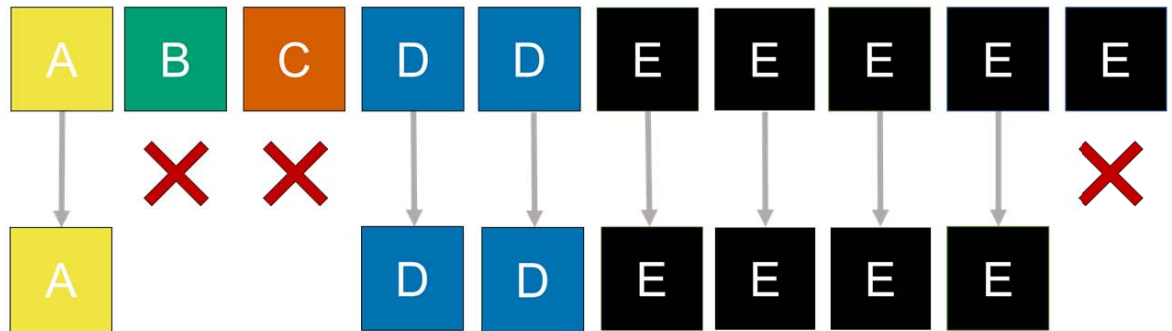


Figure 2 Rarefaction curves showing the number of unique sequence variants as a function of normalized library size for six samples (labelled A – F) of varying diversity and initial library size. Selection of unnecessarily small library sizes (I) omits many sequence variants. Rarefying to the smallest library size (II) omits fewer sequences and variants. While selection of a larger normalized library size (III) would omit even less sequences, it is necessary to omit entire samples (e.g., Sample F) that have too few sequences)

a) Without Replacement



b) With Replacement

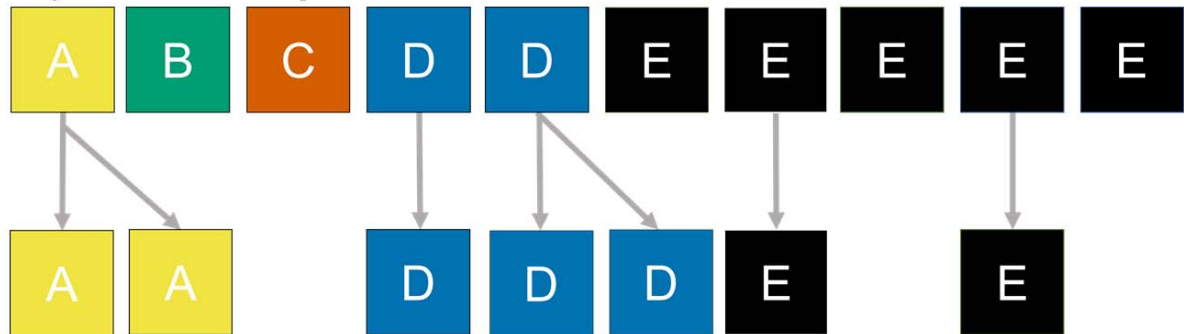


Figure 3 The mechanics of rarefying with or without replacement for a hypothetical sample with a library size of ten composed of five sequence variants (A – E). Rarefying without replacement (a) draws a subset from the observed library excluding the complementary subset, while rarefying with replacement (b) has the potential to artificially inflate the numbers of some sequence variants beyond what was observed.

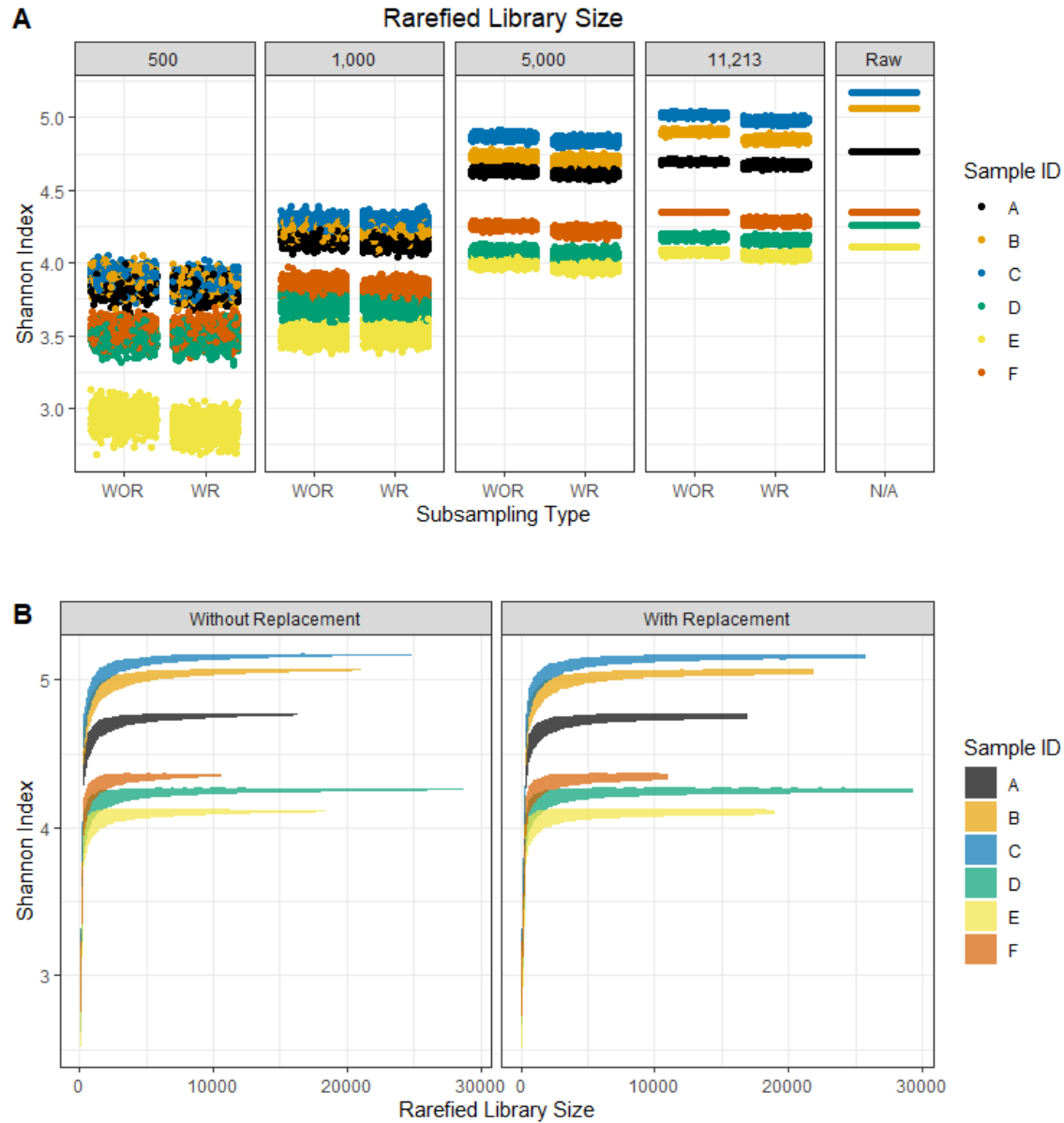


Figure 4 Effect of chosen rarefied library size and sampling with (WR) or without (WOR) replacement upon the Shannon Diversity Index. Six microbial communities were rarefied repeatedly (A) at specific rarefied library sizes of 11,213 sequences, 5,000 sequences, 1,000 sequences, and 500 sequences and (B) to evaluate the Shannon Index as a function of rarefied library size.

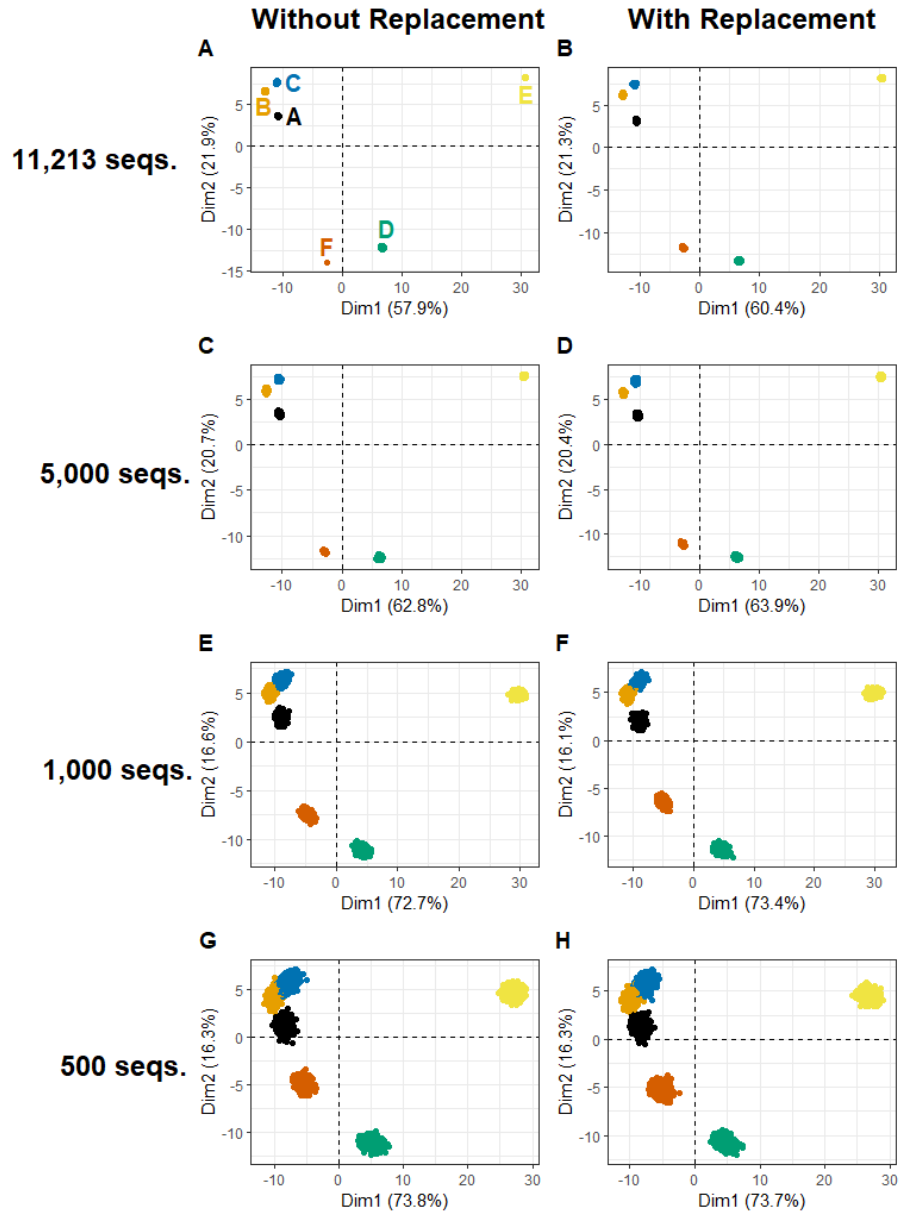


Figure 5 Variation in PCA ordinations (using the Bray-Curtis dissimilarity on Hellinger transformed rarefied libraries) of six microbial communities repeatedly rarefied with and without replacement to varying library sizes.

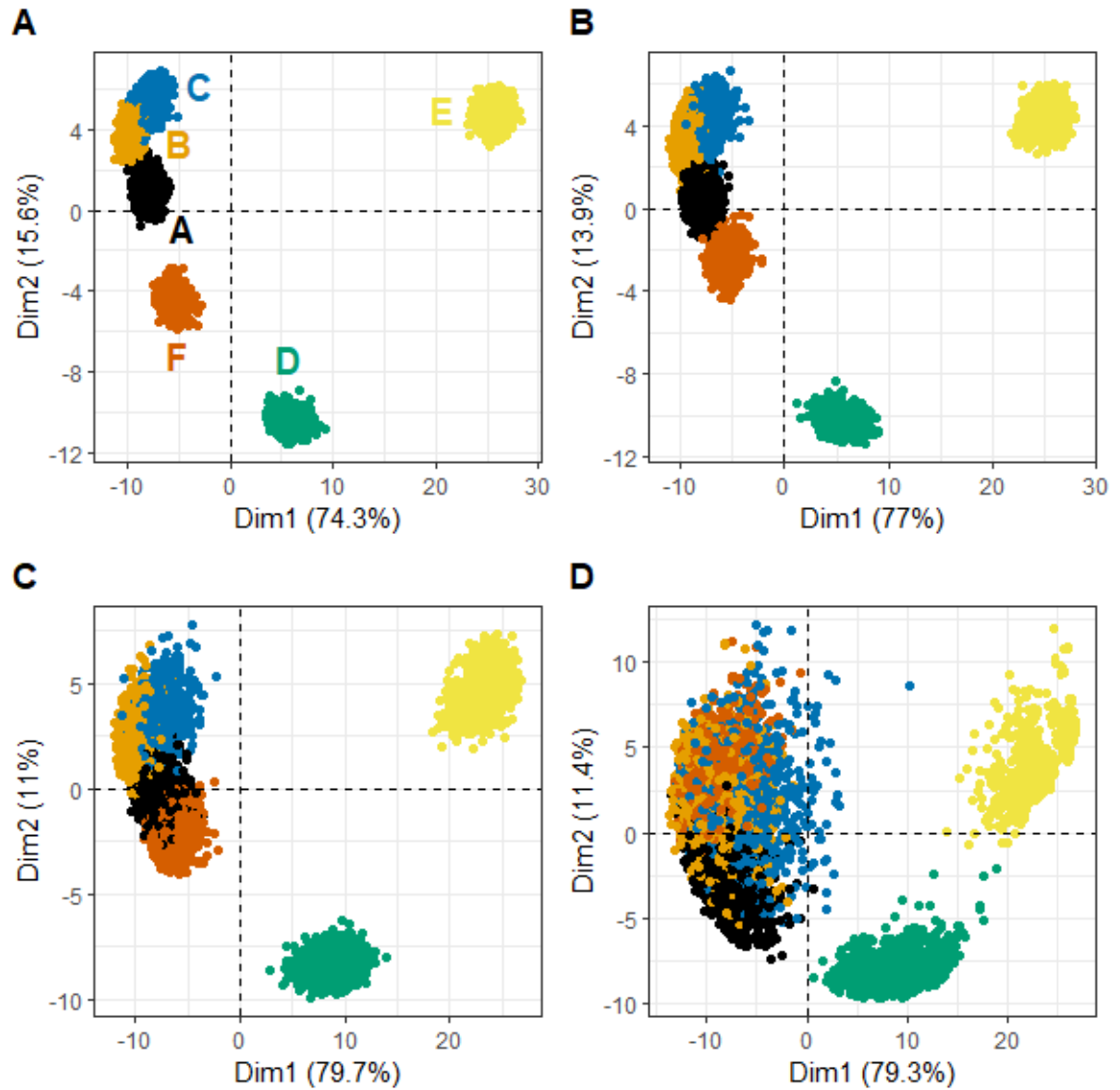


Figure 6 Variation in PCA ordinations (using the Bray-Curtis dissimilarity on Hellinger transformed rarefied microbial communities) of six microbial communities repeatedly rarefied to very small library sizes of (A) 400, (B) 300, (C) 200 and (D) 100 sequences.