

1 **How many is enough? Determining optimal count totals for**
2 **ecological and palaeoecological studies of testate amoebae.**

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15 **Keywords:** Testate amoebae, Protists, Palaeoecology, Palaeohydrology,
16 Palaeoclimate, Peatlands.

17

18 **Running title:** Optimising testate amoebae counts in palaeoecology

1 **Abstract**

2 Testate amoebae are increasingly used in ecological and palaeoecological studies of
3 wetlands. To characterise the amoeba community, a certain number of individuals
4 need to be counted under the microscope. To date, most studies have aimed for 150
5 individuals, but that sample size is not based on adequate evidence. When testate
6 amoeba concentrations are low, it can be difficult or impossible to reach this total.
7 The impacts of lower count totals have never been seriously scrutinised. We
8 investigated the impact of count size on number of taxa identified, quantitative
9 inferences of environmental variables and the strength of the links between amoebae
10 and environmental data in the context of predicting depth to water table. Low counts
11 were simulated by random selection of individuals from four existing datasets.
12 Results show progressively diminishing returns by all criteria as count size increases
13 from low numbers to counts of 150. A higher count is required to identify all taxa
14 than to adequately characterise the community for transfer function inference. We
15 suggest that in most cases, it will be a more efficient use of time to count a greater
16 number of samples to a lower count. While a count of 50 individuals may be
17 sufficient for some samples from some sites we recommend that counts of 100
18 individuals should be sufficient for most samples. Counts need only be increased to
19 150 or more where the aim is to identify relatively minor, but still potentially
20 ecologically relevant community changes. This approach will help reduce lack of
21 replication and low resolution, which are common limitations in testate amoeba-based
22 palaeoecological and ecological studies.

23

1 **Introduction**

2 Testate amoebae are a group of unicellular eukaryotic micro-organisms characterised
3 by a decay-resistant shell (Meisterfeld 2000a, b). Testate amoeba analysis has been
4 used in a variety of ecological and palaeoecological applications (Foissner 1999;
5 Mitchell et al. 2008b) and has been intensively applied in palaeoecological studies
6 from peatlands with the aim to reconstruct mire palaeohydrology and thereby, at least
7 for ombrotrophic bogs, past climatic change (Charman 2001). By analysing testate
8 amoeba community changes down the length of a core and interpreting the results
9 with a transfer function model, it is possible to quantitatively reconstruct changing
10 mire surface wetness (Booth et al. 2004; Lamentowicz et al. 2008; Sillasoo et al.
11 2007; Woodland et al. 1998) as well as sea-levels (Gehrels et al. 2002; Roe et al.
12 2002) through the Holocene. Testate amoebae are also used over shorter time periods
13 to monitor the success of peatland restoration (Buttler et al. 1996; Davis and
14 Wilkinson 2004; Jauhiainen 2002; Laggoun-Défarge et al. 2008; Vickery and
15 Charman 2004) and the impact of pollution in aquatic or terrestrial ecosystems (Balik
16 1991; Kandeler et al. 1992; Kauppila et al. 2006; Kumar and Patterson 2000; Nguyen-
17 Viet et al. 2007; Nguyen-Viet et al. 2004; Patterson et al. 1996; Patterson and Kumar
18 2002; Reinhardt et al. 1998; Scott et al. 2001; Török 2001).

19 To adequately characterise the community composition of any microfossil
20 used in palaeoecology, a minimum number of individuals needs to be counted under
21 the microscope. For different biological proxies, the minimal count required ranges
22 between 50 and over 300, largely dictated by the number of taxa typically identified
23 in a sample. Lytie and Wahl (2005) explored the effect of pollen sample size on
24 vegetation reconstruction and observed that with counts as low as 150 grains, the
25 vegetation reconstruction was accurate within >90%. Other studies suggest that

1 counts of 200 (Barkeley 1934) to 250 grains (Hill 1996) are sufficient. However, the
2 recommended sum is generally between 300 and 500 grains, and in order to obtain a
3 precise estimate for rare taxa, counts of 500 to 1000 grains are preferred (Birks and
4 Birks 1980). Heiri and Lotter (2001) investigated the impact of low counts on transfer
5 function inference for Chironomids and concluded that counts as low as 50 specimens
6 provided useful results. For charcoal, the minimum count (including exotic marker
7 grains) was estimated at 200 (Finsinger and Tinner 2005). For diatoms using the
8 random settling method, a total count of 300 has been shown to yield reasonable
9 estimates for the most abundant taxa, and increasing the count to 400 did not greatly
10 improve the accuracy (Boden 1991), although the reliability of this approach has been
11 criticized (Meng 1994). Totals of 500-1000 valves are frequently used (Lowe and
12 Walker 1997). In the case of foraminifera, the required minimum is 300-400
13 individuals (Lowe and Walker 1997).

14 For testate amoeba analysis, studies have usually aimed to count either 150 or,
15 less commonly, 100, 200, or 300 individuals (Bobrov 2005; Charman et al. 2000;
16 Tolonen 1986; Warner 1990). Whether such counts are actually sufficient has not
17 been rigorously tested. Mitchell et al. (2000) looked at the relationship between
18 testate amoeba count and number of taxa and found a plateau was reached after
19 around 100 individuals. Warner (1990) recorded the number of taxa with increasing
20 count for 8 samples and found a plateau was reached after around 60 individuals.
21 Woodland et al. (1998) showed that cumulative species diversity reached a plateau
22 after around 100 individuals.

23 Several studies have found it impossible, or at least impractical, to reach the
24 usual total. Low test concentrations have been encountered in situations such as
25 palaeoecological sequences with highly humified peats (Beyens and Chardez 1987;

1 Charman et al. 2001) and surface and sub-surface samples of fen peats (Jauhiainen
2 2002; Payne and Pates 2008). There are probably three factors contributing to these
3 problems: 1) low abundance of amoebae in the sediments on deposition, 2)
4 decomposition of tests over time and 3) a reduction in apparent test concentration due
5 to a high abundance of organic or inorganic material in the same size range as the
6 tests, which is not removed in sample preparation.

7 Low test concentrations present particular problems in palaeoecological
8 studies where an adequate count may not be achievable for parts of the sequence. This
9 leads to the question of whether some data is better than none: is it better to have
10 portions of a sequence with very low counts or to leave a gap in the palaeoecological
11 record? Some palaeoecological studies have presented records with very low counts
12 and have made palaeoenvironmental inferences based on these results, albeit with
13 caveats clearly stated (e.g. Beyens and Chardez 1987). However no study has been
14 undertaken to assess the relationship between number of testate amoeba shells
15 counted and data quality.

16 This study attempts to determine the minimum count levels required for
17 ecological and palaeoecological studies of testate amoebae by examining the impact
18 of count size on the number of taxa identified in three contrasting sites, by simulating
19 low counts using a random-selection approach with four previously established
20 modern training sets, and then applying the same approach to two palaeoecological
21 data sets.

22

23

1 **Methods**

2 General approach and aims

3

4 To determine the test count required to characterise a sample, we use four criteria: 1)

5 The number of taxa. As count size increases, the number of taxa identified should

6 increase and then stabilise as the total complement is reached. 2) Transfer function

7 inferences of environmental variables. As count increases, transfer function inferred

8 values and associated standard errors should stabilise. 3) The strength of the

9 relationship between the amoeba community and environmental variables. As the

10 amoeba community becomes better characterised with increasing count size, the

11 strength of the relationship with key environmental controls should improve. 4) The

12 inferred pattern of wet and dry periods in palaeoecological records: As count size

13 increases, the pattern should become more and more similar to the full model.

14

15 Impact of count on number of taxa identified

16

17 Previous studies have looked at the impact of count size on the number of taxa

18 identified (Warner 1990; Woodland et al 1998). However these studies only

19 considered a limited number of samples from a limited range of sites, and it is useful

20 to examine this with additional data. We present new data from the Ispani-2 peatland,

21 a percolation bog in western Georgia (Connor et al. 2007; Joosten et al.

22 2003)(10samples), the Sterling peatland, a kettle-hole mire in southern Alaska (Payne

23 et al. 2006)(10 samples) and Moidach More a blanket bog in eastern Scotland (150

24 samples). The sites represent a variety of peatland types and cover a large geographic

25 range. Samples were extracted from the surface of the Ispani-2 and Moidach More

1 sites and just below the surface (10-20cm) in the Sterling site. The normal total of 150
2 tests was counted for each sample and the number of tests required to identify each
3 additional taxon recorded.

4
5 Impact of count on species-environment relationships and predicted water table depth

6
7 To assess the impact of count size on amoeba community recorded, low-counts were
8 simulated using four data sets from previous studies of peatland testate amoebae: 1)
9 the data of Payne & Mitchell (2007) from the Elatia mires in northern Greece, 2) the
10 data of Payne et al. (2008) from the Sürmene Ağaçbaşı Yaylası peatland in north-
11 eastern Turkey, 3) the data of Mitchell et al. (1999) from peatlands of the Jura
12 Mountains (France and Switzerland) and 4) the data of Payne et al. (2006) from
13 peatlands in southern Alaska (Table 1). Samples in these studies were counted to at
14 least the usual total of 150 and recorded as percentages. Species complement varied
15 from 4 to 27 taxa. Much of the count is represented by a few taxa, and a single taxon
16 typically accounts for at least 25% of the total. Counts of 10, 20, 30..140 were
17 simulated by randomly selecting individuals from the full data set, and each
18 individual was able to be selected more than once. The transfer function models
19 calculated in the original studies were used to infer depth to water table based on
20 these simulated data sets. Standard errors were calculated using bootstrapping (1000
21 cycles). The procedure was repeated nine additional times with random re-selection of
22 individuals on each occasion, and the bias from the predicted values of the original
23 model were calculated.

24 These experiments assume that a count of 150 tests is sufficient to characterise
25 the amoeba community, To test the impact of higher totals on transfer function

1 performance, a sub-set of samples was separately analysed. For some samples from
2 the Alaska and Jura studies, amoeba concentrations were high and over 250 tests were
3 counted. Counts of 150, 160,..., 240 were simulated for these samples using the same
4 repeated random selection approach as above. Transfer function inferences using the
5 simulated data sets were compared to those of the full model for these samples.

6 Ordination was used to test the impact of count on the strength of relationship
7 between species and environmental data. The percent variance explained by the depth
8 to water table (DWT) data was tested using redundancy analysis (RDA) with each of
9 the simulated low count data-sets in CANOCO ver.4.53 (ter Braak and Šmilauer
10 1997-2004). As these analyses are labour-intensive only one cycle of data-selection
11 was used for this experiment.

13 Impact of count on palaeoecological interpretation

15 Finally, the same random data-selection approach was used to simulate low counts for
16 two palaeoecological data sets from the Praz-Rodet peatland in Switzerland (Mitchell
17 et al. 2001) and a small sub-Arctic mire in eastern Alaska, USA (termed site DLB;
18 Payne & Blackford, unpublished data). Water table changes were inferred for each of
19 the simulated data sets using the transfer functions of Mitchell et al. (1999, 2001) and
20 Payne et al. (2006). Although low counts were simulated for the palaeoecological
21 data, the full data sets were used for the transfer functions. The impact of lower
22 palaeoecological counts was evaluated by comparing the pattern of inferred low and
23 high water tables, and the correlation between the inferred DWT values of the reduced
24 versus full data sets. Reconstructions were repeated with ten sets of random

1 reselection, and standard deviations were calculated to assess the repeatability of
2 predictions.

3 4 5 **Results**

6 Impact of count size on number of taxa identified

7
8 Fig. 1 shows average number of taxa encountered with various count sizes. With the
9 Ispani-2 and Sterling samples, plateaux in the number of taxa are reached with counts
10 of around 100 and 120 individuals, respectively. With the Moidach More samples
11 there is a continued but slowing increase in taxa complement through to 150
12 individuals. In 55 of these 150 samples, new taxa are still encountered with counts
13 over 130. It is probable that 150 individuals is insufficient to identify all taxa in these
14 samples. There is no simple relationship between the total number of taxa in a sample
15 and the count required to identify all taxa. The Sterling samples are more diverse than
16 the Moidach More samples, but a count of 130 individuals would identify all taxa in
17 the Sterling samples and miss taxa in more than a third of the Moidach More samples.
18 The count required to identify all taxa depends not just on the number of taxa but the
19 relative abundances of those taxa.

20 21 Impact of count size on species-environment relationships and predicted water table 22 depth

23
24 Fig. 2 shows box plots of transfer-function inferred water table depth with various
25 count totals for four representative samples from the Sürmene Ağaçbaşı Yaylasi site

1 using ten cycles of random selection of individuals. Increasing the count generally
2 reduces the scatter of predictions and brings them closer to the full-model predicted
3 result, but not necessarily the measured value. Clearer results are obtained when using
4 all samples and considering mean bias from the full-model predicted values (Fig. 3).
5 With all data sets, increasing count gives predictions that are closer to the full model.
6 This improvement is most rapid in the range of 10-50 individuals. Counts over 50 and
7 up to 100 produce a slight further improvement, this is most noticeable with the
8 outliers in the Turkey data. Increasing the count from 100 to 140 appears to make
9 little further difference. Fig 4 shows the results if this is extended to counts of up to
10 240 using just those samples from the Jura and Alaska data sets with counts ≥ 250 . As
11 with the full data, the greatest difference is obtained by increasing the count to around
12 50 with less pronounced improvement beyond that. It is notable that increasing the
13 count beyond 150 seems to make a continued slight improvement in performance in
14 both data sets. It should, however, be noted that the data sets used to obtain this result
15 are small, with 12 and 18 samples for the Alaska and Jura data, respectively.

16 Increasing count reduces the standard error of predictions (Fig. 5). The count
17 required before values stabilise varies between data sets from 40 with the Alaska data
18 to perhaps 100 with the Turkey data. Increasing the count also increases the percent
19 variance explained in the redundancy analyses (Fig. 6). As these results are based on a
20 single cycle of random selection, there is more apparent 'noise' than with the results
21 discussed above. Values stabilise after counts of 100 in all sites. The increase is most
22 rapid with the Turkey data and least rapid with the Greece data.

23
24 Impact of count size on palaeoecological inferences

The palaeoecological reconstructions from Praz-Rodet and site DLB are shown in Fig. 7a and Fig. 7b, respectively. With low counts, there is a great deal of high-amplitude variability. Increasing the counts generally gives a record with less noise. Some features of the record are only resolved with a higher count such as the double peak at the base of the Praz-Rodet record, and the trough at 21-22 cm in the DLB record. It is clearly the case that increasing the count size gives a less noisy TI-DWT record, which agrees better with the results of the full model. However, it is perhaps surprising that counting very few tests still shows many of the major trends. Even with counts as low as 10 tests, the DLB record picks out the three distinct wet phases at 26-29 cm, 53-56 cm and 61-71 cm. In the Praz-Rodet record, counts of ten tests still show the distinctive peaks at 126, 166 and 312 cm, and troughs at 156 and 226 cm. Increasing count produces a slight decrease in boot-strapped errors, but this is minor and makes little difference to the interpretation of the results. Inferences based on a count of 10 individuals probably would not be very different from those based on the full count. The correlation between the reduced and full data further shows that counts as low as 10 already lead to a correlation coefficient of 0.6-0.75, and a correlation of 0.9 is reached with counts of 50, with little further subsequent improvement (Fig. 8). A plot of the mean standard deviation of the predictions shows high standard deviation for lower counts, representing distinct differences between individual cycles of data selection. As count size increases, the repeatability of predictions improves. The improvement produced with higher counts declines after around 40-50 individuals, but still continues to make a difference through to 140 individuals (Fig. 9).

Discussion

1 This is the first in-depth attempt to determine the minimum count sizes required for
2 ecological and palaeoecological studies of testate amoebae. We addressed the impact
3 of count size on species richness, community composition and the ecological and
4 palaeoecological information that can be obtained from the community structure data.
5 This study, therefore, has direct relevance for a range of ecological and
6 palaeoecological applications of testate amoebae (Charman 2001; Mitchell et al.
7 2008a).

8 Increasing count total increases the number of taxa identified, improves the
9 precision of transfer function prediction and reduces the standard error of those
10 predictions. As count size increased, there were progressively diminishing returns to
11 further increasing the number of tests counted. The point at which counting more tests
12 fails to produce any further improvement varies depending on what criterion is used.
13 For standard errors, there appears to be little gain from counting more than 60 tests.
14 To characterise a sample for redundancy analysis, 100 individuals seems to be
15 enough. A total of 100 tests also appears to be enough to achieve precision in transfer
16 function predictions, although slight further benefit may be obtained by counting
17 more individuals. To identify all the taxa in a sample, a count of at least 100 is
18 generally required and counts of 150 or more may be needed in samples that are
19 species-rich, or which include very rare taxa.

20 A frequent limitation of testate-amoeba based studies is low sampling
21 resolution and the lack of true replication. The sampling intensity of many ecological
22 studies may be insufficient to characterise the full range of testate amoeba
23 communities and environmental variables under consideration. In transfer function
24 studies this may lead to ‘no-analogue’ problems when the data are used for
25 environmental reconstruction. In most palaeoecological studies, only one core is

1 analysed, but it is possible that this core may not be truly representative of the site.
2 Studies that examined several cores show that differences in peat accumulation
3 patterns can be relatively important (Charman 2007). The sampling resolution of
4 cores is also frequently inadequate. It is common that some palaeoecological “events”
5 are documented by very few samples, and indeed often only a single sample.

6 Given these limitations we suggest that, when counting time is limited, it is
7 likely to be more useful to count a greater number of samples to a lower count total
8 than to count fewer samples to the usual total of 150. While a count of 50 tests may be
9 sufficient for some samples from some sites, we advocate a count of 100 tests based
10 on our analyses that examined depth to water table. This total should yield most of the
11 ecological information provided by counts of 150 tests, but for a significantly reduced
12 expenditure in counting time. The time taken to count a sample is only one
13 component of the total time invested in data generation. Additional time is required
14 for fieldwork, sub-sampling and preparation. However, in most cases the greatest
15 proportion of time is spent counting, and time savings in this would allow
16 considerably more samples to be analysed. Higher counts will still be required in
17 some situations where it is important to identify very fine environmental changes, or
18 if the focus of the study is on diversity. It is likely that even counts of 150 individuals
19 will fail to identify some taxa in some samples. Higher count totals may have more
20 value in transfer function studies that will be used for multiple inferences than for
21 individual palaeoecological studies. For most studies the small amount of information
22 lost in using a lower count will not be important, and the gain in number of samples
23 will be a worthwhile trade-off.

24 In some studies low test concentrations may make it impossible to reach a
25 count of 150, or even 50 tests in a reasonable time frame, particularly if the amount of

1 material is limited. Lower counts will inevitably reduce the accuracy and precision of
2 transfer function inferences. However ,our palaeoecological results suggest that a
3 meaningful palaeoecological signal may still predominate over random noise. Where
4 counts are very low, results must be treated with considerable caution, but it is still
5 possible for major changes to be shown.

6 Our findings are largely based on four modern training sets. Although these
7 training sets cover a large geographic range and considerable differences in amoebae
8 community structure, it is possible that our conclusions are not applicable to all such
9 samples. Our findings may also not be applicable to transfer functions that aim to
10 reconstruct different environmental variables, and particularly to testate amoebae
11 communities in other habitats that may have quite different diversity. The majority of
12 our experiments also assume that a count of 150 tests is in itself sufficient. If this is
13 not the case, then our conclusions may be erroneous.

14 Testate amoeba analysis and specifically its application for palaeohydrology,
15 is a relatively young technique compared to more established palaeoecological
16 methods such as palynology and diatom analysis. As the method becomes more
17 routinely applied it is necessary to address many of its underlying assumptions. Count
18 totals are one of these assumptions along with issues such as taphonomy, taxonomy
19 and the precise environmental controls on amoeba communities (Barber and Langdon
20 2007; Charman 2007; Charman et al. 2004; Mitchell et al. 2008a; Mitchell et al.
21 2008b; Payne 2007; Payne and Mitchell 2007; Swindles and Roe 2007). Testate
22 amoeba analysis is increasingly used in palaeoecology and ecology. Our results show
23 that most of the valuable data that lies in the structure of fossil testate amoeba
24 communities can be obtained at a reduced cost.

25

1 **Acknowledgements**

2 RJP was supported by a University of Manchester Humanities Research Fellowship, a
3 CBRL Visiting Fellowship, and a Wyndham Deedes Scholarship. We thank Matthias
4 Krebs for taking the Ispani-2 samples and Lisa Payne for assistance with data
5 analysis. EADM was funded by Swiss SNF project n° 205321-109709/1, and CCES
6 projects RECORD and BigLink. Thanks to three anonymous reviewers and Thomas
7 Whitmore for comments on previous versions of the manuscript.

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Tables & Figures

Figure 1. Impact of increasing test count on number of taxa recorded in samples from three contrasting peatlands, shown as mean and standard deviations.

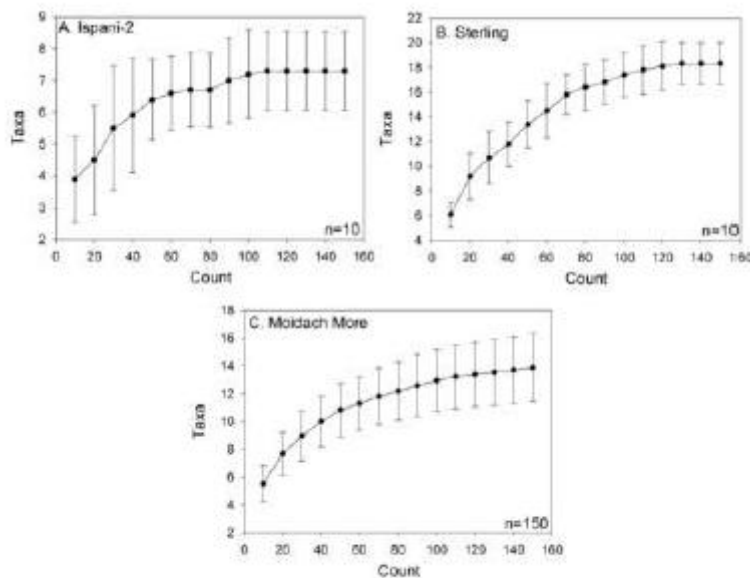
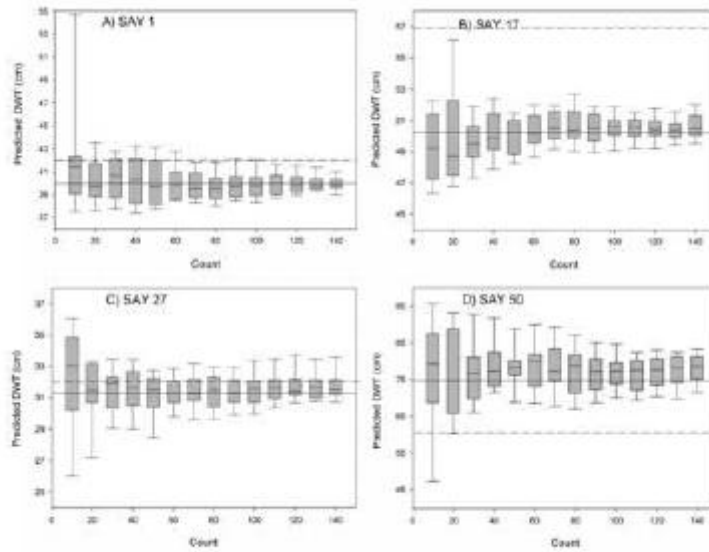
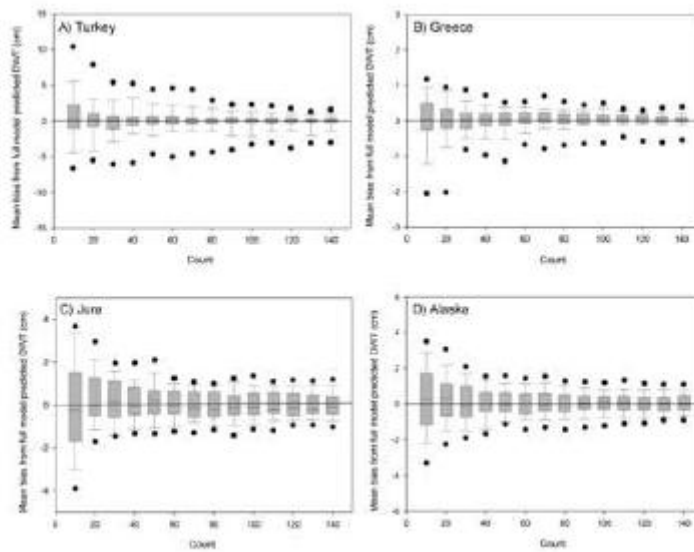


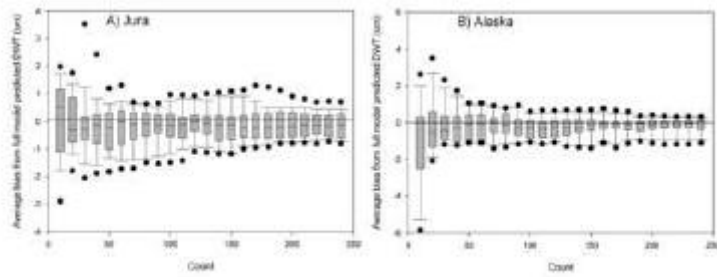
Figure 2. Box plots showing impact of increasing simulated test count on transfer function predicted depth to water table (DWT) for four samples spanning the hydrological gradient in the Sürmene Ağaçbaşı Yaylasi site. Results based on ten cycles of random re-selection of individuals. Solid horizontal line shows full model predicted value, dashed line shows measured value. Box plots show median (central line), first and third quartiles (grey box), tenth and ninetieth percentiles ('whiskers') and fifth and ninety-fifth percentiles (dots).



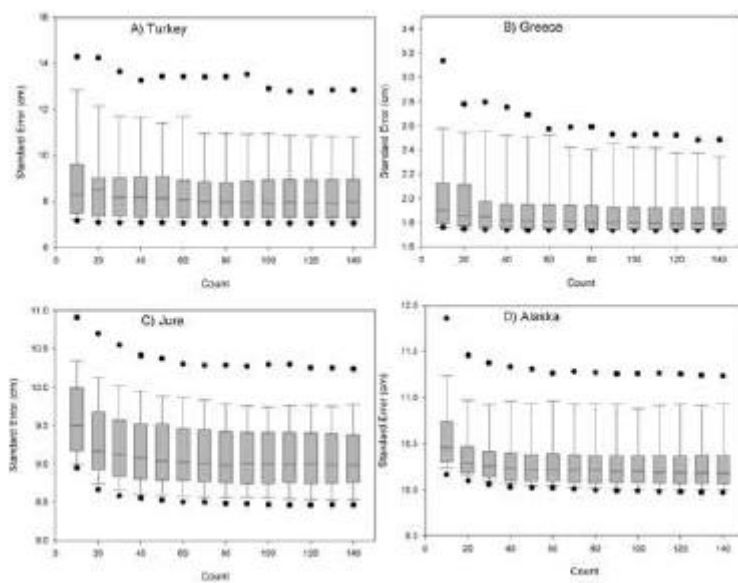
1
2 Figure 3. Mean bias from full model predicted DWT for simulated low-counts with
3 all samples from four data-sets, based on ten cycles of random re-selection of
4 individuals. Box plots as for Fig. 2.



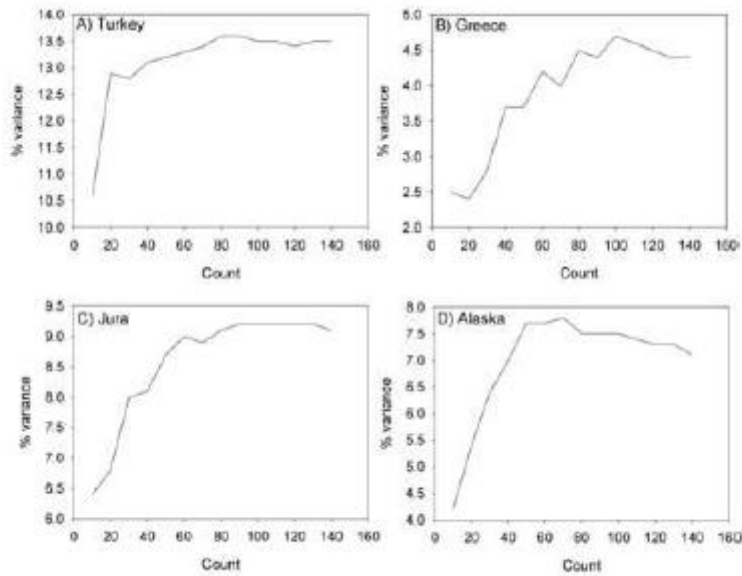
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6 Figure 4. Mean bias from full model predicted DWT with simulated low-counts
7 extended to 240 tests using 12 samples from the Alaska training set and 18 samples
8 from the Jura training set in which at least 250 tests were counted. Box plots as for
9 Fig. 2.



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2 Figure 5. Boot-strapped error estimates (mean of ten cycles of random selection) for
3 all samples from four data sets. Box plots as for Fig. 2.

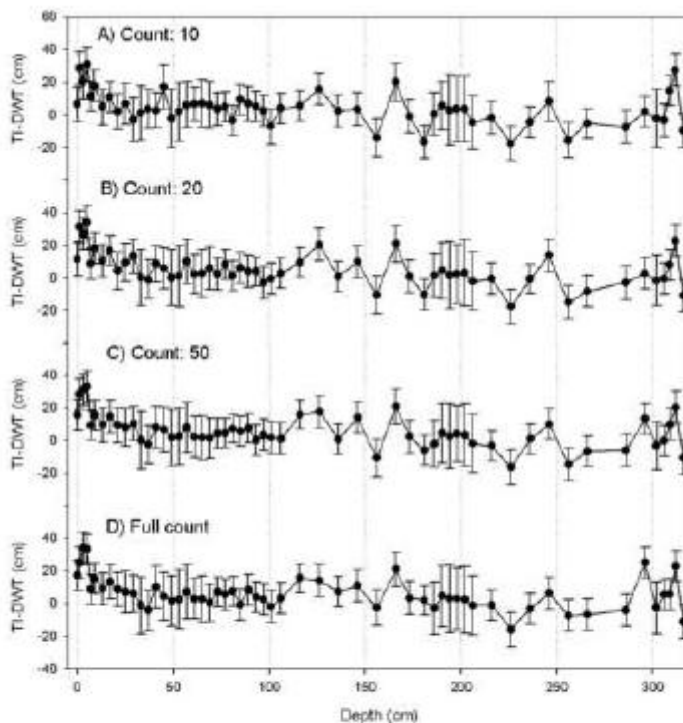


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6 Figure 6. Impact of increasing simulated count on % variance in amoebae species data
7 explained by DWT. All relationships are significant at $P < 0.001$.



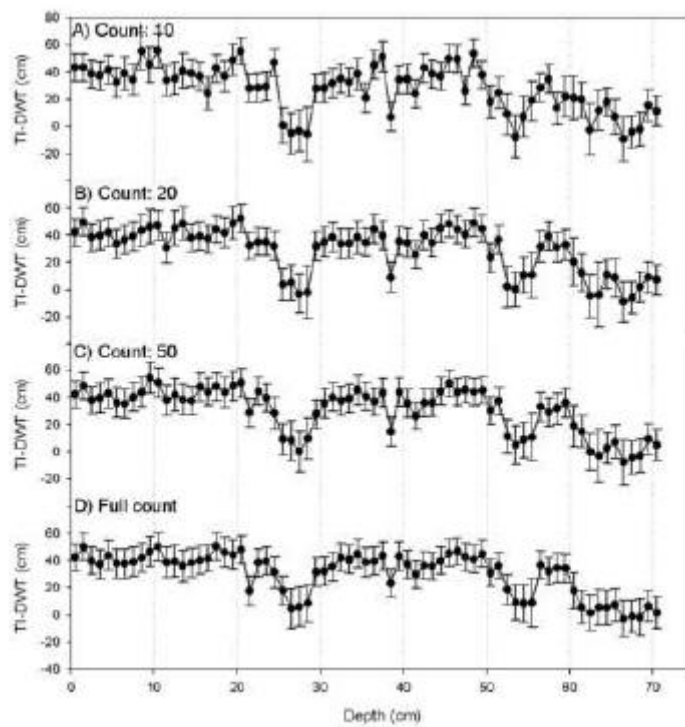
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2 Figures 7a & b. Testate amoebae inferred depth to water table (TI-DWT) with
 3 simulated counts of 10, 20 and 50 tests for palaeoecological sequences from the Praz-
 4 Rodet peatland, Switzerland (7a) and site DLB, Alaska (7b).

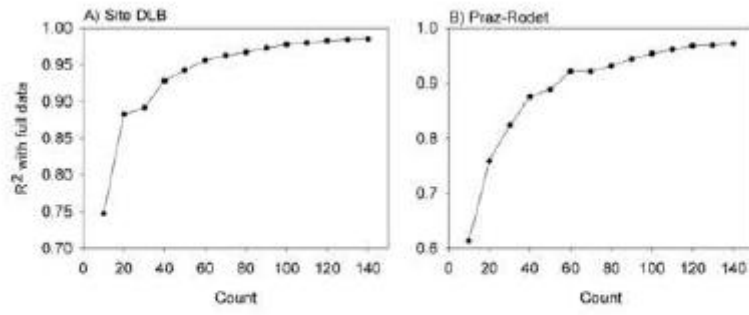


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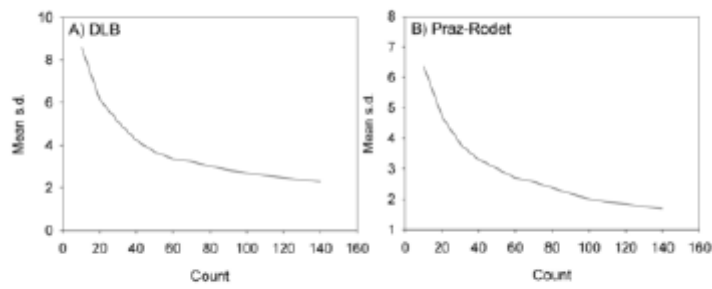
- 1 Figure 8. Relationship between count and r^2 of the regression between the inferred
- 2 DWT from the full and the reduced data sets from the Praz-Rodet peatland,
- 3 Switzerland (8a) and site DLB, Alaska (8b).



- 4
- 5 Figure 9. Mean standard deviation of predictions based on ten cycles of random data
- 6 selection for the Praz-Rodet peatland, Switzerland (9a) and site DLB, Alaska (9b).



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Table 1. Attributes of the training sets employed in this study showing number of samples, structure of transfer function model and jack-knifed root mean square error of prediction (RMSEP), Maximum Bias and R

Location	n	Model structure	RMSEP _{jack} [cm]	Max Bias _{jack} [cm]	R ² _{jack}	Reference
Alaska	91	WA-PLS (2 component)	9.7	14	0.55	Payne et al. (2006)
Greece	57	ML	1.8	2	0.82	Payne & Mitchell (2007)
Jura	48	WA-PLS (2 component)	8.0	21	0.62	Mitchell et al. (1999)
Turkey	42	ML	7.1	21	0.81	Payne et al. (2008)

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