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Mazei, Yuri, Tsyganov, Andrey N, Esaulov, Anton et al. (2017) What is the optimum sample size for the study of peatland testate amoeba assemblages? European Journal of Protistology. pp. 1-17. ISSN: 0932-4739

https://doi.org/10.1016/j.ejop.2017.09.004

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

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Abstract

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

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

Introduction

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

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

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

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

Assuming this level of fine-scale spatial variability is typical for peatlands this raises the question: what is the optimum sample size for the determination of testate amoeba assemblages in ecological studies? The sample size considered in previous studies varies considerably from a single *Sphagnum* stem up to samples of more than 25 cm^2 which may represent dozens of individual stems (Mitchell et al., 2000; Payne et al., 2006; Jassey et al., 2012). It seems plausible that different sample sizes may lead to datasets which differ in important respects. In this study we analysed surface samples spanning the range of commonly used sizes in order to assess whether and how such differences affect data quality and to make recommendations for future studies.

Material and Methods

Study site and Sampling

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

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

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

Testate amoeba analysis

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

Data analysis

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

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

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

Results

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

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

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

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

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

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

Discussion

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

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

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

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

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

Acknowledgements

Data analysis was supported by the Russian Science Foundation, grant 14-14-00891 to YuM and UK-Russia research cooperation was supported by a Royal Society International Exchange grant to RJP (IE150173). Microscopic analysis was supported by the Russian Foundation for Basic Research, grant 17-04-00320 to ANT.

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

Figure legends

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

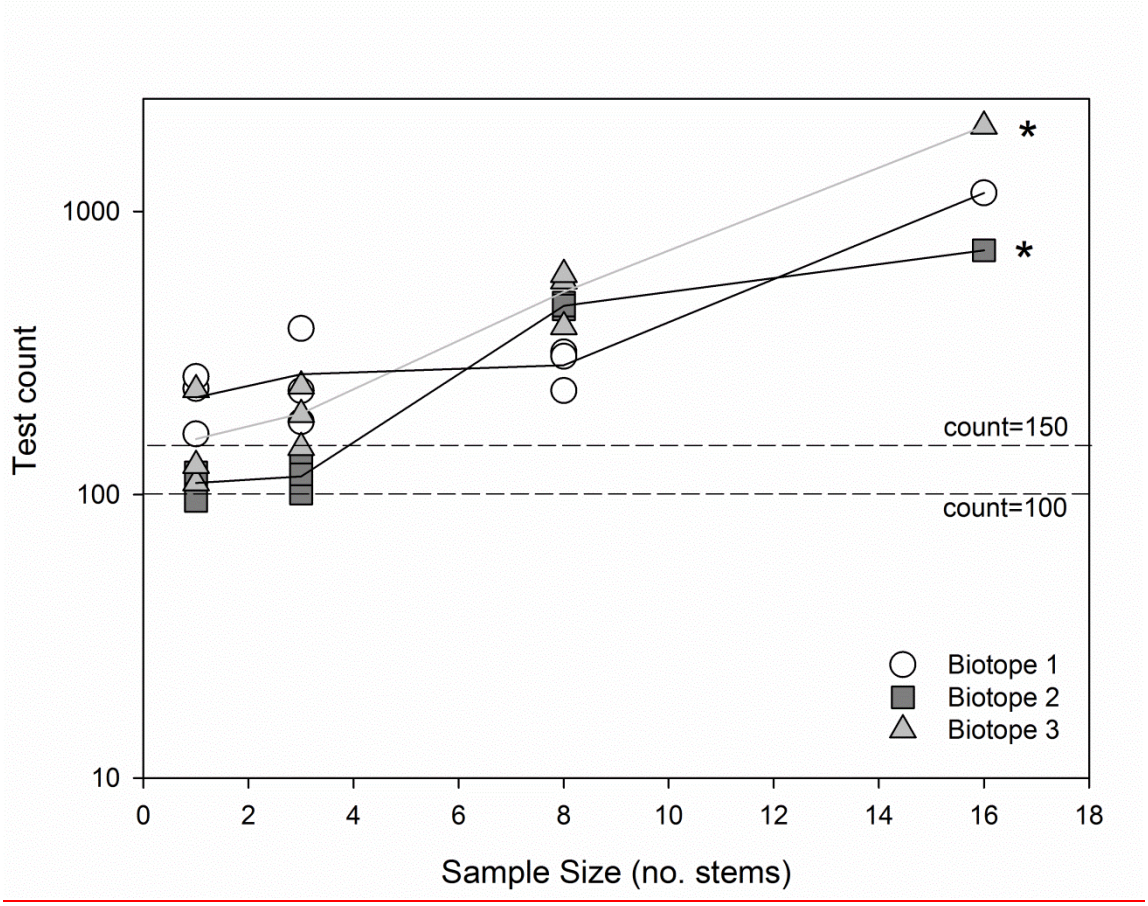


Figure 2. Change in testate amoeba assemblage metrics with increasing sample size based on original count data. Plots show: A) species richness, B) Simpson diversity, C) Bray-Curtis dissimilarity from the corresponding largest sample and D) predictions of water table depth using a transfer function model.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

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

Figure 1.

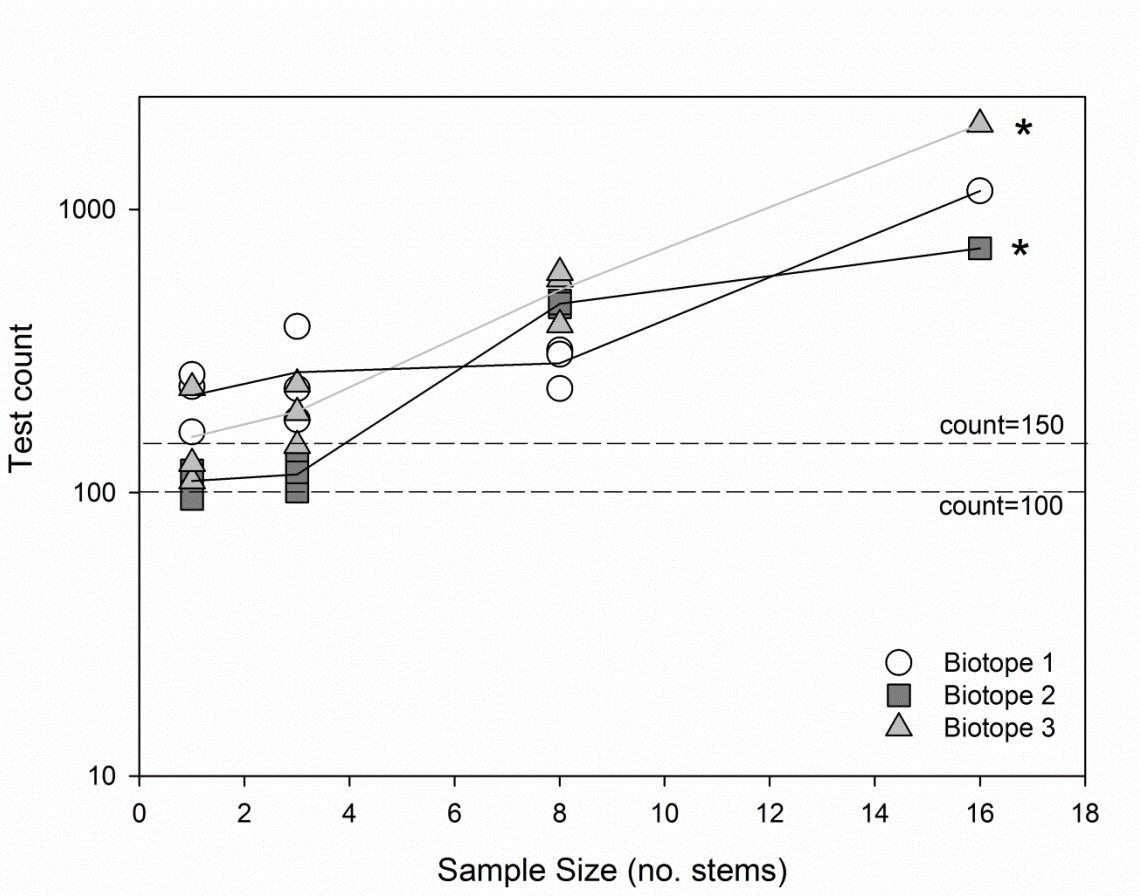

Test count
1000
100
10
count=150
count=100
*
*
Biotope 1
Biotope 2
Biotope 3
0
2
4
6
8
10
12
14
16
18
Sample Size (no. stems)

Figure 2.

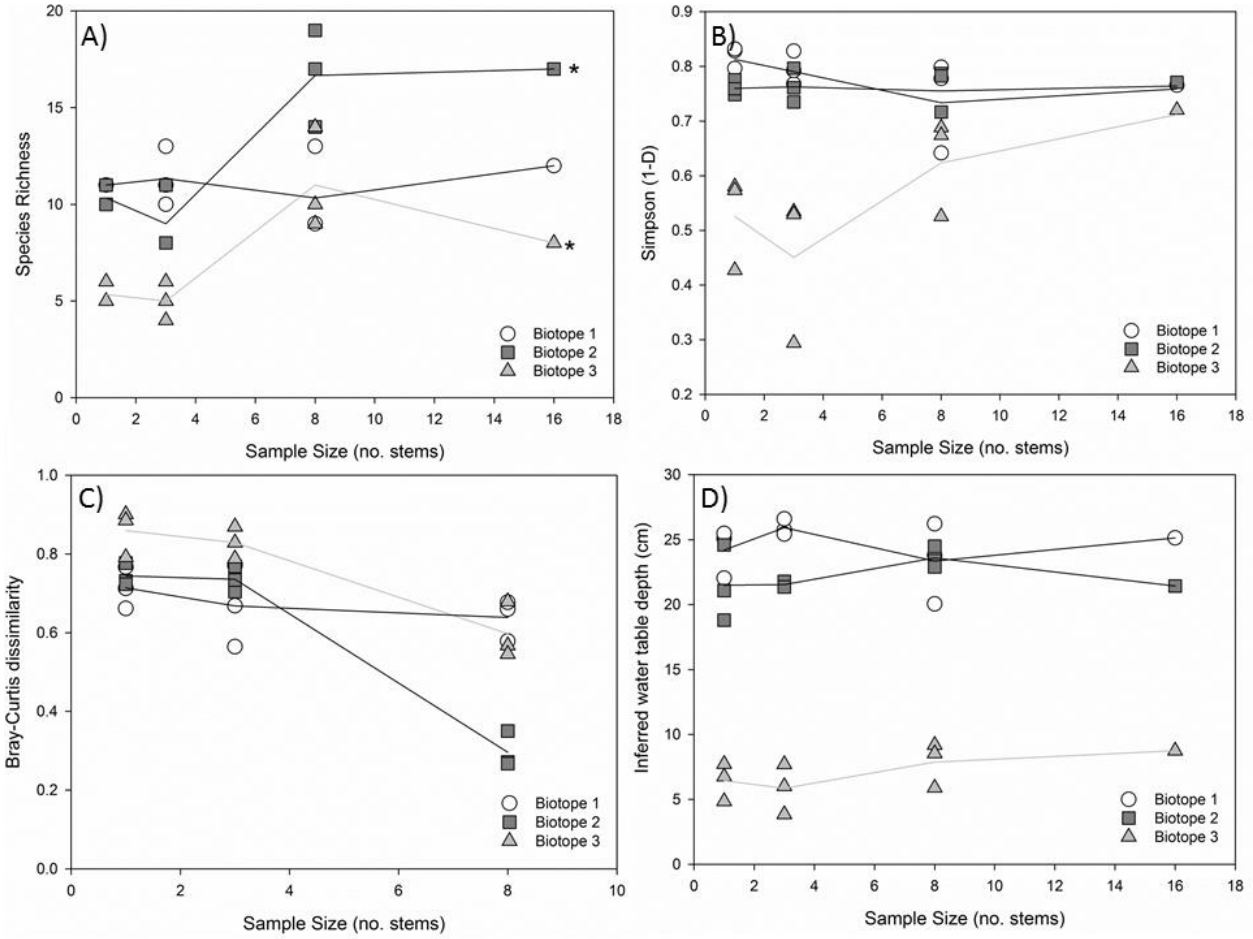
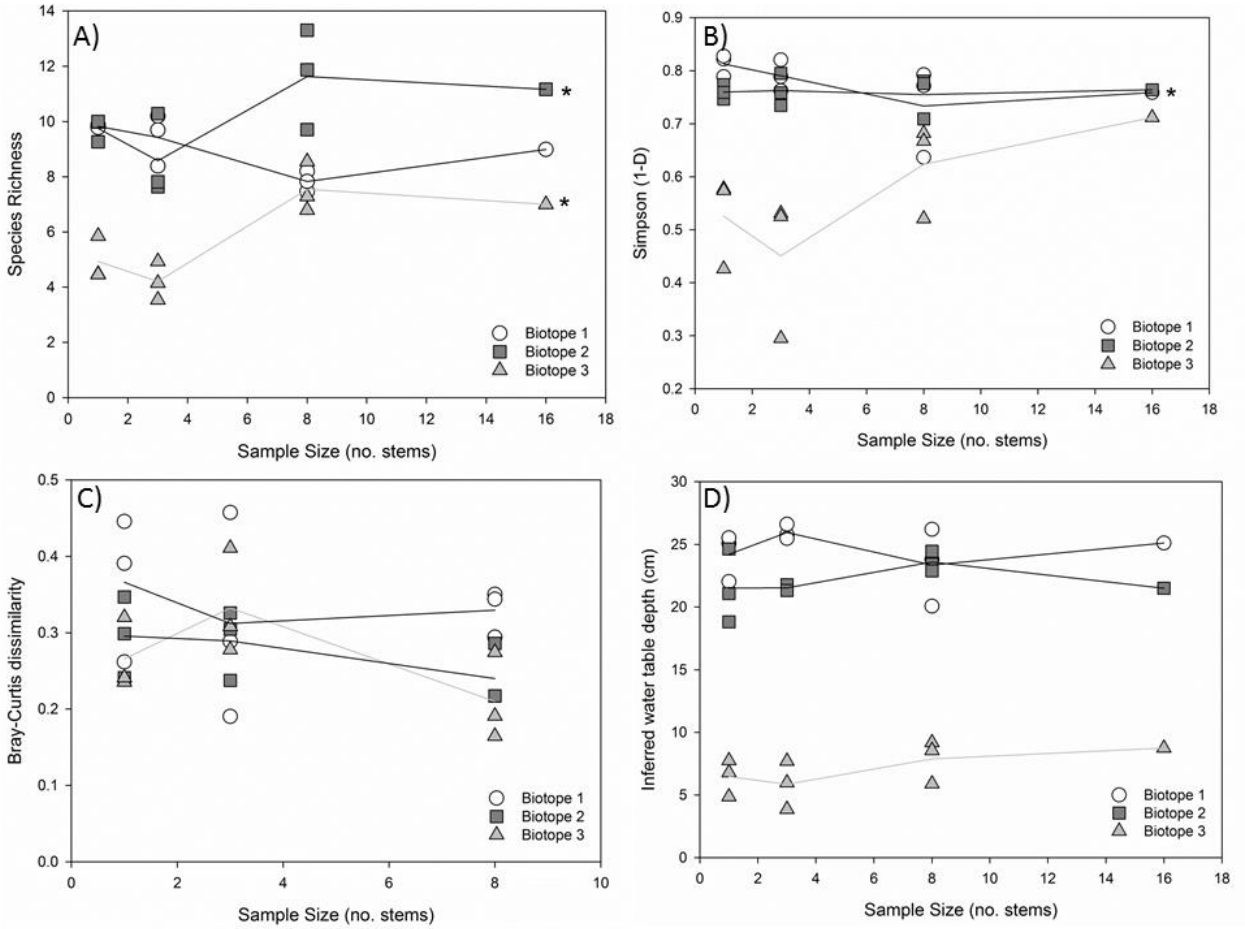

A)
Species Richness
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3
B)
Simpson (1-D)
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3
C)
Bray-Curtis dissimilarity
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3
D)
Inferred water table depth (cm)
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3

Figure 3.


A)
Species Richness
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3
*
*
B)
Simpson (1-D)
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3
*
C)
Bray-Curtis dissimilarity
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3
D)
Inferred water table depth (cm)
Sample Size (no. stems)
Biotope 1
Biotope 2
Biotope 3

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