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1 What is the optimum sample size for the study of peatland testate amoeba assemblages?

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6 Abstract

7 Testate amoebae are widely used in ecological and palaeoecological studies of peatlands, particularly as
8 indicators of surface wetness. To ensure data are robust and comparable it is important to consider
9 methodological factors which may affect results. One significant question which has not been directly
10 addressed in previous studies is how sample size (expressed here as number of *Sphagnum* stems)
11 affects data quality. In three contrasting locations in a Russian peatland we extracted samples of
12 differing size, analysed testate amoebae and calculated a number of widely-used indices: species
13 richness, Simpson diversity, compositional dissimilarity from the largest sample and transfer function
14 predictions of water table depth. We found that there was a trend for larger samples to contain more
15 species across the range of commonly-used sample sizes in ecological studies. Smaller samples
16 sometimes failed to produce counts of testate amoebae often considered minimally adequate. It seems
17 likely that analyses based on samples of different sizes may not produce consistent data. Decisions
18 about sample size need to reflect trade-offs between logistics, data quality, spatial resolution and the
19 disturbance involved in sample extraction. For most common ecological applications we suggest that
20 samples of more than eight *Sphagnum* stems are likely to be desirable.

21 **Keywords:** Testate amoebae; Sample Size; Protist; Bioindication; Transfer function; Wetland

22 Introduction

23 Testate amoebae are a polyphyletic group of protists defined by the presence of a test (Meisterfeld,
24 2002). Testate amoebae are abundant in a wide variety of habitats but are particularly abundant in
25 freshwater wetlands where they are typically the dominant group of heterotrophic protists (Gilbert et
26 al., 1998; Mitchell et al., 2008). Over recent years there has been considerable interest in the application
27 of testate amoebae as bioindicators for a wide variety of environmental changes (Payne, 2013). The
28 most widespread of these uses has been as indicators of water table depth in palaeoecological studies
29 from peatlands (Charman, 1999; Qin et al., 2013; Van Bellen et al., 2014). After numerous studies over
30 the last 25 years it is now well-established that testate amoebae taxa have differing preferences for
31 peatland surface wetness (usually expressed as water table depth). Transfer functions which attempt to
32 quantify these optima in surface samples have been widely used to produce quantitative
33 reconstructions of changing water table depth in peatlands (Payne et al., 2016).

34 As testate amoebae have become more widely studied in peatlands there has been an increasing focus
35 on the testing and refinement of methods and interpretation. Studies have focussed on questions such
36 as optimum preparation methods (Hendon and Charman, 1997; Avel and Pensa, 2013), sampling depth
37 (Roe et al., 2017), taxonomic approach (Payne et al., 2011; Mitchell et al., 2014) and sample storage
38 (Mazei et al., 2015). There are particularly important questions regarding the scaling relationships
39 between sampling effort and data quality. Several studies have looked at the relationship between the

40 number of individual tests counted under the microscope and the species richness (Warner, 1990;
41 Woodland et al., 1998; Mitchell et al., 2000) and composition (Payne and Mitchell, 2009) of the
42 assemblage identified. The influence of the size of sample analysed has been little considered despite
43 extensive consideration in other contexts (Heck et al., 1975; Azovsky, 2000).

44 Testate amoeba assemblages are known to show fine-scale spatial variation even in areas of relatively
45 homogeneous vegetation and physical environment. In the most intensive study of this topic Mitchell et
46 al. (2000) studied the testate amoeba assemblages of a *Sphagnum magellanicum* lawn in a Swiss
47 peatland. Across a macroscopically homogeneous plot of only 40×60 cm these authors showed
48 considerable variability in testate amoeba assemblages with clear spatial structuring of the species
49 composition and large variability in biomass. Some individual taxa differed in relative abundance by an
50 order of magnitude between adjacent samples. Another study of testate amoeba distribution in a
51 macroscopically homogeneous *Sphagnum angustifolium* lawn has shown species-dependent spatial
52 organisation down to a scale of 1 cm (Mazei and Tsyganov, 2007).

53 Assuming this level of fine-scale spatial variability is typical for peatlands this raises the question: what is
54 the optimum sample size for the determination of testate amoeba assemblages in ecological studies?
55 The sample size considered in previous studies varies considerably from a single *Sphagnum* stem up to
56 samples of more than 25 cm² which may represent dozens of individual stems (Mitchell et al., 2000;
57 Payne et al., 2006; Jassey et al., 2012). It seems plausible that different sample sizes may lead to
58 datasets which differ in important respects. In this study we analysed surface samples spanning the
59 range of commonly used sizes in order to assess whether and how such differences affect data quality
60 and to make recommendations for future studies.

61 **Material and Methods**

62 ***Study site and Sampling***

63 Samples for the study were collected in a mesotrophic peatland (53.125511° N, 45.841298° E) located in
64 the forest-steppe zone of the East European Plain (Penza Region, Russia) in July, 2007 (Supplementary
65 Figure 1). The study area has a continental climate characterized by mean January temperature of –12
66 °C and mean July temperatures of +20 °C. Mean annual precipitation is 500 mm yr⁻¹, at the lower end of
67 the range typical for northern peatlands (World Water and Climate Atlas, 1961–1991; New et al. 2002).
68 The vegetation of the peatland is dominated by *Carex* spp. and *Sphagnum* spp.

69 To consider how sample size-assemblage relationships may differ between microhabitats we conducted
70 sampling in three locations spanning the range of surface wetness and vegetation commonly
71 encountered in northern peatlands. Biotope 1 was the driest with vegetation cover of *Sphagnum*
72 *angustifolium* and *Polytrichum strictum* and a canopy of *Betula* sp., the measured water table depth was
73 26 cm. Biotope 2 was intermediate in wetness with open lawn vegetation of *Sphagnum palustre* and
74 *Sphagnum magellanicum* and no trees, water table depth was 12 cm. Biotope 3 was a hollow with
75 *Sphagnum squarrosum* and was the wettest of the sampling locations with a water table depth of 0 cm.
76 In each location samples of different size (1, 3 and 8 *Sphagnum* stems) were extracted from the same
77 location in three replicates and one larger sample of 16 stems was extracted giving a total of 30
78 samples. We focus on the number of *Sphagnum* stems as an index of sample size because this is easily
79 determined in the field and frequently used by analysts. Sampled stems extended to a depth of 6 cm.
80 Material sampled was *Sphagnum angustifolium* in Biotope 1, *Sphagnum palustre* in Biotope 2 and
81 *Sphagnum squarrosum* in Biotope 3. This difference in *Sphagnum* species sampled was necessitated by

82 the aim to consider a variety of assemblages. However it is important to note that this may influence
83 results because different *Sphagnum* species may contain different test densities and may grow at
84 different rates meaning that the same stem depth represents differing time periods. The samples were
85 placed in plastic flasks and stored in 4% formalin to avoid the possibility of any post-sampling change in
86 assemblage (Mazei et al., 2015).

87 ***Testate amoeba analysis***

88 Samples were prepared for testate amoeba analysis following a modified water-based technique (Mazei
89 and Chernyshov, 2011). Moss samples were suspended in deionised water and thoroughly shaken for 5
90 minutes. The suspension was carefully poured in to a Petri dish (10 cm diameter) and left to settle.
91 Testate amoebae were identified and counted by direct microscopy with a dissecting light microscope
92 (Biomed, Russia) at a magnification of 160×. Tests were identified based on Mazei and Tsyganov (2006).
93 The full volume of each sample was counted and all tests recorded, live individuals were not
94 differentiated.

95 ***Data analysis***

96 We analysed the data to determine how key properties of the identified assemblage varied with
97 increasing sample size. We considered four widely used metrics: species richness, Simpson's diversity
98 index, compositional dissimilarity and transfer function predictions of water table depth. We first
99 calculated two measures of diversity: species richness (the number of taxa recorded per sample) and
100 Simpson's diversity index (expressed as 1-D, where D is the raw index) which combines species richness
101 with a measure of species evenness. Both may be expected to increase as sample size increases and
102 more taxa are encountered. We calculated Simpson diversity using the 'diversity' function in the R
103 package vegan (Oksanen et al., 2007). Next we considered the similarity in assemblage between the
104 smaller sized samples and the largest sized sample we analysed (16 stems). We quantified compositional
105 dissimilarity using the Bray-Curtis index (Bray and Curtis, 1957). It can be expected that as sample size
106 increases the assemblage structure may become increasingly similar to that of the largest sample. We
107 calculated Bray-Curtis dissimilarity between each sample and that of the 16 stem sample using the
108 'vegdist' function in vegan. Finally we considered the predictions of a transfer function for hydrological
109 inference (Tsyganov et al., 2017). It can be expected that as sample size increases the model prediction
110 of water table depth may become both more accurate and more similar to that of the largest sample.
111 Tsyganov et al. (2017) have recently presented a transfer function for the peatlands of European Russia
112 including samples from the site considered here. We applied the optimum weighted average/inverse
113 deshrinking transfer function from that study to these samples to predict water table depth for each
114 sample. Transfer function analyses used the R package rioja (Juggins, 2009).

115 Our approach of counting all tests in the sample meant that count totals varied considerably amongst
116 samples (89-1979 tests, mean=354). As this is likely to influence many of the metrics, we used
117 rarefaction to reduce all datasets to a common count total (that of the lowest value encountered in any
118 one sample: 89 tests). Rarefaction was conducted using the function 'rrarefy' in vegan which is based on
119 sampling without replacement. We repeated this process 1000 times to give a range of plausible
120 datasets for each sample. We calculated each metric using both these rarefied datasets based on
121 consistent counts and the original dataset with variable counts. We tested for correlations between
122 sample size and each metric using Spearman's R_s .

123 Our laboratory data collection only addressed four possible sample sizes (1, 3, 8 or 16 *Sphagnum* stems).
124 To consider alternative sample sizes beyond these four we simulated alternative possibilities based on
125 the combination of analysed samples. For each biotope we randomly selected combinations of the
126 analysed samples in order to achieve each possible sample size from 1-16 stems and repeated analyses.

127 **Results**

128 In total, 29 testate amoeba taxa belonging to 10 genera were observed (Supplementary Table 1). The
129 most abundant taxa were *Arcella arenaria* (27% of the total count), *Euglypha tuberculata* (18%), *Arcella*
130 *gibbosa* (12%), *Assulina seminulum* (11%), *Corythion dubium* (11%) and *Arcella polypora* (5%)
131 (Supplementary Table 1). All the taxa, except for *Arcella gibbosa* and *Arcella polypora*, were observed in
132 more than 80% of all samples. Three taxa (*Arcella arenaria irregularis*, *Euglypha aspera*, *Euglypha*
133 *cristata major*, *Hyalosphenia minuta*) were observed in one sample only. The number of species per
134 sample varied from 4 to 19 (10 ± 0.67 , mean \pm SE, $n = 30$).

135 The most clear-cut change with increasing sample size was that the count total increased substantially
136 (Fig. 1). This is to be expected but it is interesting to note the scale of the difference. In one sample
137 based on a single stem and one sample based on three stems the count total of 100 tests recommended
138 by Payne and Mitchell (2009) was not achieved. The higher total of 150 tests used in many studies was
139 not achieved for five of nine samples based on one stem and four of nine samples based on three
140 stems..

141 The total number of testate amoeba species observed was greater than the total number of species in
142 the largest samples (16 stems). The number of identified species differed among the biotopes and was
143 greatest in biotope 2 (intermediate moisture content). Species richness increased with increasing
144 sample size (this correlation was significant in two biotopes). Similar increases were apparent when
145 considering both the raw count data (Fig. 2A) and the rarefied data based on consistent count (Fig. 3A).
146 This suggests that the trends are not solely driven by increasing count total but also represent a real
147 increase in the diversity of the assemblage identified with increasing sample size. The increase appears
148 to be greatest as sample size exceeds eight *Sphagnum* stems with relatively little further change to 16
149 stems (Supplementary Figure 2A). Trends with sample size were less apparent when considering
150 Simpson diversity with a non-significant increase in biotope 3 but no clear trend in the other two
151 biotopes in either raw or rarefied data (Fig. 2B, 3B).

152 Differences in species composition of testate amoeba assemblages were apparent with sample size. In
153 the raw data there were strong (but non-significant) declines in Bray-Curtis dissimilarity from the largest
154 samples with increasing sample size (Fig. 2C). Similar but more subtle declines could be observed in the
155 rarefied data (Fig. 3C) and general declining trends were also present in the simulated data series
156 (Supplementary Figure 2C). Although the results were non-significant they imply that assemblage
157 composition varies with sample size with larger samples tending to be increasingly similar.

158 Transfer function predictions of water table depth showed considerable variability between sample
159 locations (Fig. 2D, 3D, Supplementary Figure 2D). Predictions for biotope 3, the wettest site were
160 typically in the range of 5-7 cm, drier than the measured water table depth (0 cm) but within the
161 expected prediction accuracy of the transfer function (Tsyganov et al., 2017). Predictions for biotope 1,
162 the driest site were typically in the range 20-27 cm, close to the measured value of 26 cm. Predictions
163 for the intermediate biotope 2 were the least accurate, typically 18-25 cm, considerably drier than the

164 measured depth of 12 cm. Across all three biotopes there was little trend in predicted water table depth
165 values with increasing sample size and no trends which were significant.

166 **Discussion**

167 Our study is of limited scale considering 30 samples from three locations in a single site, not
168 differentiating live from dead individuals and considering relative abundance rather than test
169 concentrations. In future research it would be desirable to replicate our work across a greater number
170 of sites, replicate sampling within biotopes, consider concentrations as well as relative abundance and
171 live individuals as well as all tests. Nevertheless, this is the first study of the topic and the results are
172 revealing in several respects.

173 In terms of count total our data show that the smallest samples investigated may fail to identify
174 sufficient tests to reach commonly used target count totals . Results from samples containing less than
175 eight *Sphagnum* stems may fail to reach totals considered minimally adequate to produce robust
176 results. Results also imply that more taxa are likely to be located in larger samples. This is relatively
177 unsurprising as larger samples will inevitably encompass more heterogeneity with different taxa and a
178 larger total species pool. However, more surprisingly, the results here imply that this holds true even at
179 fine scales over which key environmental controls on peatland testate amoebae vary relatively little. The
180 range of sample size in this study encompasses the range of sample size encountered in the published
181 literature, implying that some differences in species richness between published studies might be due to
182 the size of the samples considered, rather than any fundamental difference in the investigated
183 assemblages. Somewhat less robustly, our results also imply that assemblage composition tends to
184 converge as sample sizes become larger. Smaller samples may reflect differences in environment at a
185 smaller spatial scale than environmental measurements and therefore introduce noise into the data.
186 Our results do not provide any direct evidence that sample size influences transfer function predictions.
187 This may be because differences in assemblage represent taxa with similar hydrological preferences or
188 that real trends are overwhelmed by noise in this relatively small dataset.

189 Overall, our results imply the strong possibility that sample size may affect data quality in peatland
190 testate amoeba studies. It is common in the literature for sample size not to be stated in the methods
191 but it seems likely that sizes used may differ sufficiently to mean that results could be inconsistent.
192 Differing sample size is probably one of several methodological factors which complicate current
193 attempts to combine and synthesise testate amoeba datasets (Amesbury et al., 2016).

194 The appropriate sample size for a study will always be a trade-off between various considerations. For
195 studies which attempt to link testate amoeba assemblages to environmental variables the appropriate
196 scale for the analysis of testate amoebae will depend on the scale at which the environmental variables
197 are investigated. The most frequently measured variable is water table depth (Payne et al., 2012) which
198 is unlikely to vary greatly over the scale of different potential sample sizes and is usually measured by
199 inserting a dipwell or digging a hole which is unlikely to be less than ~5 cm diameter. For these purposes
200 a larger sample would seem appropriate to maximise the pool of testate amoeba species identified.
201 Where the environmental variables vary and are measured at finer resolution a smaller sample may be
202 more appropriate despite the probable lower numbers of individuals and species (Mitchell et al., 2000).
203 Small sample sizes may also be appropriate in situations where there is a need to minimise disturbance.
204 This is particularly the case in experimental studies where the volume of material available is small (e.g.
205 mesocosm experiments) or where the need to re-sample over time means that sampling needs to
206 consider the possibility of disturbance to the plots (Mulot et al., 2014). Logistical constraints may also

207 become important; when sample numbers are high or sample sizes very large the resulting volume of
208 material may complicate sample transport and storage. The optimum sample size for a study is a matter
209 for researcher discretion but it is important that an informed decision is made and that such trade-offs
210 are recognised. We suggest that for many common applications a sample size of more than eight
211 *Spahgnum* stems may be desirable. Comparisons between studies should acknowledge the
212 methodological factors which may influence results of individual studies.

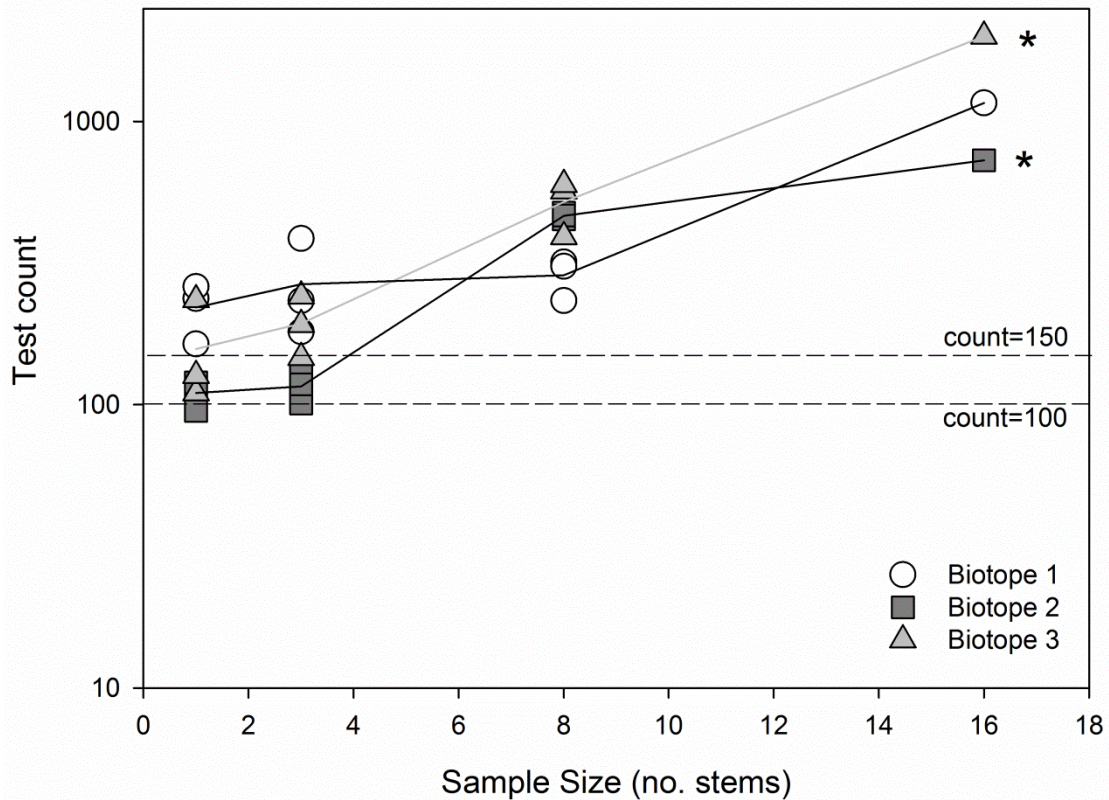
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218 Author contributions: YuM conceived the study, ASE analysed the samples, RJP, ANT and AYuT designed
219 and conducted the data analysis. RJP and ANT wrote the first draft of the manuscript; all authors
220 contributed comments and interpretation.

221 **Figure legends**

222 **Figure 1.** Change in test count with increasing sample size. Points show individual samples, lines show
223 means by biotope. Series marked with * show significant correlations with number of *Sphagnum* stems
224 analysed (Spearman's R_s , $P < 0.05$). Y-axis is shown on a logarithmic scale to facilitate visualisation of
225 differences at the lower end of the scale; common target count totals of 100 and 150 tests are marked
226 by dashed horizontal lines.



227

228 **Figure 2.** Change in testate amoeba assemblage metrics with increasing sample size based on original
229 count data. Plots show: A) species richness, B) Simpson diversity, C) Bray-Curtis dissimilarity from the
230 corresponding largest sample and D) predictions of water table depth using a transfer function model.
231 Series marked with * show significant correlations with number of *Sphagnum* stems analysed
232 (Spearman's R_s , $P < 0.05$). Points show individual samples, lines show means by biotope. Material sampled
233 was *Sphagnum angustifolium* in Biotope 1, *Sphagnum palustre* in Biotope 2 and *Sphagnum squarrosum*
234 in Biotope 3.

235 **Figure 3.** Change in testate amoeba assemblage metrics with increasing sample size based on rarefied
236 data. Plots show: A) species richness, B) Simpson diversity, C) Bray-Curtis dissimilarity from largest
237 sample and D) predictions of water table depth using a transfer function model. For each point figures
238 show mean of 1000 cycles of rarefaction. Series marked with * show significant correlations with
239 number of *Sphagnum* stems analysed (Spearman's R_s , $P < 0.05$). Points show individual samples, lines
240 show means by biotope. Material sampled was *Sphagnum angustifolium* in Biotope 1, *Sphagnum*
241 *palustre* in Biotope 2 and *Sphagnum squarrosum* in Biotope 3.

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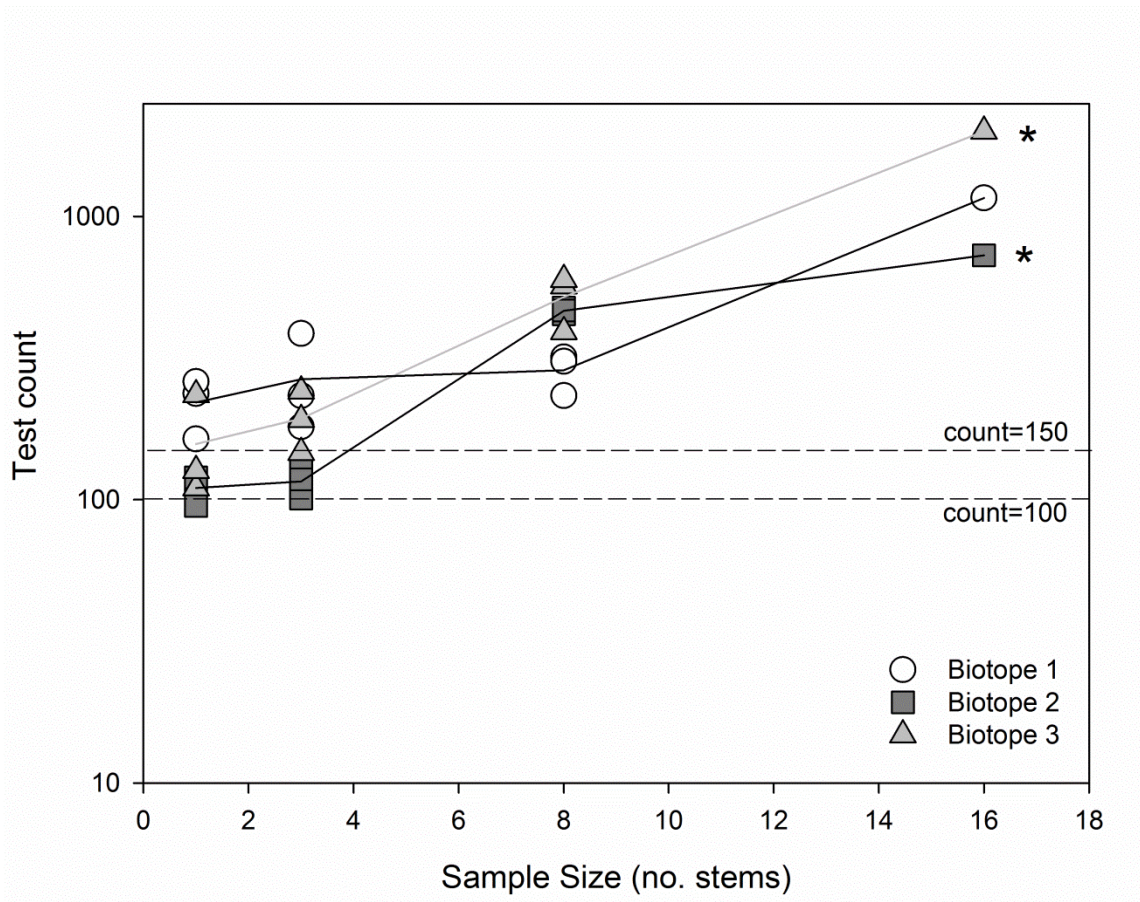
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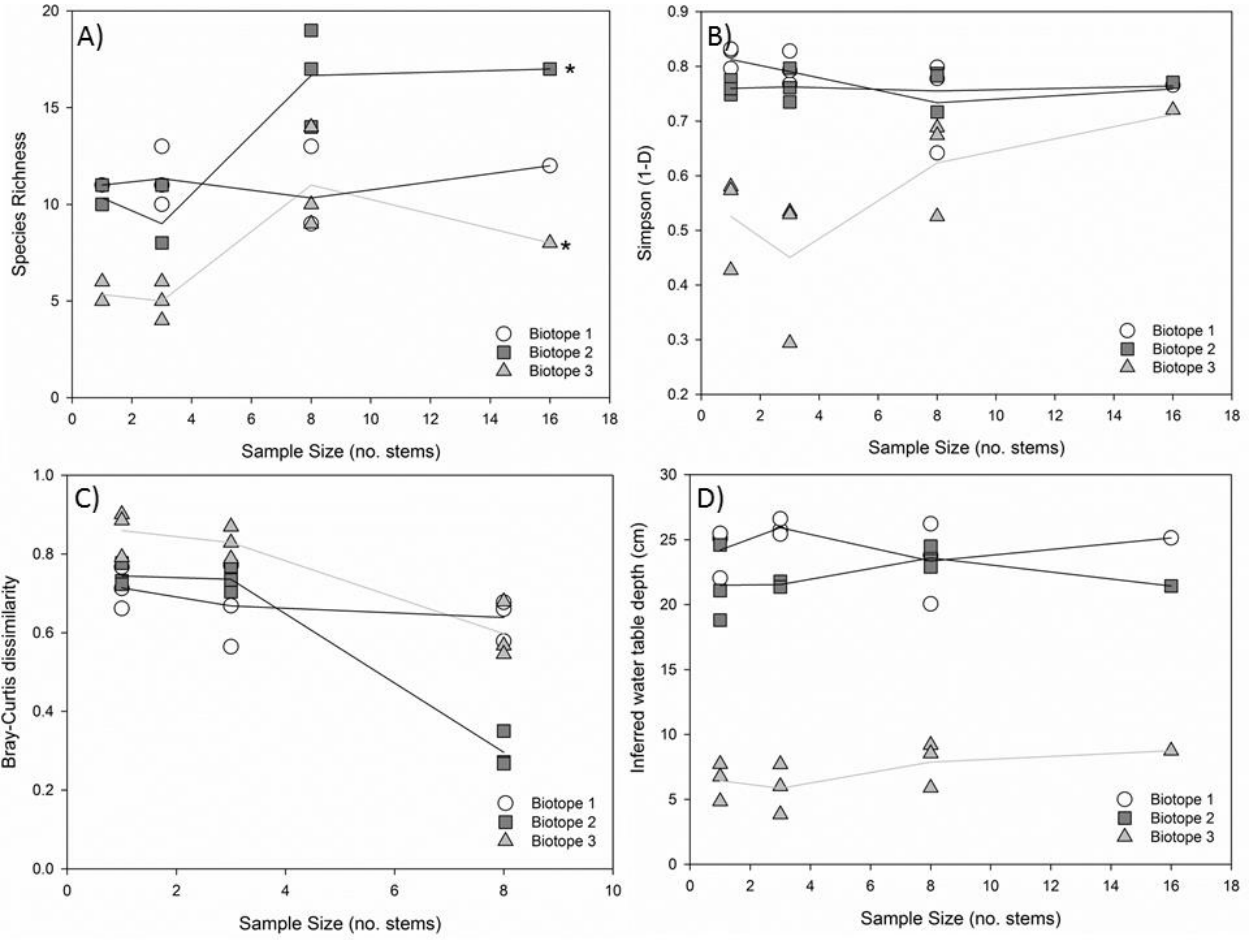
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248 **Figure 1.**



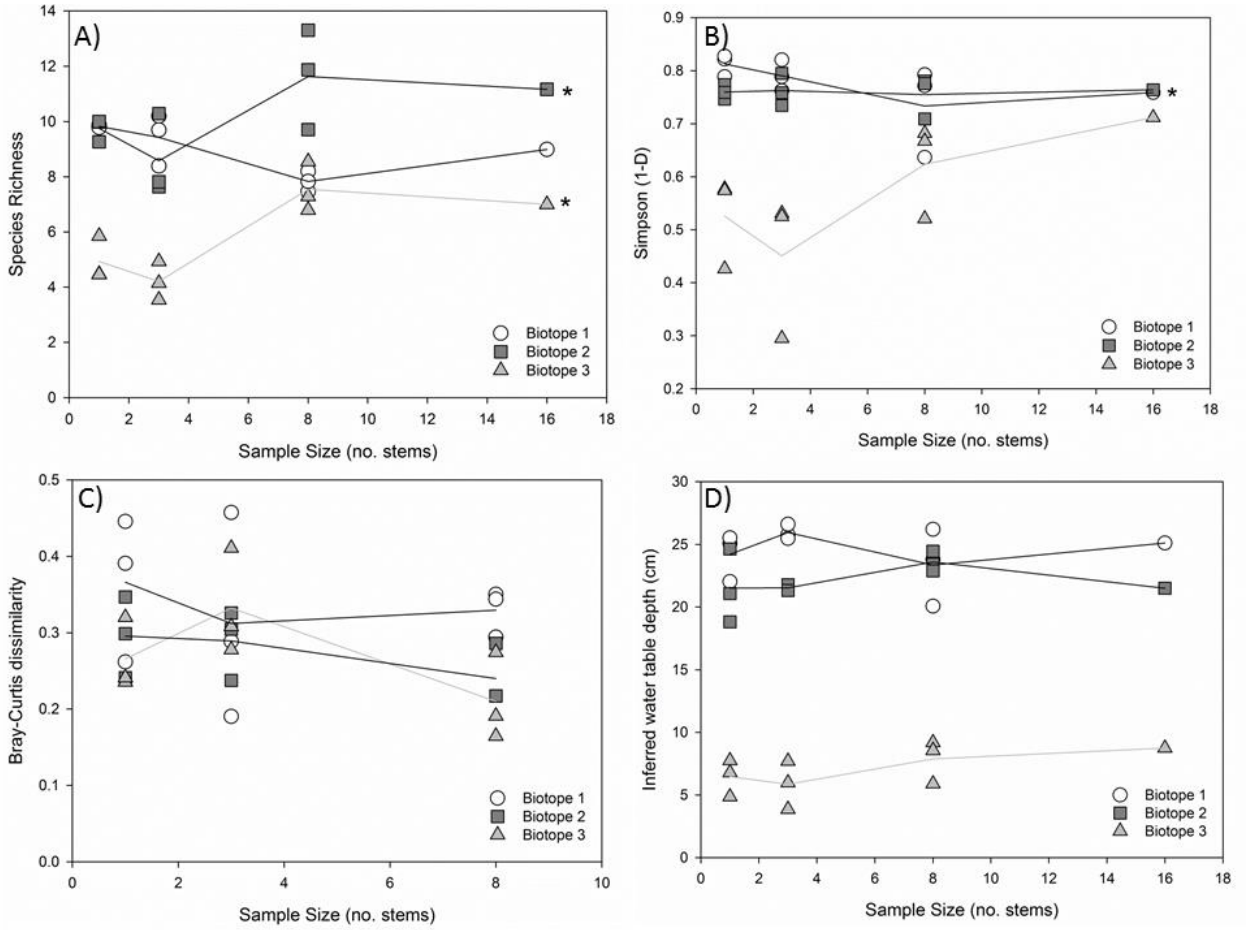
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