Determining the absolute abundance of dinoflagellate cysts in recent marine sediments: the *Lycopodium* marker-grain method put to the test

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Abstract

Absolute abundances (concentrations) of dinoflagellate cysts are often determined through the addition of *Lycopodium clavatum* marker-grains as a spike to a sample before palynological processing. An inter-laboratory calibration exercise was set up in order to test the comparability of results obtained in different laboratories, each using its own preparation method. Each of the 23 laboratories received the same amount of homogenized splits of four Quaternary sediment samples. The samples originate from different localities and consisted of a variety of lithologies. Dinoflagellate cysts were extracted, counted, and relative and absolute abundances calculated. The relative abundances proved to be fairly reproducible, notwithstanding a need for taxonomic calibration. By contrast, excessive loss of *Lycopodium* spores during sample preparation resulted in non-reproducibility of absolute abundances. Further testing of the applied methodologies shows that losses of *Lycopodium* spores can occur through decantation and/or sieving. The results of this work therefore indicate that the dinoflagellate cyst worker should make a choice of using either a proposed standard method which circumvents critical steps, adding *Lycopodium* tablets at the end of the preparation, or using an alternative method.

Keywords

Dinoflagellate cyst, concentration, *Lycopodium clavatum* tablets, spike, inter-laboratory calibration.

1. Introduction

Dinoflagellate cyst concentrations are an important component of paleoceanographical studies (e.g. Pospelova et al., 2006; González et al., 2008) and can be determined using the volumetric method (e.g. Dale et al., 2002; Holzwarth et al., 2007). In general, dinoflagellate cyst concentrations are calculated by adding a known amount of exotic markers or a "spike" to every sample according to the method described by Stockmarr (1971). The marker commonly used is *Lycopodium clavatum* Linnaeus (Stag's Horn Clubmoss or Ground Pine).

As noted by Lignum et al. (2008), the so-called 'standard' palynological processing methods are still very variable in terms of initial sample sizes, type and concentration of acids, sieve material and mesh size, sonication time and strength, number of decanting cycles, use of heavy liquid separation, etc. However, critical evaluation of the effect of different laboratory procedures on the marker grain technique for obtaining dinoflagellate cyst concentration has so far never been attempted. Although it has been reported that several processing methods such as sonication and chemical treatments can inflict damage on organic-walled microfossils to a certain extent (e.g. Schrank, 1988; Hodgkinson, 1991), the effect on palynomorph concentrations remain unknown.

This study aims to test the reproducibility of the marker-grain method, in order to understand the discrepancies in the results following different preparation techniques. A better insight results in a proposal of recommendations for a standardized method to determine absolute abundances of Quaternary dinoflagellate cysts with the marker-grain method. Similar efforts to test the reproducibility of specific laboratory techniques have been done for other microfossil groups: benthic and planktonic foraminifera (Zachariasse et al., 1978), diatoms (Wolfe, 1997), nannofossils (Herrle and Bollman, 2004) and their biomarkers (Rosell-Melé et al. 2001). It is therefore timely to carry out a similar exercise with dinoflagellate cysts.

Surface sediment samples from four localities (North Sea, Celtic Sea, NW Africa and Benguela) were sent out to 23 laboratories. The samples were processed using the palynological techniques routinely used in these laboratories. An equal amount of *Lycopodium* tablets, all from the same batch, were added to each sample. The reproducibility of both absolute and relative abundances for dinoflagellate cysts is here put to the test, and has resulted in a proposal of recommendations for a standardized method to determine absolute abundances of Quaternary dinoflagellate cysts with the marker-grain method. Two laboratories used the volumetric method (Dale, 1976) for comparison purposes. This study focuses additionally on whether it is necessary to count 300 or 400 dinoflagellate cysts and on some important taxonomic issues, since notable interlaboratorial differences in nomenclature were recorded.

2. Material & methods

Late Quaternary surface sediment samples from four sites with different lithologies were used by the 23 different laboratories involved in the project. The North Sea sample consisted of a homogenized surface sediment taken using a Reineck boxcorer (51.47° N, 3.48° E, 10 m water depth). The Celtic Sea sample was assembled through mixing multi-corer samples from Station 8, collected during several time slots from the Celtic Sea (51.05°N, 5.83°W, 86 m water depth) (Marret and Scourse, 2002). The sample from Northwest Africa was a mixture of multicores GeoB9504-4 (15.87°N, 16.67°W, 43 m water depth) and GeoB9503-3 (16.07°N, 16.65°W, 50 m water depth). The Benguela sample consists of a mixture of sediment samples collected offshore Walvis Bay, at a water depth of about 200 m during Meteor cruise M63/2. Sample details are given in Table 1. Each laboratory was given a number, followed by a letter when the laboratory used more than one processing method. Laboratory identification and

numbers were kept anonymous to eliminate accountability. A brief overview of the used methods is described in section 2.1 to 2.5, a special variation of this method in section 2.6 and the volumetric method in section 2.7. Details of the used methods are given in the supplementary data. An extensive review of preparation techniques for extraction of dinoflagellate cysts is given by Wood et al. (1996) and more recently by Riding and Kyffin-Hughes (2004).

Homogenization was done with the quartile method. The samples were oven dried at a temperature of 58°C for 24 hours. The *Lycopodium* spore tablets used are produced and distributed by the Subdepartment of Quaternary Geology, University of Lund, Sweden (http://www.geol.lu.se/kvg/eng/). Ten *Lycopodium clavatum* tablets of batch 483216, (X=18.583 per tablet, s=±1708), were dispatched with the samples, and a fixed number of tablets was added by each laboratory to each sample.

2.1 *Chemical treatment*

Hydrochloric acid (HCl) with a concentration of 6.5–36 % was added for the removal of carbonate. Some 20 to 300 ml was used depending on the intensity of the reaction. Cold HCl was used in most of the cases, although some laboratories used hot HCl with a temperature ranging between 42 and 80°C. Afterwards, the residue was left to settle (15 min to 42 h). Laboratories that used short settle times at this step, used centrifugation or sieving to concentrate the sample. Demineralised or distilled water was used for rinsing until pH reached more neutral values of 5 to 7. One to 5 decanting cycles with intervals of 3 to 24 h were needed depending on HCl-concentrations used. To avoid losing residue during decanting, some laboratories used centrifuging for the concentration of the samples. The extensive rinsing is necessary for the removal of Ca^{2+}, to avoid calcium fluoride (CaF_2) precipitation

during the following HF treatment. A few laboratories used KOH for neutralization (Laboratory 2: 1% and Laboratory 18 b: 10%).

The siliciclastic component was removed by adding 10 to 250 ml of hydrofluoric acid (HF) with a concentration ranging from 19% to 70%. Commonly a concentration between 40 and 50% was used. All laboratories used cold HF, except laboratories 12 (42°C), 2 (50°C), 6 (60°C), 10 (70°C) and 23 (80°C). Settling times varied between 12 and 144 h. A few laboratories repeated the HF treatment up to 3 times before all silicates were removed.

Without neutralising, about 10 to 300 ml HCl with a concentration of 6.5 to 36 vol% was added for the removal of formed fluorosilicates. Mostly cold HCl was used, although some laboratories used hot HCl with a temperature ranging between 42 and 100°C. The following settling time varied between 15 min to 72 h. Laboratories that used short settling times, used centrifugation. The sample was subsequently rinsed with distilled water, until pH reached 5–7. The rinsing took 1 to 6 decanting cycles with intervals of 3 to 24 h, depending on the concentrations used. To avoid losing residue during decanting, some laboratories used centrifuging for the concentration of the samples. One laboratory used KOH for the neutralisation (Laboratory 2: 1%). A few laboratories skipped the second HCl treatment and proceeded directly to the rinsing with distilled water until pH reached values of 5–7. Several of these laboratories used centrifuging and/or sieving for concentration of the samples. During rinsing toxic HF was decanted and removed.

One laboratory (Laboratory 22b) oxidised three of the samples (excluding the North-West Africa sample) with Schulze's solution (70% nitric acid saturated with potassium chlorate).

2.2. *Mechanical treatment*

Centrifugation is often used to concentrate the organic material. The rotation speed used varied from 1900 to 3500 rpm, and lasted between 5 sec to 10 min.

Heavy liquid separation for the removal of heavy minerals was carried out by a few laboratories. Labs 10 and 16 used sodium polytungstate (SPT) at specific densities to isolate the palynological fractions.

Between 13 and 1800 seconds sonication was used to break down organic matter aggregates by some laboratories. Most laboratories used sonic baths (Branson™, Sonimasse™, Sonicor™, Eurolab™). Laboratory 8 used a standard oscillating sensor.

2.3. *Sieving*

Some laboratories pre-sieved before the chemical treatment for the elimination of the coarse fraction (sieve widths 100, 106, 120, 150 µm) and/or fine fraction (sieve widths 10, 11, 15 µm). All the laboratories added the *Lycopodium* tablets before pre-sieving, except Laboratory 23.

The sieving was used after the chemical treatment to remove the fine fraction from the residue. Calgon (sodium hexametaphosphate) was used to disaggregate the material in a few cases. The sieve mesh sizes used varied from 6 to 20 µm, and meshes were made of nylon, polyester, polymer or steel. The devices used were hand, mechanical and water pressure pumps. Some laboratories sieved without using a pump.

2.4. *Staining and mounting of the slides*

Staining with a colouring agent enhances contrast for optical microscopy purposes and might be useful for the detection of pre-Quaternary specimens (Stanley, 1966). Safranin-O, Fuchsin

329 or Bismark Brown was used by a few laboratories. Not every laboratory stained the residue.
330 Finally a few drops of a copper sulphate solution, thymol or phenol were often added to the
331 residue for the inhibition of fungal growth.
332 Slides were mounted on a heated metal plate (65°C) using a pipette, by strewing or a mix of
333 both methods. The mounting medium was usually glycerin jelly, but sometimes thymol,
334 Elvacite, Eukitt, UV adhesive, or Canada balsam was used. Although sealing is not *per se*
335 necessary (Poulsen et al., 1990), nail polish or paraffin wax was used for sealing the margins
336 of the cover slip protecting the residue from degradation by dehydration.
337
338 2.5 *Counting of the palynomorphs and calculation of absolute abundances*
339
340 Dinoflagellate specimens were counted only when they comprised at least half of a cyst. The
341 same criterion was used for other palynomorphs, also counted by some of the laboratories.
342 Initially 300 dinoflagellate cysts were counted, and subsequently an extra 100 specimens were
343 added. The purpose was to check whether it is necessary to count 300 or 400 dinoflagellate
344 cysts to obtain representative relative and absolute abundances. Indeterminate dinoflagellate
345 cysts were grouped as Indeterminate spp., and were not taken into account for the calculation
346 of the relative abundances, since every observer had a different concept of what counts as an
347 indeterminate dinoflagellate cyst, and this would introduce observer bias into the relative
348 abundances. Raw counts together with a summary of the methodology used are available as
349 supplementary data to this article.
350 Absolute abundances of dinoflagellate cysts are calculated following the equation by
351 Benninghoff, 1962:
352
353 $$c = \frac{d_c \times L_t \times t}{L_c \times w}$$

where

c = concentration = number of dinoflagellate cysts / gram dried sediment.

d_c = number of counted dinoflagellate cysts

L_t = number of *Lycopodium* spores / tablet

t = number of tablets added to the sample

L_c = number of counted *Lycopodium* spores

w= weight of dried sediment (g)

Maher (1981) devised an algorithm to calculate confidence limits on microfossil concentrations. A slight correction to this algorithm was made, since the current study used sediment weight instead of sediment volume. The confidence limits calculated based on this algorithm have a 0.95 probability (Z=1.95). It should be noted that these confidence limits are similar to the total error on concentration proposed by Stockmarr (1971) (Appendix 2). These confidence limits can then be used in a statistical test to check whether microfossil concentrations are the same in two different samples (Maher, 1981). To investigate the reproducibility of results from the different laboratories, the coefficient of variation (or relative standard deviation) of all counts of a particular sample can be compared. Ideally, the results should fall within the confidence limits of Maher (1981), and thus the coefficient of variation calculated from these confidence limits can be used as a comparison.

2.6. Special methods: the maceration tank method (with HF) and the washing machine method (without HF)

The maceration tank method (Poulsen et al., 1990; Desezar and Poulsen, 1994) was used for HF treatment by Laboratory 20a. Other processing steps are similar to those used by the other

laboratories and are detailed in Poulsen et al. (1990) and Desezar and Poulsen (1994). Each sample is tightly wrapped in filter cloth (25 cm x 25 cm) with a mesh size of 10 µm, and the filter bags are packed in rubber foam for protection. The samples are placed inside the maceration tank and HF is conducted to the tank in PVC tubes. The samples are treated with cold HF for 7-8 days, after which the HF is drained out through a bottom-stop cock and led via PVC tubes directly to a waste-container for used hydrofluoric acid.

With the washing machine method, used by Laboratory 20b, no HF is used. Each sample is tightly wrapped in filter cloth (25 cm x 25 cm) with a mesh size of 10 µm and and the filter bags are packed in rubber foam for protection . The samples are placed in a standard household washing machine and washing occurs with a standard household washing powder, after which carbonates are removed with citric acid at 65°C. Next the samples are again given a normal wash with a standard household washing powder. Finally the remaining minerals are removed by heavy liquid separation. This method cleans the samples very efficiently from most of the amorphous material. Furthermore, since HF is not used, siliceous constituents (e.g. diatoms) are not destroyed. Heavy liquid separation with zinc dibromide ($ZnBr_2$) was used at specific densities (2.3, 2.0 and 1.8 g/ml) to remove heavy minerals. In order to test the influence of the specific density of the $ZnBr_2$, the NW African sample from 20b, was separated at specific densities at 1.8, 2.0 and 2.3 g/ml.

2.7. *Volumetric method*

For comparison with the marker-grain method, the volume aliquot method was performed by laboratories 6 and 8 following Dale (1976). This method was not used for the North Sea sample because of the difficulty associated with counting a fixed volume of this sample with very low abundances.

3. **Results**

3.1. *Relative abundance of dinoflagellate cysts*

A cause for the quantitative and qualitative disparities between assemblages recorded by the laboratories may be due to the different processing methods employed. It is obvious that aggressive agents could destroy the more sensitive cysts. To check this dependence of preservation on methodology, it is warranted to group species according to their resistance to degradation. It is hereby assumed that both mechanical and chemical degradation have similar effects on an assemblage. The grouping proposed here is similar to the grouping described in Zonneveld et al. (2001). Cysts not referred by these authors were added to a particular group based on the assumption that comparable morphology (e.g. wall thickness, resistance of structures against folding) is indicative of similar resistance to decay.

Extremely sensitive cysts: round brown cysts (RBC), spiny brown cysts (SBC), cysts of *Alexandrium* spp., cysts of *Gymnodinium* spp., *Stelladinium* spp., *Lejeunecysta* spp., *Selenopemphix* spp., *Tuberculodinium vancampoae, Polykrikos* spp., *Xandarodinium xanthum* and *Dalella chathamense.*

Moderately sensitive cysts: *Lingulodinium machaerophorum*, *Operculodinium* spp., *Pyxidininopsis reticulata*, *Spiniferites* spp., *Quinquecuspis concreta, Trinovantedinium applanatum* and *Votadinium* spp.

Resistant cysts*: Nematosphaeropsis labyrinthus*, *Impagidinium* spp., *Operculodinium israelianum*, *Pentapharsodinium dalei*, *Polysphaeridium zoharyi*, *Ataxiodinium choane* and *Bitectatodinium* spp.

Within these three groups, it is also interesting to note which taxa demonstrate the largest variation, and the results of the individual taxa will also be described in the following paragraphs. It is evident from the dataset that some species were not recorded by some observers. One obvious example is *Dubridinium* spp., which was often counted as RBC, or in some cases not counted at all. To partly reduce this observer bias, we decided to group species into genera or even larger groups (Appendix 1). Averages of relative abundances were only calculated when at least 300 dinoflagellate cysts were counted. The counts from oxidized samples (Laboratory 22b) were also excluded, since all heterotrophic cysts were destroyed. The *average results of the four samples are shown in Table 2. Representative cysts from the four samples are shown in Plate I to IV.*

3.2. Absolute abundances of dinoflagellate cysts

The cyst concentration (absolute abundance) in the North Sea sample ranges from 570 to 3,304 cysts/g, excluding the outliers. Laboratory 1a makes an overestimation (8,342 cysts/g) and Laboratory 22b produced very low numbers (278 cysts/g). The average is 1,516 cysts/g with a standard deviation of 698 cysts/g (coefficient of variation, V=46%). The average coefficient of variation from the confidence limits of Maher (1981) is 20%. The volumetric method was not used for the North Sea sample (Table 2).

The cyst concentration (absolute abundance) in the Celtic Sea sample ranges from 1,240 to 5,284 cysts/g, excluding the outliers. Laboratories 14 and 1a overestimate with 75,633 and

10,961 cysts/g respectively, while Laboratory 20a, 2 and 20b gives respectively low values of 1,053, 731 and 501 cysts/g. The average is 2,583 cysts/g, with a standard deviation of 1,342 cysts/g (V=52%). The average coefficient of variation from the confidence limits of Maher (1981) is 25%. Results obtained by the volumetric method give estimates that are much lower than with the marker grain method. For the Celtic Sea these values (1,160 cysts/g (Laboratory 6) and 1,167 cysts/g (Laboratory 8)) are even below the lowest value obtained by the marker grain method (Table 2).

The cyst concentration (absolute abundance) in the NW Africa sample ranges from 4,606 to 38,357 cysts per gram, excluding the outliers: labs 11, 1a and 14 produced very high numbers (168,899, 167,651 and 129,236 cysts/g, respectively). The average is 19,441 cysts/g, with a standard deviation of 9,148 cysts/g (V=47%). The average coefficient of variation from the confidence limits of Maher (1981) is 23%. As before, the volumetric method gave lower estimates but within the range of the marker grain method (11,600 cysts/g (Laboratory 6) and 9,992 cysts/g (Laboratory 8)) (Table 2).

The cyst concentration (absolute abundance) in the Benguela sample ranges from 30,130 to 298,972 cysts/gram, excluding the outliers. Laboratory 1c produced an overestimated value of 1,455,988 cysts/g, while laboratories 20b and 8 give values as low as 18,472 and 15,910 cysts/g, respectively. The average is 144,299 cysts/g with a standard deviation of 84,159 cysts/g (V=58%). The average coefficient of variation from the confidence limits of Maher (1981) is 21%. The volumetric method used by Laboratory 6 yields 53,200 cysts/g (within the range above) and 8,492 cysts/g by Laboratory 8. The volumetric estimate by Laboratory 8 is considered to be an underestimation caused by the destruction of fragile cysts by sonication (see Discussion) (Table 2).

3.3. *Reworked dinoflagellate cysts*

About 7% of the recorded dinoflagellate cysts in the North Sea sample were reworked. The pre-Quaternary cysts recorded in the North Sea sample were *Wetzeliella* spp. (dominant), *Glaphyrocysta* spp., *Cordosphaeridium* spp., cf. *Oligosphaeridium* spp. and cf. *Cribroperidinium* spp. In terms of absolute abundances, reworking shows the same trends as *in situ* dinoflagellate cyst absolute abundances. Very high absolute abundances were recorded in the sample oxidized by Laboratory 22b. This indicates that the robust pre-Quaternary cysts are more resistant to oxidation. Reworking is very low (less than 1%) in the samples from the Celtic Sea, NW Africa and Benguela.

3.4. *Other palynomorphs*

Chlorophycean palynomorphs such as *Cymatiosphaera* sp. (not present in Celtic Sea), *Pediastrum* sp., *Pterospermella* sp. (not present in Benguela), *Tasmanites* sp., *Botryococcus* sp. (not present in Benguela), *Mougeotia* sp. (only North Sea), *Concentricystes circulus* (only NW Africa), *Gelasinicysta* sp. indet. (only NW Africa) are recorded in low numbers in all samples, except the North Sea sample.

Faunal remains such as microforaminiferal linings, scolecodonts, tintinnids, planktonic crustacean eggs and invertebrate mandibles were encountered in almost every sample. Planktonic crustacean eggs are very abundant in the North Sea sample.

Pollen and spores are abundant in the North Sea sample. The assemblage is dominated by pollen (90%). Non-bisaccate pollen include *Quercus*, *Corylus*, *Betula*, *Alnus*, Poaceae, Cyperaceae and Chenopodiaceae, whereas bisaccate pollen comprise mainly *Pinus* and *Picea.* Some *Cedrus* pollen is recorded. Reworked pollen and spores are present in low numbers.

The Celtic Sea sample is dominated by pollen (94%). Non-bisaccate pollen comprises mainly Poaceae, *Quercus*, Ericaceae and Chenopodiaceae. Bisaccate pollen is mainly *Pinus* pollen. Reworked pollen and spores are very rare.

The sample from NW Africa is also dominated by pollen (95%). Non-bisaccate pollen comprise mainly Poaceae, *Quercus*, Ericaceae and Chenopodiaceae. The bisaccate pollen are mainly *Pinus* pollen. Reworked pollen and spores are very rare.

The Benguela assemblage is dominated by pollen (99%). Non-bisaccate pollen includes mainly Poaceae, Asteraceae and Caryophyllaceae. Bisaccate pollen is mainly *Pinus* pollen. No reworked pollen and spores were recorded.

Hyphae and fruiting bodies were counted as fungal remains in order to check whether the samples were infected by fungi. No samples showed significant abundances.

The recorded incertae sedis algae include *Cyclopsiella*, *Halodinium* sp., *Hexasterias problematica* (not present in Northwest Africa), *Micrhystridium* sp. (Celtic Sea and Benguela), *Palaeostomocystis subtilitheca* (North Sea and Celtic Sea), *Radiosperma corbiferum* (Celtic Sea and Benguela) and *Sigmopollis* sp. (NW Africa). These were more abundant in both North Sea and Celtic Sea samples.

Other organisms occurring are the organic linings of calcareous dinoflagellate cysts, thecamoebians (North Sea, Celtic Sea), chrysomonad cysts (North Sea, Celtic Sea) and diatoms. Diatoms can still be present when low concentrations of HF are used, possibly combined with heavy liquid separation, which enhances the abundance of diatoms with low densities (Laboratory 1c; 9; 17). Laboratory 20b has good recovery of diatoms, since the samples are not treated with HF.

4. **Discussion**

4.1. Is a 300 or 400 dinoflagellate cyst count sufficient to reach reliable diversities and absolute abundances?

There is no general agreement on the number of cysts which should be counted to obtain reliable data for diversity and absolute abundance studies. The majority of palynologists usually count 300 cysts per sample, which can provide up to 98% confidence (Germerad et al., 1968). To check whether it is necessary to count 300 or 400 dinoflagellate cysts, results from counting 300 cysts, plus an additional 100 cysts are compared using absolute abundances, species diversity and the Shannon-Wiener Index for all samples (Table 3). The comparison shows that the disparities in the results are insignificant: averages of absolute abundances, species richness and the Shannon-Wiener Index show limited changes compared to the associated standard deviations. The statistical test of Maher (1981) indicates that all absolute abundances derived from the 300 dinoflagellate cyst count statistically produce the same concentration as from the 400 dinoflagellate cyst count. It can thus be concluded that a 300 dinoflagellate cyst count is sufficient for generating reliable diversities and absolute abundance data in Quaternary studies.

4.2. *Reproducibility of relative abundances*

The standard deviations of the relative abundances observed in the grouping based on cyst preservation are always lower than 11.2%. These relatively small standard deviations suggest that changes in the relative abundance counts are caused by observer bias rather than by differences in methodology. Indeed, the highest standard deviations in the taxonomical groupings are with the taxa RBC, SBC and *Lejeunecysta* s.l. and since it can be assumed that the potential for preservation of these taxa is similar, it is likely that the disparities in the

552 counts are the result of observer bias. The high standard deviation for RBC is probably caused
553 by the high numbers of the morphologically similar *Dubridinium* spp. and the unfamiliarity of
554 many observers with *Dubridinium* spp. Furthermore, an unambiguous definition of a round
555 brown cyst is still lacking. The same is true for the spiny brown cysts, and several poorly
556 defined species fall within this group. All other standard deviations are lower than 10%,
557 which we consider an acceptable range for completely independent dinoflagellate cyst counts.
558 Another possible reason for observer bias could be related to the use of different illumination
559 techniques for routine counting of dinoflagellate cysts. Comparison of the use of phase
560 contrast to interference contrast illumination to count dinoflagellate cysts on the same slides
561 by Laboratory 15 revealed that phase contrast emphasizes the transparent cysts (*Spiniferites*
562 s.l., *Operculodinium* s.l., *Nematosphaeropsis labyrinthus*, etc.), whilst interference contrast
563 emphasizes the brown heterotrophic cysts (RBC, SBC, etc.). Despite the observer bias, there
564 is no doubt that dinoflagellate cyst relative abundance counts by one single observer are
565 repeatable.
566
567 4.3. *Explanation of outliers in absolute abundances*
568
569 The overestimates can each be explained by examining specific methodologies employed by
570 particular labs. Labs 1a and 1c lost an excessive amount of *Lycopodium* spores due to the use
571 of sieving at 20 µm as shown by Lignum et al. (2008). Labs 11 and 14 experienced problems
572 with settling after centrifugation and were not confident that the final residues were suitable
573 for quantitative analysis.
574 The underestimates by Laboratory 22b are due to the use of oxidation, which causes
575 preferential destruction of dinoflagellate cysts. Due to the low amounts of material used in the
576 exercise, the maceration tank and washing machine method (laboratory 20a and laboratory

20b) did not function optimally and yielded atypical resultsthat should not be regarded as representative results for the technique. This would be mainly related to cysts getting attached to the large filter cloth (25x25 cm) used in this technique (see Discussion, assumption eight).Furthermore, one of the samples from NW Africa (Laboratory 20b) was separated at specific gravities of 1.8, 2.0 and 2.3 g/ml. At the specific gravities of 1.8 and 2.3 g/ml, there were almost no dinoflagellate cysts in the slides, whereas ten times more dinocysts were noted at the specific gravity of 2.0 g/ml. Further investigation needs to be carried out to evaluate the effect of heavy liquid separation at different specific gravities.

For Laboratory 8, the use of a sonic oscillator resulted in destruction of sensitive cysts, again yielding underestimates.

4.4. *Reproducibility and accuracy of absolute abundances, excluding the outliers*

Total cyst count is less dependent on taxonomical expertise, and thus probably less influenced by the observer bias. The different laboratories participating in the current inter-calibration exercise used different processing techniques(see supplementary data). The reproducibility of estimates of absolute cyst abundances, as expressed as coefficient of variation in Table 2, shows that there are quite some differences among the 23 laboratories: the coefficients of variation are relatively large (46–58%) and nearly twice as high as the coefficients of variations (20–25%) which are calculated from Maher (1981). Our results suggest that the determination of absolute abundances is mainly dependent on processing methodology. In this light the accuracy also needs to be considered: a better understanding of what is causing the variation can only be achieved when correct absolute abundances of dinoflagellate cysts have been determined. To estimate whether the absolute abundances give an accurate picture of the true absolute abundances of the dinoflagellate cysts, results from the marker-grain method are

compared with independent methods. When compared to the volumetric method, absolute abundances calculated using the marker-grain method, are 44–63 % higher (Table 2). In a similar study, de Vernal et al. (1987), noted systematically higher concentrations from the marker-grain method compared to the results from the volumetric method, and they suggested that significant losses of *Lycopodium* spores (close to 33% on the average) took place during labratory procedures. On the other hand, in a study on Paleogene sediments, Heilmann-Clausen (1985), found marker-grain estimates varying between 70% and 129% of volumetric estimates and on average 2% lower concentration was calculated from the marker-grain method. Our study confirms the observation of de Vernal et al. (1987), and even shows larger deviations. It should also be noted, that counts from strew slides made from unprocessed samples show much lower abundances than the average absolute abundances from the marker grain method. From these observations, it can be concluded that with most preparation techniques there are significant losses of *Lycopodium* spores, and this is most probably the reason for overestimation of the absolute abundances using the marker-grain method. Furthermore, there was no evidence of significant loss of dinoflagellate cysts during the labratory preparations, except when oxidation or very long or destructive sonication was used (see below). Thus, in order to understand what causes the differences in absolute abundances, one needs to consider which of the underlying assumptions need to be questioned. Ten assumptions need to be considered.

1)"Drying samples does not cause decay. "

Although drying is often done in palynological preparation, it should be avoided in organic rich sediments, where drying causes formation of selenite (gypsum, $CaSO_4.2H_2O$), by reaction of calcium carbonate with sulphuric acid, usually derived from pyrite decay. The formation of sulphuric acid significantly affects extremely sensitive dinoflagellate cysts. In this case, to calculate the weight of the samples, wet volumes should be used, corrected with

627 dry bulk densities. In our samples, gypsum crystals were not observed. The homogenized
628 samples were oven dried before subdivision into smaller batches and dispatching to individual
629 laboratories. This was done to avoid differential drying. However, not all laboratories
630 processed the samples exactly at the same time. Samples were dispatched in March 2007, and
631 were processed within the following year. The possibility exists that samples that were
632 processed at a later stage dried out more. Clustering of amorphous organic matter around the
633 cysts seems to occur in more dried out samples (most obvious around *Lingulodinium*
634 *machaerophorum* specimens in Plate III), but there were no clear signs that this process
635 caused changes in the assemblage.
636 2)"Samples are homogenous."
637 Heterogenous samples processed in a similar way do not give reproducible results. One
638 quasi-replica was done by Laboratory 21 (a and b). The only difference in preparation was the
639 addition of some soap during sieving (Table 4). Following the test by Maher (1981), for every
640 studied sample, the microfossil concentration in the replicas is the same. It can thus be
641 concluded that the samples are well-mixed and are homogenous. Furthermore, there are few
642 differences between both samples in terms of relative abundances.
643 3)"A single *Lycopodium* tablet from batch 483216 contains 18,583 ± 1,708 spores."
644 This reference is given by the supplier (Lund University), and these numbers were calibrated
645 using a Coulter counter. Lignum et al. (2008) also used a Coulter counter for verification and
646 obtained 16,971 ± 1,251 *Lycopodium* spores. We dissolved one tablet in distilled water and
647 sieving on a 0.25 µm Millipore filter. The filter was cut into two pieces, mounted on a slide
648 and counted under a transmitted light microscope. On this filter, 16,993 *Lycopodium spores*
649 were counted, which falls within the range proposed by the supplier and Lignum et al. (2008).
650 A similar exercise has been done for another batch by Stabell and Henningsmoen (1981)
651 which found similar results. This assumption is thus acceptable.

652 4) ”No degradation of the palynomorphs occurs caused by chemical treatment such as
653 oxidation or acid treatments by HF and HCl.”
654 Since *Lycopodium* spores are acetolysed during the manufacturing process, they can
655 withstand this treatment. Effects of chemicals on *Lycopodium* show that colour changes are
656 only caused by acetolysis or HCl treatment (Sengupta, 1975). On the other hand, it has been
657 shown that acetolysis or oxidation selectively destroys the cysts of the Polykrikaceae and
658 Protoperidiniaceae (Reid, 1977; Marret, 1993). KOH treatment causes destruction of the
659 Protoperidiniaceae after five minutes (de Vernal et al., 1996, and Mertens, pers. observations)
660 and causes swelling of the palynomorphs. Likewise, methods using H_2O_2 (Riding et al., 2007)
661 result in the destruction of protoperidiniacean cysts (Riding, pers. comm., Hopkins and
662 McCarthy, 2002; Mertens, pers. obs.). This has also been demonstrated for Late Cretaceous
663 peridinioid dinoflagellate cysts (Schrank, 1988). Oxidation with Schulze’s solution by
664 Laboratory 22b resulted in the nearly complete destruction of the RBC, SBC and other
665 heterotrophs in all samples, and led to the relative enrichment of resistant pollen and reworked
666 non-peridinioid dinoflagellate cysts. Cold HF and HCl have never been reported to destroy
667 dinoflagellate cysts. However, hot rinses with HCl after the HF treatment were particularly
668 harmful to recent peridinoid cysts (Dale, 1976). Palynomorphs treated with warm HF clearly
669 showed traces of deterioration: destruction of delicate structures with fragmentation along
670 sutures and changes in wall texture with a thickening of the robust structures (Plate I, 11, 16,,
671 Plate III, 6). It can be concluded that chemical degradation is minimized when only cold
672 hydrochloric and hydrofluoric acid are used.
673 5) ”Sonication causes no mechanical degradation of the pollen and spores or dinoflagellate
674 cysts.”
675 The extensive use of ultrasound will not harm any dinoflagellate cysts according to
676 Funkhouser and Evitt (1959), however, other authors report differential damage (e.g.

Hodgkinson, 1991). This has not yet been checked in a quantitative manner for dinoflagellate cysts. The use of a sonic oscillator, although dependent of frequency (Marceau, 1969) is extremely damaging: the sonication by Laboratory 8 resulted in the destruction of RBC and SBC in the Benguela sample (Plate IV, 20). An ultrasonic bath appears relatively harmless, unless used for too long, i.e. more than 6 minutes. Laboratory 18a used an ultrasonic bath for 30 minutes, and this resulted in extensive damage to the cysts. Many cysts were fragmented, often with broken or even lost spines and were often clustered (Plate I, 17, Plate III,, 13, Plate IV, 11). In addition microforaminiferal linings were often fragmented.

6) ”Centrifuging causes no mechanical degradation of the palynomorphs.”

No visible signs were noted that this technique causes degradation of the cysts.

7)”Sieving causes no loss of palynomorphs.”

Lignum et al. (2008) demonstrated that sieving should be done with a sieve mesh widthsmaller than 15 µm. Our results confirm this observation. Laboratories using nylon sieve with widths of 20 µm (Laboratory 1a, 1c) showed extremely high absolute abundances. This suggests that significant losses of *Lycopodium* spores occurred during the sieving process - even larger than the 20% that is proposed by Lignum et al. (2008). No significant loss of cysts was documented in this study.It is possible that cysts of *Pentaspharsodinium dalei* pass through 20 µm sieves, this species was present in such low abundances in the studied samples to significantly affect relative or absolute abundances.

8) ”Decantation causes no loss of palynomorphs.”

An experiment was done to determine how many *Lycopodium* spores were lost during decanting and sieving. One gram of the NW Africa sample together with one *Lycopodium* tablet, was processed with a HCl/HF/HCl cycle, followed by sieving on a nylon mesh of 10 µm. After every decantation, the decanted fluid was filtered through a 0.25 µm Millepore filter. What remained on the filter was counted under a transmitted light microscope. Only

Lycopodium spores were left on the filters, next to some amorphous organic matter (Table 5). The number of spores will be dependent of the size of the filter used. Apparently 24% of the *Lycopodium* spores were lost during decanting. This is not surprising, since it is a well-known fact that *Lycopodium* spores have very good floating capacity (e.g. Salter et al., 2002). An extra 1.3% was left on the filter and 1% got stuck to handling material (spatula, tube). In the slides only 43.4% of the *Lycopodium* spores were found. An additional 30.2% spores were unaccounted for, and could have been lost during sieving and/or could have been obscured in the slides to some extent. Because we did not expect any significant losses to occur during sieving, we did not capture sieved material during this experiment. However, we tested sieving a complete *Lycopodium* tablet on 10 µm and capturing on a 0.25 µm sieve. We found losses to be 0.79% when gently pouring the dissolved tablet over the sieve and subsequent washing, 0.97% when using a hand pump to facilitate sieving and 2.01% when using a pipette tip. Lignum et al. (2006) recorded losses up to 5.8 ± 1.2 % for 15 µm meshes. It can thus be assumed that only a small part of the missing spores were pushed through the 10 µm nylon sieve. Presumably, by being obscured, spores are often concealed and this plays a more significant role in explaining the missing amount of spores. Also, it is possible that due to the texture of the exines of *Lycopodium* spores, the spores get more easily caught in the sieves than smoother palynomorphs. However, this loss can be easily checked by the observer.

9)”Pre-sieving causes no losses.”

It is unclear to what extent presieving causes loss of *Lycopodium* spores, although it is evident that it should be avoided in samples from high productivity areas, where high production of amorphous organic matter forms large clusters in the sediment, which can be discarded with the large fraction. However; it can be easily checked whether *Lycopodium* spores were lost.

10) Heavy liquid separation causes no loss of *Lycopodium* spores. It has been noted that density separation with heavy liquids, can cause incorporation of mineral particles modifying the density (de Vernal et al., 1996). Litwin & Traverse (1989) recommend pyrite to be removed prior to density separation. The results of this study do not show any obvious difficulties with this processing step, although for definite clarity further study is suggested.

From these considerations it can be concluded that a significant amount of *Lycopodium* spores are lost, mainly during decanting and sieving. There is little evidence that there is loss of dinoflagellate cysts during these manipulations.

4.5. Recommendations

The exercise demonstrated that relative abundances are reproducible, but underlined the urgent need for a taxonomic intercalibration process. Counting 300 dinoflagellate cysts is sufficient both in terms of diversity and absolute abundances. Absolute abundance calculations of dinoflagellate cysts are dependent on processing methodology, since *Lycopodium* spores are being lost during different processing steps. In this respect, there are three possible choices the Quaternary worker can make:

Standardize methodology for the extraction of dinoflagellate cysts.

Since samples can be reproducible when one fixed methodology is followed (see 4.3), a standard methodology is suggested (Figure 1). We consider that there are critical steps that must be avoided in this standard method when preparing samples for dinoflagellate cyst work: the use of oxidation, KOH, warm acids, acetolysis, mesh sizes larger than 15 µm, decanting (substituted by sieving) and sonication longer than 1 minute. During sieving, care should be

taken to avoid *Lycopodium* spores being forced through the sieve. A certain degree of freedom is allowed in the number of HCl and HF cycles, length of ultrasonication (0-60 seconds), duration of sieving and sieve mesh size (6-14 µm), Care should be taken to neutralize HF by diluting at least ten times before sieving. Further studies are required to fine-tune the method by focusing on designated issues.

Adding *Lycopodium* tablets at the end of processing.

The marker grain method is based on the assumption that there is no selective loss of fossil and exotic pollen during the procedures. However, this assumption has never been checked. Our study suggests that predominantly *Lycopodium* spores are lost, and that losses of dinoflagellate cysts are negligible. Therefore the addition of *Lycopodium* tablets at the end of the preparation could be suggested, thus severely limiting the loss of *Lycopodium* spores. However, this goes against the well-accepted idea of spiking with an internal standard before the start of preparation.

Alternative methods.

There are alternative methods that can be used, but it is unknown if these methods yield better results. The use of microbeads was introduced by Ogden (1986), but often results in much higher abundance estimates, apparently because of difficulty in sustaining an even suspension of the particles in the stock solution: the higher specific gravity of microspheres causes them to settle three to four times more rapidly than pollen grains (McCarthy, 1992). Other marker-grain methods, such as the *Eucalyptus globulus* marker-grain method (Matthews, 1969), has also been used (e.g. de Vernal et al., 1987). However, it is not known

whether these methods give more reliable results. The aliquot method gives more accurate results than the *Lycopodium* method in our study, but unfortunately not much is known about the precision of this method.

5. Conclusions

Based on an interlaboratory comparison, this study proposes a new standard method to determine absolute abundances of dinoflagellate cysts. Alternatively, *Lycopodium* tablets can be added at the end of the preparation or an alternative method (e.g. microbeads or aliquot method) can be used.

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947 Appendix 1: Species list

948

Species name	***Grouped under***	***North Sea***	***Celtic Sea***	***NW Africa***	***Benguela***
Achomosphaera andalousiensis Jan du Chêne 1977	*Spiniferites* s.l.	x	x	x	
Cysts of *Alexandrium affine* (Ioue & Fukuyo 1985) Balech 1985	Cyst of *Alexandrium* spp.		x		x
Cysts of *Alexandrium tamarense* (Lebour 1925) Balech 1985	Cyst of *Alexandrium* spp.	x	x		
Ataxiodinium choane Reid 1974	*Ataxiodinium choane*	x	x	x	
Bitectatodinium spongium Zonneveld 1997	*Bitectatodinium* spp.		x	x	x
Bitectatodinium tepikiense Wilson 1973	*Bitectatodinium* spp.	x	x	x	x
Tectatodinium pellitum Wall, 1967 emend. Head 1994	*Tectatodinium* spp.				x
cf. Tectatodinium pellitum Wall, 1967 emend. Head 1994	*Tectatodinium* spp.	x			
Brigantedinium cariacoense (Wall 1967) Lentin and Williams 1993	Round Brown Cyst	x	x	x	x
Brigantedinium majusculum Reid 1977 ex Lentin and Williams 1993	Round Brown Cyst	x	x		
Brigantedinium simplex Wall 1965 ex Lentin and Williams 1993	Round Brown Cyst	x	x	x	x
Cyst of Protoperidinium americanum (Gran & Braarud 1935) Balech 1974	Round Brown Cyst	x	x	x	x
Dalella chathamense McMinn & Sun 1994	*Dalella chathamense*				x
Diplopelta? symmetrica Pavillard 1993 (Dale et al. 1993)	Spiny Brown Cysts			x	
Dubridinium ulsterum Reid 1977	Round Brown Cyst	x		x	x
Dubridinium caperatum Reid 1977	Round Brown Cyst	x	x	x	x
Echinidinium aculeatum Zonneveld 1997	Spiny Brown Cysts	x	x	x	x
Echinidinium bispiniformum Zonneveld 1997	Spiny Brown Cysts			x	x
Echinidinium delicatum Zonneveld 1997	Spiny Brown Cysts	x	x	x	x
Echinidinium granulatum Zonneveld 1997	Spiny Brown Cysts	x	x	x	x
Echinidinium transparantum Zonneveld 1997	Spiny Brown Cysts	x		x	x
Echinidinium cf. *transparantum* Zonneveld 1997	Spiny Brown Cysts	x	x		x
Cyst of *Gymnodinium catenatum* Graham 1943	Cyst of *Gymnodinium* spp.	x	x	x	x
Cyst of *Gymnodinium microreticulatum* Bolch et al. 1999	Cyst of *Gymnodinium* spp.	x	x		
Cyst of *Gymnodinium nolleri* Ellegaard & Moestrup 1999	Cyst of *Gymnodinium* spp.	x	x	x	x
Impagidinium aculeatum (Wall 1967) Lentin and Williams 1981	*Impagidinium* spp.		x		
Impagidinium pallidum Bujak 1984	*Impagidinium* spp.		x		
Impagidinium paradoxum (Wall 1967) Stover and Evitt 1978	*Impagidinium* spp.	x	x		x
Impagidinium patulum (Wall 1967) Stover and Evitt 1978	*Impagidinium* spp.	x	x	x	
Impagidinium sphaericum (Wall 1967) Lentin and Williams 1981	*Impagidinium* spp.	x	x		x
Impagidinium strialatum (Wall 1967) Stover and Evitt 1978	*Impagidinium* spp.				x
Impagidinium velorum Bujak 1984	*Impagidinium* spp.	x		x	
Islandinium? cezare de Vernal et al. 1989 ex de Vernal in Rochon et al. 1999	Spiny Brown Cysts	x			
Islandinium minutum Harland and Reid in Harland et al. 1980	Spiny Brown Cysts	x	x	x	x
Leipokatium invisitatum Bradford 1975	*Lejeunecysta* s.l.		x		
Lejeunecysta diversiforma (Bradford 1977) Artzner and Dörhöfer 1978	*Lejeunecysta* s.l.				x
Lejeunecysta mariae Harland in Harland et al. 1991 ex Lentin and Williams 1993	*Lejeunecysta* s.l.	x			
Lejeunecysta oliva (Reid 1977) Turon and Londeix 1988	*Lejeunecysta* s.l.	x	x	x	x
Lejeunecysta paratenella (Benedek 1972) Zonneveld & Marret xxx	*Lejeunecysta* s.l.	x	x		x
Lejeunecysta sabrina (Reid 1977) Bujak 1984	*Lejeunecysta* s.l.	x	x	x	x
Lingulodinium machaerophorum (Deflandre and Cookson 1955) Wall 1967	*Lingulodinium machaerophorum*	x	x	x	x
Nematosphaeropsis labyrinthus (Ostenfeld 1903) Reid 1974	*Nematosphaeropsis labyrinthus*	x	x	x	x
Operculodinium centrocarpum sensu Wall and Dale (1966)	*Operculodinium* s.l.	x	x	x	x
Operculodinium israelianum (Rossignol 1962) Wall 1967	*Operculodinium israelianum*	x	x	x	x
Operculodinium janduchenei Head et al. 1989	*Operculodinium* s.l.	x	x	x	x
Operculodinium sp. II? Marret, 1994	*Operculodinium* s.l.				x
Operculodinium sp. A of Vink (2000)	*Operculodinium* s.l.			x	
Cyst of *Pentaspharsodinium dalei* Indelicato & Loeblich III 1986	Cyst of *Pentaspharsodinium dalei*	x	x	x	x
Polykrikos kofoidii Chatton 1914	*Polykrikos* spp.	x	x	x	x
Polykrikos schwartzii Bütschli 1873	*Polykrikos* spp.	x	x	x	x
Polysphaeridium zoharyi (Rossignol 1962) Bujak et al. 1980	*Polysphaeridium zoharyi*	x	x	x	x
Pyxidinopsis reticulata (McMinn & Sun 1994) Marret & de Vernal 1997	*Pyxidinopsis reticulata*	x			
Quinquecuspis concreta (Reid, 1977) Harland, 1977	*Quinquecuspis concreta*	x	x	x	x
Selenopemphix crenata Matsuoka and Bujak, 1988	*Selenopemphix* s.l.				x
Selenopemphix nephroides Benedek 1972; emend. Bujak in Bujak et al., 1980;	*Selenopemphix* s.l.	x	x	x	x
Cyst of Protoperidinium nudum (Meunier 1919) Balech 1974	*Selenopemphix* s.l.	x	x	x	x
Selenopemphix quanta (Bradford 1975) Matsuoka 1985	*Selenopemphix* s.l.	x	x	x	
Spiniferites belerius Reid 1974	*Spiniferites* s.l.	x	x	x	x
Spiniferites bentorii (Rossignol 1964) Wall and Dale 1970	*Spiniferites* s.l.	x	x	x	x
Spiniferites bulloideus (Deflandre & Cookson 1955) Sarjeant 1970	*Spiniferites* s.l.	x	x		x
Spiniferites delicatus Reid 1974	*Spiniferites* s.l.	x	x	x	x
Spiniferites elongatus Reid 1974	*Spiniferites* s.l.	x	x		x
Spiniferites elongatus Reid 1974	*Spiniferites* s.l.	x			
Spiniferites hyperacanthus (Deflandre and Cookson 1955) Cookson and Eisenack 1974	*Spiniferites* s.l.	x	x	x	x
Spiniferites lazus Reid 1974	*Spiniferites* s.l.	x	x		x
Spiniferites membranaceus (Rossignol 1964) Sarjeant 1970	*Spiniferites* s.l.	x	x	x	x
Spiniferites mirabilis (Rossignol 1964) Sarjeant 1970	*Spiniferites* s.l.	x	x	x	x
Spiniferites pachydermus Rossignol 1964	*Spiniferites* s.l.	x	x	x	
Spiniferites ramosus (Ehrenberg 1838) Loeblich and Loeblich 1966; emend. Davey and Williams 1966	*Spiniferites* s.l.	x	x	x	x
Stelladinium reidii Bradford 1975	*Stelladinium* spp.	x	x	x	
Stelladinium stellatum (Wall and Dale 1968) Reid 1977	*Stelladinium* spp.	x	x	x	x
Trinovantedinium applanatum (Bradford 1977) Bujak and Davies 1983	*Trinovantedinium applanatum*	x	x	x	x
Tuberculodinium vancampoae (Rossignol 1962) Wall 1967	*Tuberculodinium vancampoae*	x		x	x
Votadinium calvum Reid 1977	*Votadinium* spp.	x	x	x	x
Votadinium spinosum Reid 1977	*Votadinium* spp.	x	x		x
Xandarodinium xanthum Reid 1977	*Xandarodinium xanthum*	x	x	x	x

949

950

951 Appendix 2 : error calculation according to Stockmarr (1971)
952
953 According to Stockmarr (1971) total error is $e = \sqrt{e_1{}^2 + e_2{}^2 + e_3{}^2}$
954 where
955 e_1 = error on number of spores in marker tablets
956 $e_2 = \frac{\sqrt{cysts\ counted}}{cysts\ counted}$ = error on dinoflagellate cysts counted
957
958 $e_3 = \frac{\sqrt{spores\ counted}}{spores\ counted}$ = error on the number of spores counted
959

960 Figure captions
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962 Figure 1
963
964 Flow-chart of the proposed standardized method. AOM stands for amorphous organic matter.
965
966
967

968 Plate captions

969 Plate I : Cysts extracted from the North Sea sample using different methodologies. Labs are
970 sorted from high (upper left corner) to low abundances (lower right corner). (1) Lab 1a. (2)
971 Lab 20a. (3) Lab 13. (4) Lab 12. (5) Lab 19. (6) Lab 2. (7) Lab 11. (8) Lab 21a. (9) Lab 21b.
972 (10) Lab 22a. (11) Lab 10a. (12) Lab 18b. (13) Lab 1b. (14) Lab 16. (15) Lab17. (16) Lab
973 10b. (17) Lab 18a. (18) Lab 5. (19) Lab 4. (20) Lab 22b, oxidized. All scale bars are 20 μm.
974

975 Plate II : Cysts extracted from the Celtic Sea sample using different methodologies, sorted
976 from high absolute abundances (upper left corner) to low absolute abundances (lower right
977 corner) (1) Lab 14. (2) Lab 1a. (3) Lab 13. (4) Lab 3. (5) Lab 19. (6) Lab 12. (7) Lab 1b. (8)
978 Lab 15b. (9) Lab 1c. (10) Lab 21b. (11) Lab 21a. (12) Lab 11. (13) Lab 5. (14) Lab 4. (15)
979 Lab 16. (16). Lab 23. (17) Lab 17. (18) Lab 18a. (19) Lab 20a. (20) Lab 2. All scale bars are
980 20 μm.
981

982 Plate III : *Lingulodinium machaerophorum* extracted from the NW Africa using different
983 methodologies, sorted from high (upper left corner) to low absolute abundances (lower right
984 corner). (1) Lab 11. (2) Lab 1a. (3) Lab 14. (4) Lab 13. (5) Lab 19. (6) Lab 10b. (7) Lab 21a.
985 (8) Lab 1b. (9) Lab 12. (10) Lab 17. (11) Lab 21b. (12) Lab 6. (13) Lab18a. (14) Lab 18b.
986 (15) Lab 1c. (16) Lab 15b. (17) Lab 22a. (18) Lab 4. (19) Lab 5. (20) Lab 20b. (21) Lab 16.
987 (22) Lab 8. (23). Lab 23. (24) Lab 3. All scale bars are 20 μm.
988

989 Plate IV : *Dubridinium* spp. extracted from the Benguela sample using different
990 methodologies, sorted from high (upper left corner) to low absolute abundances (lower right
991 corner). (1) Lab 1c. (2) Lab 3. (3) Lab 19. (4) Lab 11. (5) Lab 13. (6) Lab 1a. (7) Lab 21a. (8)
992 Lab 21b. (9) Lab 6. (10) Lab 16. (11) Lab 18a. (12) Lab 18b. (13) Lab 1b. (14) Lab 23. (15)
993 Lab 10b. (16) Lab 17. (17) Lab 10a. (18) Lab 5. (19) Lab 2. (20) Lab 8. Destructive
994 ultrasonication. All scale bars are 20 μm.
995

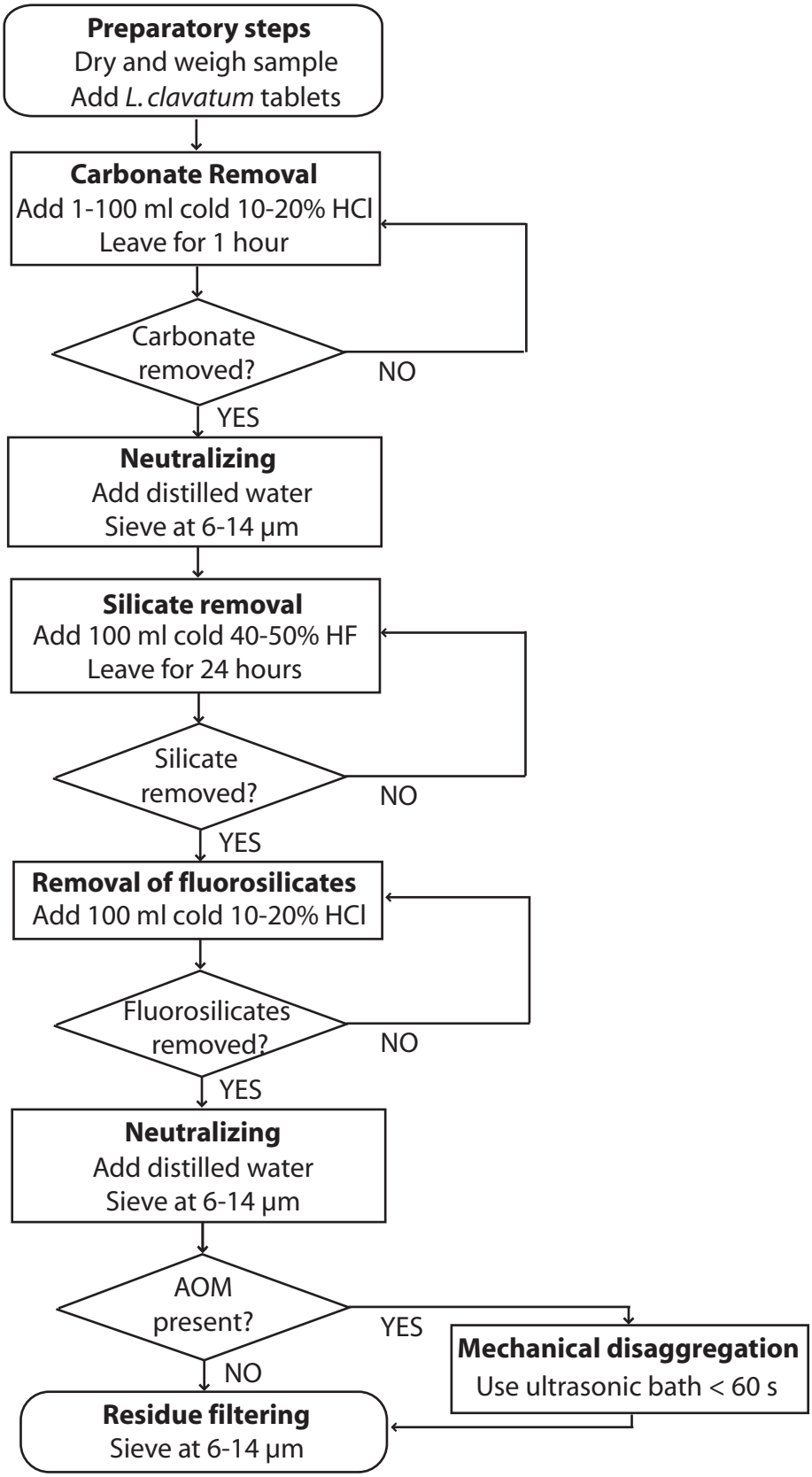

Preparatory steps
Dry and weigh sample
Add L. clavatum tablets
Carbonate Removal
Add 1-100 ml cold 10-20% HCl
Leave for 1 hour
Carbonate removed?
NO
YES
Neutralizing
Add distilled water
Sieve at 6-14 µm
Silicate removal
Add 100 ml cold 40-50% HF
Leave for 24 hours
Silicate removed?
NO
YES
Removal of fluorosilicates
Add 100 ml cold 10-20% HCl
Fluorosilicates removed?
NO
YES
Neutralizing
Add distilled water
Sieve at 6-14 µm
AOM present?
YES
NO
Mechanical disaggregation
Use ultrasonic bath < 60 s
Residue filtering
Sieve at 6-14 µm

Table captions

Table 1: Description of the samples.

Table 2: Average percentages of the four samples for the different taxa.

Table 3: Comparison between the marker-grain method and the volumetric method.

Table 4: Comparison between the average results after counting 300 dinoflagellate cysts, and counting 400 dinoflagellate cysts.

Table 5: The results of the counts of samples processed and counted by Lab 21, processed with one processing technique. According to the statistical test by Maher (1981), the results are reproducible.

Table 6: Results of an experiment to look into the effects of manipulations on loss of *Lycopodium* spores. Shown is the number of *Lycopodium* spores lost during each manipulation. It is supposed that one tablet contains 18583 spores, so the % is calculated by dividing the number of counted spores by 18583 spores.

Tables

Table 1

Sample	*Lithology*	*Dry weight (g)*	*Number of tablets added*	*# spores added*	*Stdev spores*
North Sea	Fine-medium sand	10	3	55749	2959
Celtic Sea	Fine silty sand	10	1	18583	1708
NW Africa	Clay	2	2	37166	2416
Benguela	Clay	1	4	74332	3417

Table 2

Species name	***North Sea***	***Celtic Sea***	***NW Africa***	***Benguela***
Round brown cysts (RBC)	35.8 ± 16.0	10.0 ± 7.7	3.4 ± 2.3	62.7 ± 17.0
Spiny brown cysts (SBC)	15.5 ± 12.5	1.7 ± 3.3	2.3 ± 2.4	8.5 ± 8.5
cysts of *Alexandrium* spp.	0.2 ± 0.3	0.5 ± 0.9	-	0.1 ± 0.5
cysts of *Gymnodinium* spp.	0.3 ± 0.6	0.3 ± 0.6	0.0 ± 0.1	0.0 ± 0.1
Stelladinium spp.	0.3 ± 0.3	0.2 ± 0.2	0.3 ± 0.3	0.1 ± 0.4
Lejeunecysta spp.	9.5 ± 12.0	1.5 ± 1.6	0.4 ± 0.5	1.4 ± 1.6
Selenopemphix spp.	5.5 ± 1.7	4.8 ± 2.1	1.0 ± 0.6	6.5 ± 6.3
Tuberculodinium vancampoae	0.0 ± 0.1	-	0.1 ± 0.3	0.0 ± 0.1
Polykrikos spp.	6.9 ± 3.5	5.7 ± 3.8	1.2 ± 0.8	1.1 ± 0.8
Xandarodinium xanthum	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.1
Dalella chathamense	-	-	-	0.0 ± 0.1
Extremely sensitive cysts (total)	74.3 ± 7.4	24.8 ± 11.2	15.4 ± 8.2	80.6 ± 9.9
Lingulodinium machaerophorum	1.5 ± 2.5	0.7 ± 0.9	86.2 ± 4.7	0.2 ± 0.5
Operculodinium spp.	2.8 ± 1.9	12.3 ± 3.7	0.5 ± 0.7	8.4 ± 6.6
Pyxidininopsis reticulata	0.0 ± 0.2	-	-	-
Spiniferites spp.	9.8 ± 3.5	51.8 ± 10.7	3.3 ± 1.1	5.5 ± 3.2
Quinquecuspis concreta	3.3 ± 2.1	2.3 ± 2.0	0.1 ± 0.1	1.0 ± 1.5
Trinovantedinium applanatum	0.2 ± 0.4	1.2 ± 1.0	0.2 ± 0.3	0.3 ± 0.4
Votadinium spp.	5.8 ± 6.6	0.5 ± 0.7	0.0 ± 0.1	0.7 ± 0.7
Moderately sensitive cysts (total)	23.6 ± 7.2	68.9 ± 10.7	90.3 ± 4.2	16.2 ± 9.7
Nematosphaeropsis labyrinthus	0.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	2.1 ± 2.0
Impagidinium spp.	0.3 ± 0.6	0.15 ± 0.3	0.0 ± 0.1	0.0 ± 0.1
Operculodinium israelianum	0.2 ± 0.2	0.0 ± 0.1	0.4 ± 0.7	0.4 ± 0.7
Pentapharsodinium dalei	0.4 ± 0.5	2.6 ± 3.5	0.0 ± 0.1	0.2 ± 0.5
Polysphaeridium zoharyi	0.4 ± 0.6	0.1 ± 0.3	0.1 ± 0.5	0.2 ± 0.7
Ataxiodinium choane	0.0 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	-
Bitectatodinium spp.	0.6 ± 1.1	3.3 ± 2.0	0.1 ± 0.2	0.2 ± 0.6
Resistant cysts (total)	0.5 ± 0.6	6.2 ± 3.8	0.7 ± 0.9	3.1 ± 2.5

Table 3

Method	*Variable / sample*	*North Sea*	*Celtic sea*	*NW Africa*	*Benguela*
Marker grain method	Average (cysts/g)	1516	2583	19441	144299
	St dev (cysts/g)	698	1342	9148	84159
	Coefficient of variation (%)	46	52	47	58
	Coefficient of variation (%) Maher (1981)	20	25	23	21
Volumetric method	Average (cysts/g)		1163	10796	53200
	St dev (cysts/g)		5	1137	0
	Coefficient of variation (%)		0	11	0
Difference	Cysts/g	-	1420	8645	91099
	%		55	44	63

Table 4

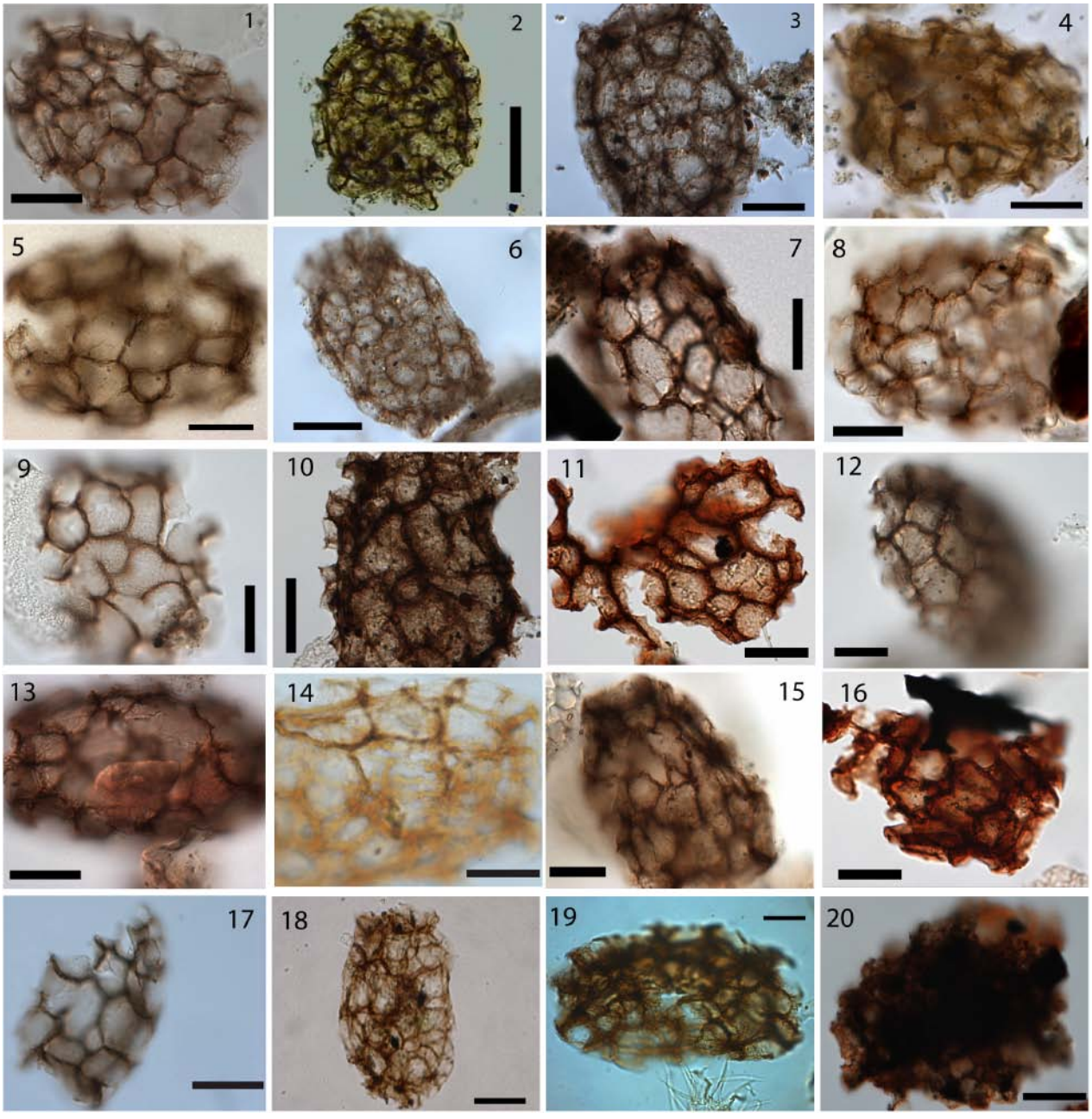
Variable / sample	*North Sea 300 cysts*	*North Sea 400 cysts*	*Celtic sea 300 cysts*	*Celtic sea 400 cysts*	*NW Africa 300 cysts*	*NW Africa 400 cysts*	*Benguela 300 cysts*	*Benguela 400 cysts*
Average (cysts/g)	1539	1546	2792	2670	33798	33684	141825	142612
Stdev	767	711	1474	1236	43286	42193	87324	88779
Coefficient of variation (%)	50	46	53	46	128	125	62	62
Species richness	22.00	22.85	24.26	25.26	14.75	16.50	19.13	20.22
Stdev	4.67	4.79	5.61	6.02	3.64	4.12	4.94	5.27
Shannon-Wiener index	2.25	2.25	2.29	2.29	0.70	0.72	1.94	**1.92**
Stdev	0.41	0.41	0.30	0.32	0.22	0.23	0.35	**0.33**

Table 5

Lab number	*Variable / sample*	*North Sea*	*Celtic sea*	*NW Africa*	*Benguela*
21a	Dinoflagellate cysts/g	1547	2581	27851	172078
	95% confidence limits (Maher, 1981)	1265-1885	2092-3327	21612-32060	138365-206955
21b	Dinoflagellate cysts/g	1447	2723	24929	170888
	95% confidence limits (Maher, 1981)	1166-1785	2117-3354	19294-28216	135585-200884

Table 6

	Counted *Lycopodium* spores	%
HCl treatment		
First decantation	916	4.9
Second decantation	267	1.4
Third decantation	2485	13.4
HF/HCl treatment		
First decantation	6	0.0
Second decantation	143	0.8
Third decantation	650	3.5
Left on filter (not washed off)	242	1.3
Left in tube + stuck on spatula	187	1.0
Found on slides	8067	43.4
Total	12963	69.8
Missing spores	5620	30.2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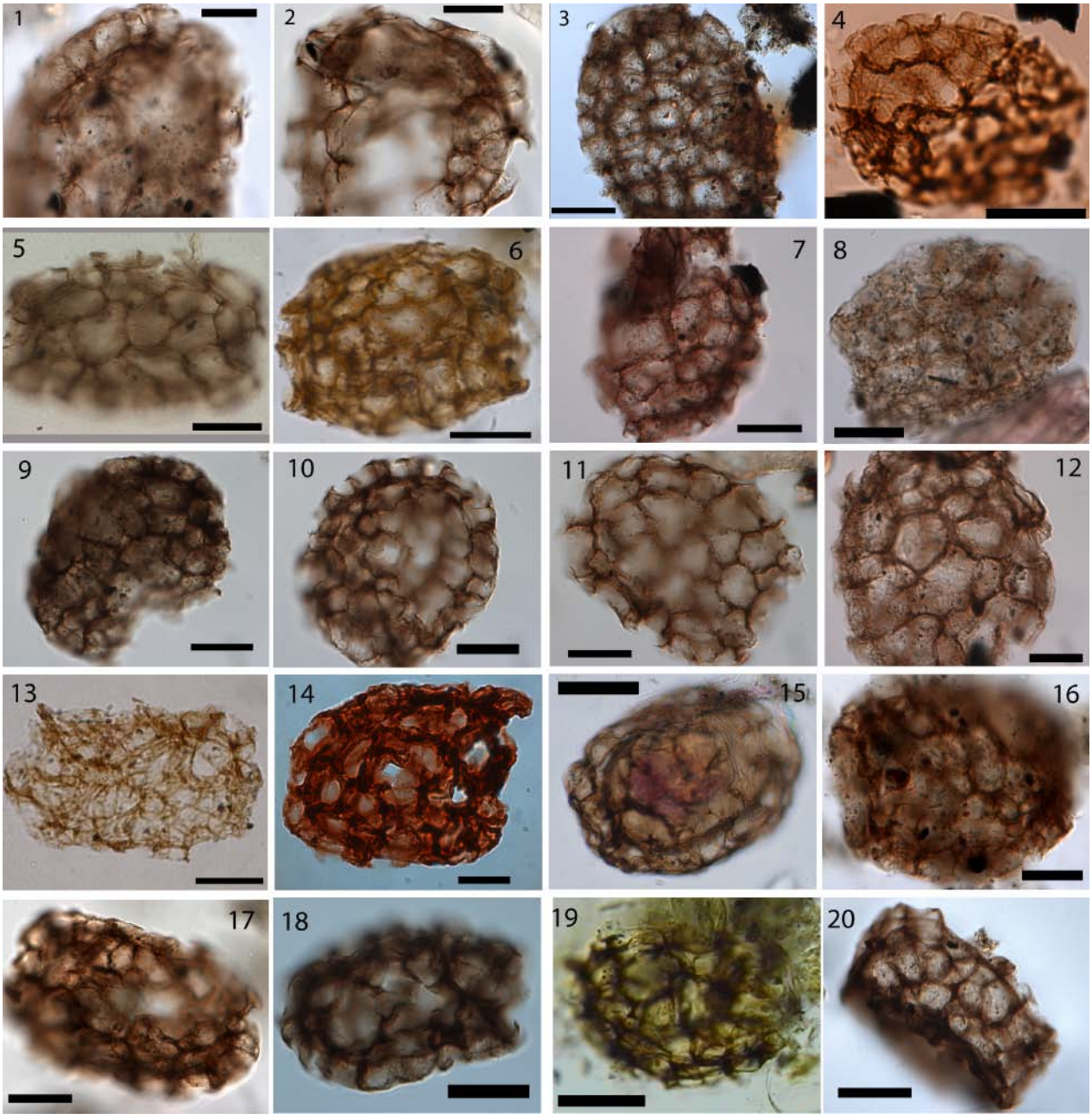
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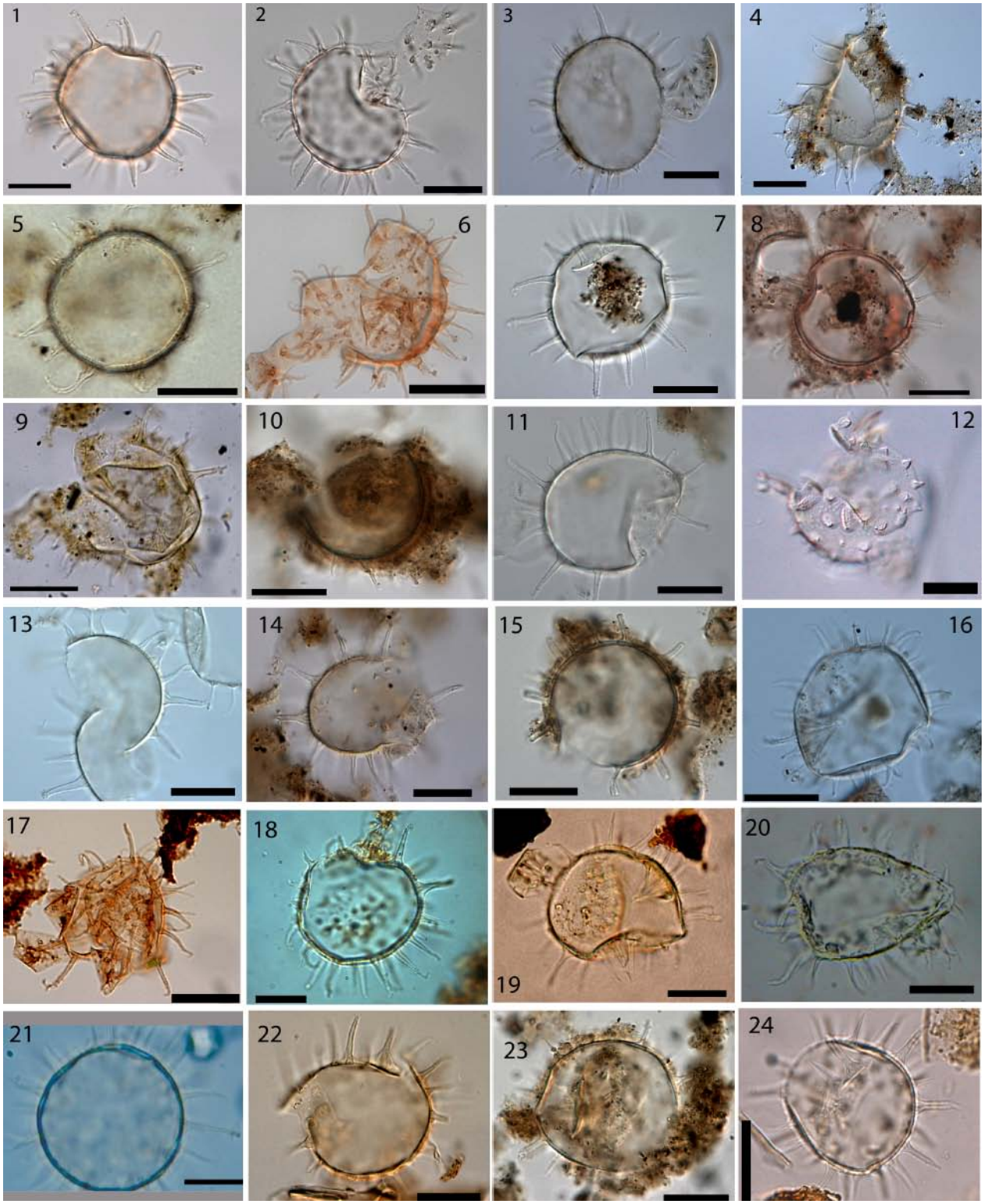
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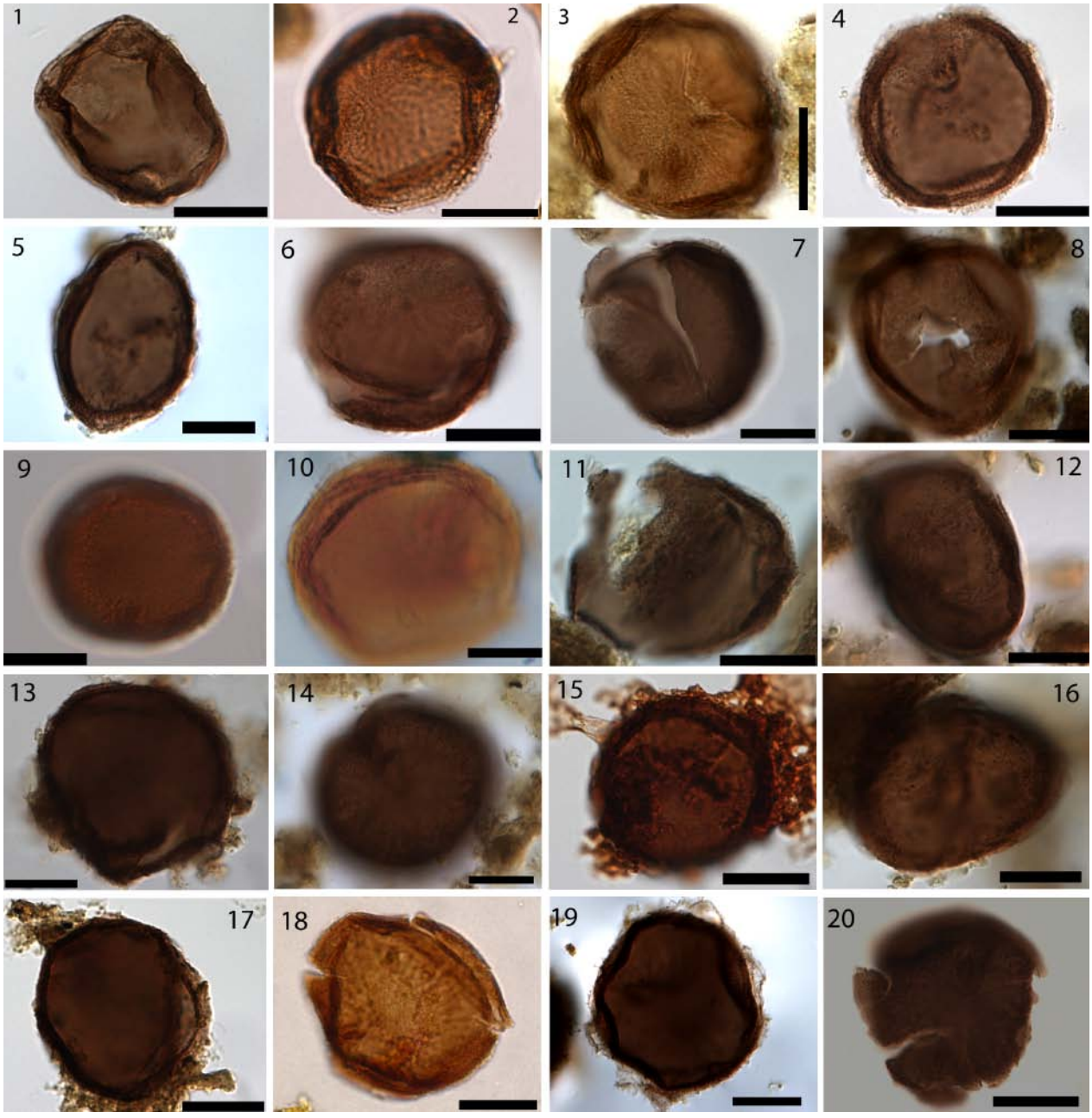
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