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Testing of palynological processing techniques: an example using Silurian palynomorphs from Gotland

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ABSTRACT – Well-preserved acritarchs and prasinophyte algae recovered from the Lower Wenlock of Gotland (Sweden) were used to test the effects of centrifuging in heavy liquid, treatment with nitric acid to remove pyrite, and the loss of material through a 7 μm sieve during washing of a sample. The centrifuge test showed that the relative proportions of different genera stabilized after three heavy liquid separations and the number of acritarchs extracted fell consistently by about 35% at each separation. Treatment with nitric acid yielded a slightly lower number of acritarchs per gram of sediment, but it appears to have yielded more herkomorph and sphaeromorph acritarchs. Treating samples with nitric acid renders them more comparable with those not containing pyrite. The loss of specimens whilst washing through a 7 μm sieve proved to be minimal. It was concluded that three heavy liquid separations should routinely be carried out, in conjunction with treatment with nitric acid and regular tests to examine material passing through a 7 μm sieve. *J. Micropalaeontol.* **21**(1): 81–86, May 2002.

INTRODUCTION

The foundation to any palynological research is processesing technique. The method must be reliable, consistent and accurate, so that biostratigraphical or palaeoecological data are meaningful. Although the processing method is often specifically tailored to the sample being analysed and the equipment available, it is important to maintain some constant factors and to quantify the losses experienced. Consistency of processing technique is the most important factor, so that samples can be compared.

Several publications outline techniques used in extracting palynomorphs from rock. These reviews include Funkhouser & Evitt (1959), Gray (1965a, b), Barss & Williams (1973), Forster & Flenley (1989), Litwin & Traverse (1989) and Wood *et al.* (1996). Colbath (1985) is the only author who used Silurian material to compare some of the extraction techniques available.

This paper focuses on the errors that may be introduced during the extraction of palynomorphs from rock. The palynomorph assemblages from the Lower Wenlock of Gotland (Sweden) provide a large number of species and relatively high numbers of palynomorphs per gram of sediment, with a very low thermal alteration and only occasional pyrite growth. These factors mean that the material is ideally suited to the testing of processing procedures.

The small size of palynomorphs and the techniques used for their removal from the mineral fraction mean that specimens are likely to be lost or gained (as contamination) during the processing of the samples. Specimens may be lost when the organic residue is separated from the mineral fraction using centrifuge separation with sodium polytungstate or other heavy liquid. Pyrite present in the fossils will increase the mass of the specimens, and the mineral residue may clump around specimens (individuals with numerous processes may act as debris traps). Specimens may be lost through the sieve when washing the sample (regular tests can monitor any loss). A 7 μm sieve was used because processing at 5 μm is not time-effective. General human errors will also provide a source of loss, as some acritarchs may remain on the surface of equipment through inadequate recovery. Errors may also arise in the recording of data. A sample may gain specimens if it incorporates acritarchs that remain on the surface of equipment from the processing of previous samples.

Pyrite is present in the samples collected from Gotland. The pyrite grains slightly distort the specimens and may disguise some of their diagnostic features, such as plugged processes. This is not usually a problem, however, as the specimens are not totally enveloped in pyrite and can be identified accurately (Fig. 1a, b). The main problem created by pyrite is encountered during the separation process. Separation by heavy liquid depends upon the different densities of the acritarchs and the mineral portion of the sample. Any specimens with an increased mass due to the presence of pyrite (specific gravity 4.95–5.35) are, therefore, more likely to be forced into the lower part of the centrifuge tubes along with the mineral fraction. Even after treatment with nitric acid some specimens (especially those with large cavities such as *Schismatosphaeridium* (Fig. 1b)) still contain pyrite grains. This may be due to the grains being protected from the nitric acid by the acritarch vesicle, or possibly insufficient time in the acid, or insufficient acid concentration. The presence of these specimens in the slides shows that a number were not pulled down with the mineral fraction; this may be due to their large relative size compared to their weight. Problems may result from any variations of pyrite content from sample to sample, with more specimens possibly lost in pyrite-rich horizons.

The purpose of the processing technique is to provide samples where variations of species abundance and diversity, and numbers of palynomorphs per gram of sediment can be recorded. The processing should reflect these requirements and should aim to give the most accurate representations of the true values. It is important that the processing technique should be such as to minimize any artificial errors or biases in these observations.

Processing technique

- Wash and scrub the sample to remove lichen, fungi and other recent contaminants.

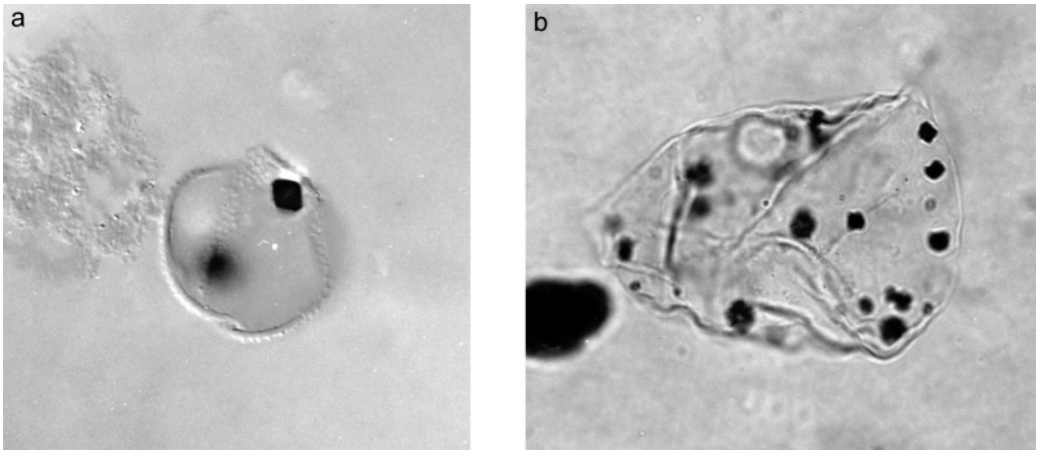

Fig. 1. (**a**) *Dictyotidium alveolatum* (Shultz) ×750, DG00LK1.259, 1 (S29, 3). Showing two large pyrite grains in the vesicle, prior to nitric acid treatment. (**b**) *Shizmatosphaeridium algerense* (Eisenack, Cramer & Díez) ×750, DG00LK1.259, 1 (P28). Showing typical extent of pyritization prior to nitric acid treatment. The specimens are held in the repository at the Department of Geology, University of Leicester, accession number LEIUG 121390.

- Dry the sample in an oven to remove all moisture.
- Crush the sample to pea-sized fragments.
- Weigh sample for processing (40 g) and put in to a polypropylene container with a screw top lid.
- Add HCl (32%) to remove the carbonate minerals, then decant three times to remove Ca and Mg.
- Add HF (40%) to the sample to remove the silicate mineral.
- Once the mineral portion of the sample has broken down, the acids are diluted with water until the sample bottle is full. When the mineral and fossil material has settled to the bottom of the bottle pour off the dilute acid, whilst ensuring no sample is lost. Repeatedly dilute the acid and pour off until the sample is neutralized. This needs to be carried out after each acid stage.
- Separate the sample by centrifuge at 2400 rpm for 14 minutes to remove the water.
- Pour the water off the sample.
- Mix the sample in a 50 ml centrifuge tube with 7% nitric acid to the 30 ml mark and leave for 10 minutes at room temperature to remove the pyrite.
- Concentrate the sample by centrifuging at 2400 rpm for 14 minutes.
- Pour the nitric acid off the sample.
- Mix the sample with sodium polytungstate (S.G. 2.0) to the 30 ml mark on the centrifuge tubes and separate off the organic residue by centrifuging for 14 minutes at 2400 rpm.
- Remove the organic residue from the sample by pipetting or pouring off the topmost sodium polytungstate into a 7 μm nylon square mesh sieve.
- Wash the residue with 1.5 l of deionized water and pipette into a 5 ml container. Then, from the thoroughly mixed residue, remove three portions of 0.05 ml and place onto 22 × 22 mm cover-slips, adding a few drops of cellosize to disperse the palynomorphs.
- Pass the remaining residue through a 53 μm sieve to remove the chitinozoans and large acritarchs, and then a 7 μm sieve, and mount the 53 μm and 7 μm residues separately, adding a few drops of cellosize.
- At room temperature slowly evaporate the water from the cover-slips to avoid particle clumping.
- Mount the cover-slips onto glass slides using Petropoxy 154. A drop of Petropoxy is placed on the dry cover-slip, which is then overturned, slowly lowered onto the glass slide and heated to 120°C until the Petropoxy has set.
- Thoroughly wash the equipment before and after each use.

TESTING THE PROCESSING TECHNIQUES

Before the samples can be analysed for numbers of palynomorphs per gram of sediment and species diversity, it is important that the possible sources of error in the processing technique are minimized and quantified. To this end a series of tests was carried out on three samples, DG00LK1.258, 257 and 255.

- To test for the effectiveness in removing acritarchs from the mineral residue at each separation by heavy liquid, and for any species biases in these separations, the sample DG00LK1.258 was separated by centrifuging at 2400 rpm for 14 minutes in sodium polytungstate. The residue was pipetted off and the resulting acritarchs sieved, mounted and logged. This provided a record of the numbers of palynomorphs recovered per gram of sediment after the first separation and the number and type of species that were seen at this stage. To quantify the number of acritarchs not recovered during the first separation, the dense residue at the bottom of the centrifuge tube was again mixed with sodium polytungstate and re-separated. The material removed from the second separation was logged in the same way as the first. This was repeated a further four times.
- To test the impact of the use of nitric acid for the removal of pyrite on the final assemblage composition, the sample DG001.257 was prepared using nitric acid before it was separated by centrifuging. The resulting slides were then logged for numbers of palynomorphs per gram of sediment and number of individuals of each species present. As a control, a further portion of the DG00LK1.257 was then

processed using the same technique, but without nitric acid preparation and logged in the same manner. The results for the two samples were compared to quantify the effectiveness of the nitric acid treatment.

- To test the risk of loss of material through the 7 μm sieve, the fraction of sample DG00LK1.255 (along with samples 258 and 257) less than 7 μm was mounted and analysed. This was carried out by saving the washings from the 7 μm sieve and leaving them to settle in a sample bottle so that the water and sodium polytungstate could be poured off. The remaining material was then mounted onto cover-slips. This enabled the acritarchs penetrating the sieve mesh to be counted and analysed for preferential removal of particular species.

RESULTS OF REPEAT SEPARATION

To obtain meaningful results the following questions must be answered: does the apparent diversity of a sample change between repeat separations? Is there a consistent gradual increase in the numbers of acritarchs per gram of sediment calculated, or are there an optimum number of separations after which the change in number of acritarchs per gram of sediment calculated becomes negligible? At first, sample DG00LK1.258 was separated three times, but the results proved inconclusive. To test that a consistent and representative sample was present when three separations had been carried out, it was decided to repeat the test, but mounting the recovered residues for six separations.

The number of acritarchs per gram of sediment was calculated by reducing the volume of the organic residue from a sample of known weight (usually 40 g) to 5 ml. From this 5 ml three representative 0.05 ml portions were removed after thorough mixing. These 0.05 ml portions were mounted onto glass slides using the methods outlined above and the number of acritarchs in each was recorded and the average calculated. This average figure, which represents the number of acritarchs in 1% of the sample, was then multiplied by 100 and divided by the weight of the sample to give the number of acritarchs per gram of sediment.

The first separation of sample DG00LK1.258 yielded 2620 acritarchs per gram of sediment, the second 1708, the third 1081, the fourth 733, the fifth 801 and the sixth 264 (Fig. 2). For separations 1 to 4 there was a drop of around 35% in each separation, the fifth separation has slightly more than the fourth, but the drop resumes in the sixth.

These data suggest that mixing of the mineral residue to release the acritarchs from the mineral is extremely important. After one separation the calculated number of acritarchs per gram is a considerable underestimate of the population in that sample. The cumulative addition of the residues from repeated separations reduces this error. The mixing between separations should re-suspend as much of the mineral matter as possible back into the sodium polytungstate, even though it can be difficult to disperse the lumps of sample.

The assemblage seen in sample DG00LK1.258 is dominated by *Micrhystridium stellatum* at an average across the six separations of 24.2%, with 11.38% *Veryhachium trispinosum*, *Leoisphaeridia* (small (<29 m) thin walled) at 8.6% and *Diexallophasis gotlandica* at 7.38%.

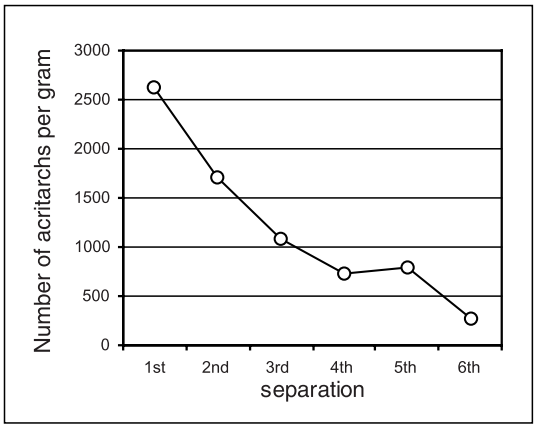

Fig. 2. Total number of palynomorphs per gram of sediment recorded in each separation procedure when carried out 1–6 times, sample DGOOLK1.258.

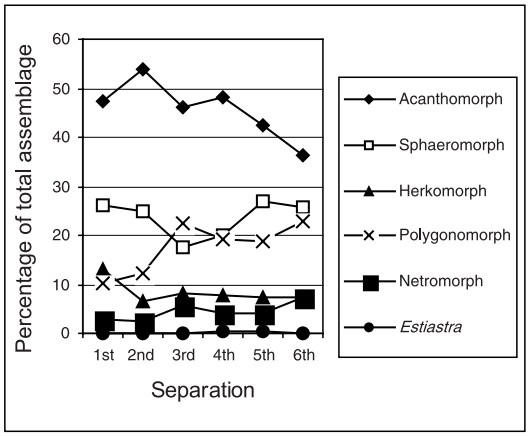

Fig. 3. Percentage abundance of each acritarch subgroup recorded in each of the six separations, sample DGOOLK1.258.

The relative proportion of each acritarch subgroup (subgroups of Downie *et al.,* 1963) changes by only a small amount between the first separation and the sixth (Fig. 3). The variation is greatest in the acanthomorph group which, at its highest, makes up 53.9% of the assemblage, in the second separation, falling to a low of 36.3% in the sixth. The sphaeromorph acritarchs begin and end the test at around 25% of the whole assemblage, but drop to 17.4% and 20.1% at the third and fourth separations respectively. The polygonomorph acritarchs appear to reach a plateau at around 22% of the whole assemblage in the third to sixth separation, after a rise from 10.4% in the first separation to 12.1% in the second. The herkomorph prasinophytes vary little after their drop from 13.2% to 6.4% from the first to the second separation, and the netromorphs maintain a gradual rise from 2.8% of the assemblage in the first separation to 7.4% in the sixth. The rarer '*Estiastra* subgroup' shows almost no variation.

The relative frequencies of genera (Fig. 4) have also been plotted, ignoring those taxa which fall below 2% of the total assemblage. The <2% data plot as noise and patterns are difficult to distinguish.

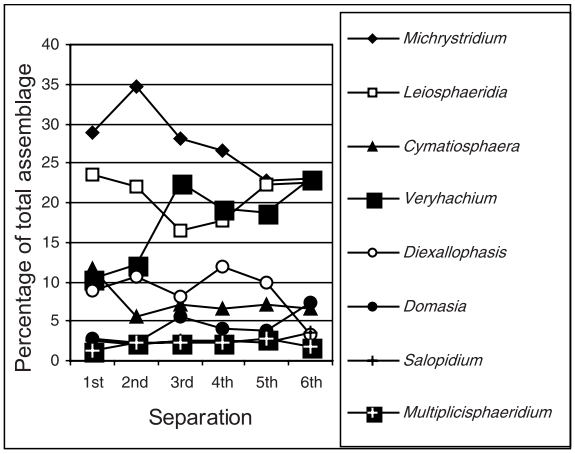

Fig. 4. Percentage abundance of each acritarch genus recorded in each of the six separations, sample DGOOLK1.258.

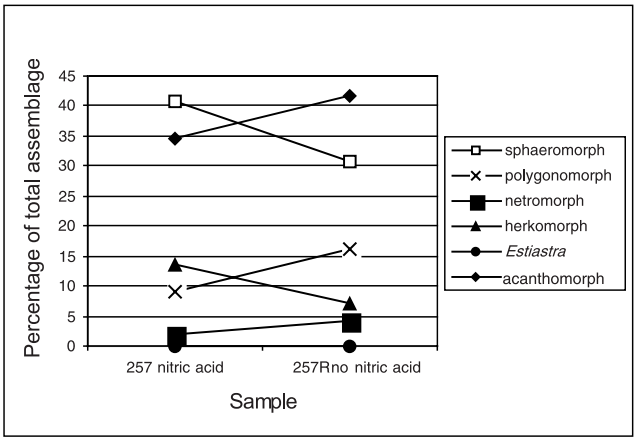

Fig. 5. Percentage abundance of each acritarch subgroup recorded in sample DGOOLK1.257 when the sample was treated with nitric acid and when nitric acid was not used during processing.

The relative frequencies of *Micrhystridium*, *Leiosphaeridia* and *Veryhachium* show very similar plots to those seen for the acanthomorphs, the sphaeromorphs and the polygonomorphs respectively. This is not surprising as they are the dominant species in these subgroups. However, *Diexallophasis* appears to maintain a near constant percentage abundance until it falls from 9.8% in the fifth separation to 3.4% in the sixth. *Cymatiosphaera* maintains a level of around 7% of the assemblage, after a fall from 11.6% recorded in the first separation. *Domasia*, *Salopidium*, and *Multiplicisphaeridium* vary little from separation to separation, with *Domasia* only rising from 2.8% to 3.4% and *Salopidium* and *Multiplicisphaeridium* maintaining a percentage of around 2.6%.

Very few species present in the population are not represented in the first three fractions (six very rare species recorded in this test). The main drawback of the heavy liquid separation procedure is that all the acritarchs in the sample are not removed by the third separation. A more accurate representation of numbers of acritarchs per gram of sediment would be achieved if separations 1–6 were put together, but this increase in accuracy does not justify the extra time needed to complete six separations. The rarer species found when the sample was separated six times rather than three are also probably not significant enough to justify the extra time involved.

The recovery of acritarchs does not seem to be affected by any clumping of mineral matter around spinose genera, as the acanthmorph subgroup (Fig. 3) actually shows a decrease in percentage removal as more separations are carried out and not an increase, which might be expected if they were mostly held in the mineral fraction during the initial separations.

RESULTS FROM NITRIC ACID TESTING

Sample DG00LK1.257 was treated with 7% nitric acid for ten minutes. Only 7% nitric acid was used to prevent the sample from being unnecessarily over-oxidized. A repeat sample of DG00LK1.257 was processed in an identical way, but without the nitric acid treatment. It is essential the sample is thoroughly mixed with the nitric acid, as lumps of mineral clustered around any palynomorphs may protect the pyrite from attack. Samples not mixed properly may expose fewer pyrite grains to digestion and yield fewer palynomorphs. It is estimated that treatment with 7% nitric acid removed 30–40% of the observed pyrite.

The sample treated with nitric acid yielded 4442 acritarchs per gram of sediment and the repeat sample (that had not undergone treatment with nitric acid) yielded 5325 acritarchs per gram of sediment. The yield of acritarchs in the sample treated with nitric acid is lower. The difference may be due to the nitric acid destroying or fragmenting the palynomorphs that contain pyrite while the pyrite is being digested. If the acritarch contains a split allowing the nitric acid to flow inside the vesicle, the reaction of the nitric acid with the pyrite may be violent enough to break up the vesicle. When calculating numbers of acritarchs per gram of sediment, specimens were only counted if more than half of the individual is preserved. Incomplete specimens, broken up by the reaction between nitric acid and pyrite would, therefore, not increase the number of palynomorphs per gram of sediment calculated and could possibly decrease it if the broken specimens form fragments that are all less than half the original size.

If treatment with nitric acid has no effect, the acritarch subgroups should have a near-identical percentage abundance in both the treated and un-treated samples. This is not the case, with the sphaeromorphs reducing in frequency from 40.9% to 30.8% and the herkomorphs reducing from 13.7% to 7.0% between the sample treated with nitric acid and the sample not treated with nitric acid (Fig. 5). In contrast, the percentage abundance of acanthomorphs increases from 34.5% to 41.8% between the sample treated with nitric acid and the sample not treated with nitric acid, the polygonomorphs increase from 9.1% to 16.1% and the netromorphs from 2.1% to 4.4%. These differences may be due to natural variability, or to the destruction of some individuals in some subgroups by nitric acid. It is important to emphasize that the increase in the percentage abundance of acanthomorphs, polygonomorphs and netromorphs in the sample not treated with nitric acid may just be a reflection of the relative decrease in the number of sphaeromorphs and herkomorphs extracted.

To investigate preferential pyrite growth in specific morphologies, the percentage of individuals containing pyrite in each subgroup was recorded after three separations of sample DG00LK1.258. The sphaeromorphs and the acanthomorphs

contained the most pyrite, with 32.9% and 32.2% of the specimens showing its presence, 19.3% of the herkomorphs, 14.4% of the polygonomorphs and 1.2% of the '*Estiastra* subgroup' contained pyrite grains.

RESULTS OF 7 μm SIEVE TEST

The <7 μm fractions from samples DG00LK1.258, 257 and 255 were logged after three separations. Sample DG00LK1.258 yielded 30 acritarchs (0.7 <7 μm acritarchs per gram of sediment), of which 29 were *Leiosphaeridia* spp., with one *Veryhachium wenlockium*. DG00LK1.257 yielded 33 acritarchs (0.8 <7 μm acritarchs per gram of sediment), of which 24 were *Leiosphaeridia* spp., the remainder consisting of *Domasia trispinosa*, *Veryhachium trispinosum*, *Diexallophasis gotlandica*, *Micrhystridium stellatum* and *Cymatiosphaera ledburica*. DG00LK1.255 yielded 23 acritarchs (0.6 <7 μm acritarchs per gram of sediment), of which 12 were *Leiosphaeridia* spp., 5 were *Veryhachium wenlockium*, 3 were *Micrhystridium stellatum*, 2 were *Domasia trispinosa* and one was *Micrhystridium irevikensis*.

No species were identified that had not been recorded in the >7 μm fraction, indicating that the lost data have little influence on the final diversity results, and the loss in numbers of acritarchs is minimal. The acritarchs recorded are species that tend to be small and their size range would be likely to include <7 μm specimens smaller than 7 μm. DG00LK1.257 included specimens of *Diexallophasis gotlandica* and *Cymatiosphaera ledburica* which are larger than 7 μm; this is probably a result of contamination from the >7 μm fraction, or even from another sample.

DISCUSSION

The results from the repeat separation test show that the relative frequency of genera in the assemblages stabilizes by the third separation. The variation seen in the fourth, fifth and sixth separations is probably due to low numbers remaining after the first three separations. A number of acritarchs do seem to remain after the third separation, but this does not appear to have an effect on the number of species recorded from a sample. The total number of acritarchs per gram of sediment is affected by factors such as sedimentation rate, preservation potential and current activity, as well as phytoplankton abundance. The difficulty in obtaining any extra meaningful interpretations from the sample that was separated six times, suggests it is not time-effective to spend overly long periods obtaining and recording data. It can be concluded that carrying out three separations provides a sufficiently representative assemblage and further separations do not significantly add to the accuracy and value of results.

Treatment with nitric acid caused a decrease in the number of acritarchs recovered per gram of sediment. The results show that herkomorph and sphaeromorph acritarchs are recovered more successfully when the sample is treated with nitric acid. The data suggest that acanthomorph and polygonomorph acritarchs are recovered more successfully if the sample is not treated with nitric acid, but this may well fall within the statistical limits of natural variation. The acanthomorph acritarchs show a fairly high occurrence of pyrite growth, suggesting that nitric acid reacting with the pyrite may have a detrimental effect on the occurrence of this subgroup. The polygonomorph acritarchs, which have a low occurrence of pyrite growth, show a decreased yield when treated with nitric acid.

From this test it can be concluded that treatment with nitric acid probably had little detrimental influence on the acritarchs recorded in sample DG00LK1.257. This may be due to the relatively low amount of pyrite present. The real influence of pyrite presence and nitric acid treatment on the recovery of palynomorphs should ideally be tested on many samples of various types and should involve quantification of the pyrite present. It is suggested that to reduce problems associated with pyrite, nitric acid treatment should be routinely adopted in sample processing. This will make the species diversity and numbers of acritarchs per gram of sediment of pyrite-rich and pyrite-poor samples more comparable. The degree of possible destruction of acritarchs during the growth of pyrite grains larger than the specimens cannot be calculated and must be considered as a factor when analysing pyrite-rich horizons.

The <7 μm sieve test data indicate that the loss of acritarchs during washing of the sample through a 7 μm sieve is minimal and can be considered as a minor factor that can be monitored by regular testing.

CONCLUSIONS

Two important points that emerged from Colbath's (1985) investigations still remain: consistency of technique and the trade-off between the accuracy of results and the time incurred in obtaining them. 100% repeatability of results is unlikely to be achieved; repeat sampling of modern biological communities gives a certain percentage of random variation (Watkins *et al*., 1990; Muylaert *et al*., 2000). Population variability and errors associated with repeat sampling, such as differential preservation and uneven distribution of palynomorphs within the rock sample mean that repeatability is never perfect. Variations in apparent diversity may result from the recording of species represented by very few individuals, which may be seen in one sample, but not the next. Consistency in the processing technique reduces errors as much as possible and the accuracy of estimates of the number of palynomorphs per gram of sediment and of the number of species present are very important. It is very unlikely every acritarch can be extracted from a rock sample. Even if every acritarch could be extracted, the time incurred in recording all the individuals could not be justified for the increase in the accuracy of the results. Three separations, nitric acid treatment and well-monitored sieving, provides a justifiable compromise between the time spent processing and the precision of the results. A similar conclusion may apply to other palynological assemblages.

This study has suggested that the losses incurred at various stages in the processing technique are significant, but can be reduced and quantified. Although palynological samples from different localities and time periods will yield different numbers of palynomorphs per gram of sediment and different species, tests should be carried out as appropriate to quantify the loss of data.

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REFERENCES

Barss, M.S. & Williams, G.L. 1973. Palynology and nannofossil processing techniques. *Geological Survey of Canada, Paper*, **73-26**: 1–25.

Colbath, G.K. 1985. A comparison of palynological extraction techniques using samples from the Silurian Bainbridge Formation, Missouri, U.S.A. *Review of Palaeobotany and Palynology*, **44**: 153–164.

Downie, C., Evitt, W.R. & Sarjeant, W.A.S. 1963. Dinoflagellates, Hystrichospheres, and the classification of the acritarchs. *Stanford University Publications, Geological Sciences*, **7**(3): 3–16.

Forster, M. & Flenley, J.R. 1989. Application of density gradient centrifugation to palynology. *School of Geography and Earth Resources, University of Hull, Miscellaneous Sciences*, **35**: 1–20.

Funkhouser, J.W. & Evitt, W.R. 1959. Preparation techniques for acid-insoluble microfossils. *Micropaleontology*, 3, **5**: 369–375.

Gray, J. 1965a. Palynological Techniques. *In* Kumel, B. & Raup, D. (Eds), *Handbook of Paleontological Techniques*, 471–481. W.H. Freeman & Co., San Francisco and London.

Gray, J. 1965b. Extraction Techniques. *In* Kummel, B. & Raup, D. (Eds), *Handbook of Paleontological Techniques*, 530–587. W.H. Freeman & Co., San Francisco and London.

Litwin, R.J. & Traverse, A. 1989. Basic guidelines for palynomorph extraction and preparation from sedimentary rocks. *In* Feldman, R.M., Feldman, R.M., Chapman, R.E. & Hannibal, J.T. (Eds), *Palaeo-techniques*. Paleontological Society Special Publication, **4**: 87–98.

Muylaert, K., Sabbe, K. & Vyverman, W. 2000. Spatial and Temporal Dynamics of Phytoplankton Communities in a Freshwater Tidal Estuary. *Estuarine, Coastal and Shelf Science*, **50**: 673–687.

Watkins, J.L., Morris, D.J., Ricketts, C. & Murray, A.W.A. 1990. Sampling biological characteristics of Krill: effect of heterogeneous nature of swarms. *Marine Biology*, **107**: 409–415.

Wood, G.D., Gabriel, A.M. & Lawson, J.C. 1996. Palynological techniques – processing and microscopy. *In* Jansonius, J. & McGregor, D.C. (Eds), *Palynology: Principles and Applications*. American Association of Stratigraphical Palynologists Foundation, **1**: 39–50.