The use of pre-treatments in palynological processing

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ABSTRACT

A sample of palynomorph-rich Upper Carboniferous mudstone from Scotland was separately pre-treated overnight with acetone, two detergent solutions, formic acid, household bleach (two methods), methylated spirits and white spirit prior to palynological preparation using sodium hexametaphosphate [$(NaPO_3)_6$]. The aim of this study was to identify effective methods of pre-treatment that would increase palynomorph yields using the $(NaPO_3)_6$ method. Pre-treatment generally increased the mass of sample that was broken down by the $(NaPO_3)_6$ technique. Detergent one (carpet cleaner), formic acid, household bleach and white spirit allowed the disaggregation of more rock than without any pre-treatment. However, formic acid produced a lower concentration of yield of Carboniferous miospores than with no pre-treatment. Pre-treatment with acetone, detergent two (industrial detergent) and methylated spirits actually decreased the weight of rock that was disaggregated with $(NaPO_3)_6$. Despite this, all these three pre-treatments improved the palynomorph yield as compared to with no pre-treatment. Moreover, all the pre-treatments except formic acid improved palynomorph productivity. The effectiveness of pre-treatments was demonstrated by the increased absolute numbers of indigenous palynomorphs extracted. However, the concentrations of miospores per gram of rock are more significant. Acetone, both detergent solutions, methylated spirit and white spirit significantly improved the amounts of palynomorph extracted. Household bleach was found to lighten and selectively destroy relatively delicate palynomorphs; this reagent should be used with caution, and only with robust material. In the subsample soaked overnight in 5% bleach solution, all the exotic *Lycopodium* spores added were destroyed. By contrast in the subsample treated with 2.5% bleach solution for six hours, a small proportion of the exotic *Lycopodium* spores survived. This study indicates that the $(NaPO_3)_6$ method using either detergent or white spirit as a pre-treatment is highly effective at extracting palynomorphs from clay-rich lithotypes. However the concentration of palynomorphs obtained is generally lower than those from mineral acid digestions.

Keywords: palynomorph preparation techniques; pre-treatment; sodium hexametaphosphate; Carboniferous; United Kingdom (Scotland).

1. Introduction

The pre-treatment of samples for palynology is not new. Raistrick (1934, p. 143) for example reported that high rank coals macerate more effectively in Schultze's solution if the sample is pretreated by soaking in cold pyridine for 24

hours. Van Cleave and Ross (1947) subsequently noted that pre-treatment of palynomorph residues with a suitable detergent may help the penetration of stain. Samples of sedimentary rock or unconsolidated sediment for palynological analysis are sometimes soaked in water or surface-active substances such as detergent solution, ethanol or other reagents prior to the main (acid-based) processing procedure. This is to attempt to deflocculate or soften the sample material so that the subsequent processing proceeds quickly and effectively. Organic pre-treatment reagents such as acetone, methylated spirits and white spirit penetrate the interstices of the sample material and start to break it down by the pressure developed. A wetting agent may aid this penetration. Alternatives to pre-treatment are to use the power of crystallisation of, for example, sodium salts to physically break up the sample material or simply not to pre-treat (Faegri et al., 1989, p. 76).

In this study, the effects of seven reagents for the pre-treatment of an extremely palynomorph-rich Upper Carboniferous mudstone before processing using $(NaPO_3)_6$ were tested. The pre-treatment reagents used were acetone, two detergents (a domestic carpet cleaner and Decon 90), formic acid, household bleach (sodium hypochlorite solution – two methods), methylated spirits and white spirit. These were chosen because it was felt that they could all potentially soften and/or partially disaggregate the sample material and hence expedite clay deflocculation with $(NaPO_3)_6$. Formic acid, methylated spirits, sodium hypochlorite and white spirit have been used to extract calcareous and phosphatic microfossils (Armstrong and Brasier, 2005).

2. Background

The present authors have recently developed methods of preparing palynomorphs from sedimentary rocks and sediments without using aggressive mineral acids such as hydrochloric acid (HCl) and hydrofluoric acid (HF). These acids dissolve carbonate and silicate minerals respectively, and acid digestion is the standard method of extracting palynomorphs (e.g. Gray, 1965a,b; Doher, 1980; Phipps and Playford, 1984; Wood et al., 1996; Batten, 1999; Green, 2001; Brown, 2008). The non-acid techniques involve the use of sodium hexametaphosphate [$(NaPO_3)_6$], and hydrogen peroxide (H_2O_2) (Riding and Kyffin-Hughes, 2004, 2006; Riding et al., 2006; 2007).

Sodium hexametaphosphate, sometimes abbreviated to SHMP, is a hexamer which is prepared by melting monosodium orthophosphate followed by rapid cooling. Alternative names include Calgon, glassy sodium and Graham's Salt. It hydrolyzes in aqueous solution to sodium trimetaphosphate and sodium orthophosphate. The pH of $(NaPO_3)_6$ is neutral (7), and it is not an oxidising agent. This substance has a wide range of applications, and is used as a detergent, a powerful deflocculant or a dispersent for clay and soil and a water softener. It is also used as a food additive, and has the E-number E452i. Sodium hexametaphosphate is a relatively non-hazardous substance, however significant ingestion may cause an allergic reaction. It reduces the coherence of the clay fraction because phosphate ions are strongly adsorbed onto the particles of clay, which are broken up to sub-10 μm particles due to the high ionic charges. This allows the dispersed clay to be separated from the organic fraction by sieving.

By contrast, H_2O_2 is a strong oxidising agent, weakly acidic and slightly viscous. Pure H_2O_2 is pale blue, but it becomes colourless when diluted. It is used in

the chemical industry, for bleaching, disinfecting and as a propellant. The major hazards pertaining to H_2O_2 are its corrosive and oxidising properties, especially at high concentrations (i.e. >50%). Additionally, because it dissociates to form water and oxygen, it can form potentially explosive mixtures if allowed to mix with combustible materials. To minimise this phenomenon, a stabiliser is normally added to commercially-supplied H_2O_2 to decrease the dissociation rate. Riding and Kyffin-Hughes (2007, p. 21, 22) described the health and safety issues surrounding H_2O_2 in detail. By contrast with $(NaPO_3)_6$, H_2O_2 disaggregates clay-rich materials physico-chemically. Because H_2O_2 spontaneously dissociates into oxygen and water, it causes the physical disintegration of clays by 'deposit swelling'. This is the action of the oxygen bubbles which are generated within the matrix of the sample material when H_2O_2 dissociates. The expansion pressure of the dissociated H_2O_2 which has soaked into the sample material breaks up the rock/sediment. Hydrogen peroxide is also a powerful oxidising agent, and this helps to simultaneously extract palynomorphs by breaking down amorphous organic material (Riding et al., 2007, pl. 2, 3). Naturally, this reagent must be used carefully because it can damage or destroy palynomorphs by oxidation (Hopkins and McCarthy, 2002).

The $(NaPO_3)_6$, and H_2O_2 procedures therefore differ from HCl and HF digestion in that the mineral fraction is broken up and sieved off, rather than being dissolved or etched away. Both $(NaPO_3)_6$, and H_2O_2 appear to work well on most clay-rich materials. These procedures are however markedly less effective on carbonate lithotypes (Riding and Kyffin-Hughes, 2004, figs. 4E, 4F). Furthermore, H_2O_2 appears to be superior to $(NaPO_3)_6$ for preparing relatively indurated mudstones (Riding et al., 2007). The avoidance of using HCl and HF is important because these acids are hazardous to laboratory personnel and to the wider environment. Furthermore, the costs of installation and maintenance of acid-safe laboratory facilities are relatively high.

Riding and Kyffin-Hughes (2004, 2006) recommended the pre-treatment of samples with a strong detergent for several hours prior to preparation with $(NaPO_3)_6$. This pre-treatment appears to soften the sample material, and allow a greater surface area for deflocculation with the $(NaPO_3)_6$. The purpose of this study is to test seven different pre-treatment reagents prior to palynomorph preparation using $(NaPO_3)_6$.

3. Material and methods

In this study, a sample of Upper Carboniferous (Westphalian B) mudstone was prepared. The material is from British Geological Survey (BGS) offshore borehole number 74/13, which was rotary-drilled 17 km east of Fife Ness in the Forth Approaches, offshore southeast Scotland, United Kingdom (Owens and Marshall, 1978, p. 19, figs. 1, 3). This borehole was fully cored, with excellent recovery achieved. The location of the borehole is 56° 18.10'N; 02° 19.30'W (Fig. 1). The material used is a composite sample of conventional core between 19.00 and 17.55 m, and was registered as BGS sample MPA 57940. This Upper Carboniferous mudstone is known to be extremely rich in well-preserved spores and pollen (Riding et al., 2007).

The composite sample was air-dried, crushed to approximately 1 mm fragments and thoroughly manually homogenised. Ten 5 g subsamples of this sample were measured, and eight of these were separately mixed with 50 ml of the pre-treatment reagents (acetone, two detergent solutions, formic acid, household bleach

[two methods], methylated spirits and white spirit), and left to stand overnight. It was anticipated that each of the pre-treatment regimes would soften the sample material and/or commence deflocculation of the clay. This would then enable the $(NaPO_3)_6$ to more efficiently break down the clay fraction, thereby releasing significantly more palynomorphs. Two control subsamples were prepared. The first of these was prepared simply using the $(NaPO_3)_6$ method of Riding and Kyffin-Hughes (2004; 2006) with no pre-treatment. The second control subsample was prepared using the standard HCl/HF digestion method (e.g. Gray, 1965b; Doher, 1980; Phipps and Playford, 1984; Wood et al., 1996; Green, 2001) without oxidation, and again with no pre-treatment. The hydrochloric and hydrofluoric acid treatments lasted until the respective reactions were complete. By contrast, the $(NaPO_3)_6$ subsamples were treated for 20 minutes only.

To allow the relative effectiveness of each of the pre-treatment reagents, the concentrations of palynomorphs were calculated. The exotic marker method using *Lycopodium clavatum* tablets as a spike was used for this (Benninghoff, 1962; Stockmarr, 1971). Ten *Lycopodium* tablets were added to each of the nine subsamples prior to the preparation procedure; including the pre-treatment phase. At least 350 Carboniferous pollen and spores were counted (Table 1). Damaged palynomorphs were counted. Fragments which are c. 50% were counted and aggregated into the count; however any small portions (<25%) were disregarded. The absolute abundances of Carboniferous miospores were calculated using the equation of Benninghoff (1962), i.e.:

$$c = \frac{m_c \times L_t \times t}{L_c \times w}$$

This is where:
c = the number of indigenous (i.e. Carboniferous) miospores per gram of dry rock (= concentration)
m_c = the number of indigenous (i.e. Carboniferous) miospores counted
L_t = the number of *Lycopodium* spores in each tablet (i.e. 18,583)
t = the number of tablets added to the sample (i.e. 10)
L_c = the number of *Lycopodium* spores counted
w = the weight of dry sediment processed in grams

It should be noted that it has been demonstrated that exotic *Lycopodium* spores may be lost during preparation, largely during the decantation and sieving stages(Mertens et al., 2009). Selected low-magnification photomicrographs of the residues are presented in Figs. 2-11. The remaining sample material, organic residues, microscope slides, primary data and illustrated material are housed in the collections of the British Geological Survey (BGS), Keyworth, Nottingham NG12 5GG, United Kingdom.

4. Results

The sample produced highly abundant and well-preserved spores and pollen which are mid/dark brown in colour (Figs. 2-11). Spores are more abundant than

pollen. This unit is a freshwater deposit, and no marine microplankton are present. The assemblage is of Middle Pennsylvanian-Duckmantian (Late Bashkirian-Early Moscovian or Atokan) age, and is dominated by *Lycospora pusilla* (Ibrahim 1932) Somers 1972 together with common *Crassispora* spp. and *Florinites* spp. The occurrences of *Endosporites globiformis* (Ibrahim 1932) Schopf et al. 1944 and *Florinites junior* Potonié & Kremp 1956 are indicative of the *Microreticulatisporites nobilis-Florinites junior* (NJ) Biozone of Clayton et al. (1977). Other miospores observed are entirely consistent with the NJ Biozone, and include *Cirratriradites saturni* (Ibrahim 1932) Schopf et al. 1944, *Cristatisporites indignabundus* (Loose 1932) Staplin & Jansonius 1964, *Grumosisporites varioreticulatus* (Neves 1958) Smith & Butterworth 1967, *Raistrickia fulva* Artüz 1957, *Raistrickia saetosa* (Loose 1932) Schopf et al. 1944, *Raistrickia superba* (Ibrahim 1933) Schopf et al. 1944, *Reinschospora triangularis* Kosanke 1950, *Simozonotriletes intortus* (Waltz 1938) Potonié & Kremp 1954, *Triquitrites bransonii* Wilson & Hoffmeister 1956, *Triquitrites sinani* Artüz 1957, *Vestispora cancellata* (Dybovà & Jachowicz 1957) Wilson & Venkatachala 1963 and *Vestispora costata* (Balme 1952) Spode in Smith & Butterworth 1967.

As previously mentioned, the acid preparation was allowed to proceed until the reactions were complete, but the $(NaPO_3)_6$ treatments were given 20 minutes. The prepared residues from the ten subsamples studied were examined and the indigenous Carboniferous pollen and spores and the exotic *Lycopodium* spores were counted. These data, together with the dry weight of sample macerated, the concentration of indigenous palynomorphs (based on the actual weight of rock broken down and on 5.0 g) and the calculated number of indigenous palynomorphs are presented as Table 1. The actual weights of the subsamples prepared are considered to be highly significant (see below).

Following both the acid and $(NaPO_3)_6$ preparations, the residues were sieved to remove the >500 μm fraction. This largely comprises undigested or undeflocculated rock as appropriate. Unsurprisingly, the acid digestion gave the lowest amount (0.9 g) of undigested rock residue. The remaining $(NaPO_3)_6$ preparations deflocculated between 1.5 and 4.0 g of the initial 5.0 g used (Table 1); hence the undeflocculated residues using $(NaPO_3)_6$ were between 1.0 and 3.5 g. The concentrations of indigenous palynomorphs based on the actual weight of rock broken down, and on the full 5.0 g of each subsample are presented in Table 1. This strategy was adopted to emphasise the difference in palynomorph concentrations if the actual weight of rock disaggregated or dissolved is taken into account. Many quantitative studies do not allow for any unprocessed raw sample material which potentially can liberate palynomorphs. Moreover, this methodology clearly demonstrate that the $(NaPO_3)_6$ method normally does not fully break down relatively indurated lithotypes.

The results of this study are discussed in the remainder of this section, subsample by subsample. Generally, the eight overnight pre-treatments did not cause any discernible physical changes to the sample material. However, it was notable that, except for formic acid, when the material was mixed with $(NaPO_3)_6$, it generally disaggregated significantly faster than material which had no pre-treatment. Prolonged soaking in pre-treatment reagents however can cause physical changes. For example, in another experiment which is not described in detail here, a subsample of this Carboniferous mudstone was completely disaggregated after soaking for one week in white spirit.

4.1. The control subsample prepared by hydrochloric/hydrofluoric acid digestion

In order to make comparisons with the seven $(NaPO_3)_6$ preparations, a subsample was prepared using the standard mineral acid digestion technique. This subsample received no pre-treatment, and the residue was not oxidised following hydrofluoric acid treatment. The sample was crushed to pea-sized fragments and treated separately with hydrochloric acid and hydrofluoric acid to remove the carbonate and silicate minerals respectively. The acid digestion proved highly effective; 4.1 g of the initial 5.0 g of rock was eliminated. Following the acid treatment, the organic concentrate was sieved using a 10 μm mesh to remove the fine material which tends to obscure the palynomorphs. The sample prepared in this way produced 341,746 palynomorphs per gram and 1,401,158 grains in total (Table 1). The concentration is significantly higher than that obtained by Riding et al. (2007) for similar material using the volume aliquot method described by Dale (1976) and Harland (1989). A sample of this Carboniferous unit was prepared from borehole 74/13 at 18.07 m, and a palynomorph concentration of 54,600 grains per gram was determined (Riding et al. (2007, table 1). The reasons behind this apparent underestimation are not clear. The volume aliquot method requires accurate measurements, but the disparity noted here is well beyond confidence limits and experimental error. Another reason may be that this mudstone unit exhibits significantly variable palynomorph concentrations because the sample material in this study is from between 19.00 and 17.55 m in BGS borehole 74/13.

It seems most likely that this anomaly is largely due to significant losses of palynomorphs during the various laboratory procedures. This will affect aliquot methods more that the exotic *Lycopodium* spore method, which uses a ratio (Stockmarr, 1971). De Vernal et al. (1987) noted that concentrations of palynomorphs determined using the weight aliquot method are 33% lower than those worked out with the marker-grain method. However, in a similar test, Mertens et al. (2009) found that exotic *Lycopodium* spores are prone to losses during preparation.

The preparation is of a reasonable standard, however moderate levels of amorphous organic material (AOM) are present (Fig. 2). This AOM could be removed by oxidising the residue with nitric acid or Schultze's solution. However, the $(NaPO_3)_6$ preparations were not separately oxidised, hence it was decided to maintain consistency and not to oxidise the HCl/HF preparation.

4.2. The control subsample prepared with sodium hexametaphosphate

So that the subsamples prepared using $(NaPO_3)_6$ with pre-treatments can be objectively assessed, a control subsample was processed. This was using the $(NaPO_3)_6$ method without any pre-treatment prior to the addition of flakes of $(NaPO_3)_6$ (Riding and Kyffin-Hughes (2004, appendix 2; 2006, appendix 3). The treatment proved moderately effective, but 3.0 g of the initial 5.0 g of sample was not broken down after soaking overnight (Table 1). The 2.0 g sample prepared in this way produced 104,427 palynomorphs per gram; this represents 208,853 grains in the subsample prepared (Table 1). This concentration compares with 341,746 pollen/spores per gram using HCl/HF. In this highly productive lithotype, the fact that the preparation is somewhat less efficient in terms of absolute extraction has no bearing in terms of normal palynological analysis. The effectiveness disparity does not bias the relative proportions of the taxa in the sample. In fact, because palynologists routinely study

only a miniscule proportion of the grains extracted from any one sample, this is hardly ever likely to be a serious problem. The 'efficiency gap' using $(NaPO_3)_6$ would only be a problem with extremely organic-lean samples such as the Neoproterozoic material from Australia studied by Grey (1999). The fact that $(NaPO_3)_6$, cannot entirely disaggregate relatively indurated lithotypes such as the Carboniferous mudstone tested here emphasises the need for an effective pre-treatment regime. In a previous study, Riding et al. (2007) used the volume aliquot method for quantitative assessments. However these authors did not undertake a quantitative study of the mudstone used in this work using $(NaPO_3)_6$, so a meaningful comparison between the volume aliquot method and the *Lycopodium* spore spiking method for this sample cannot be made in this case.

The $(NaPO_3)_6$ preparation proved very clean, and was largely devoid of AOM (Fig. 3). This phenomenon was also noted by Riding and Kyffin-Hughes (2004; 2006, pl. 4) and it appears that $(NaPO_3)_6$ can disaggregate AOM, in addition to clay minerals. This reagent is not an oxidising agent and it seems likely that $(NaPO_3)_6$ breaks up AOM using ionic charges, i.e. in a similar way to how it disaggregates clays. This phenomenon is extremely useful in that it potentially negates the need to use hydrochloric, hydrofluoric and nitric acids in palynological preparation.

4.3. The subsample pre-prepared with acetone

Acetone (CH_3COCH_3) is a colourless, flammable liquid ketone. It is miscible with most liquids, and is used as nail polish remover and in paint thinners. Acetone is a solvent for most plastics, and should always be used in glass containers. It auto-ignites at 465ºC, and acetone pre-treatments should be done in a fume cupboard. This substance is not highly toxic but it can be harmful by inhalation, ingestion or absorption.

It was thought that acetone may help to soften the sample material. However, because acetone is a volatile substance, the vessel should be monitored and topped-up if the pre-treatment is done over several days. In another experiment, during the course of several days of pre-treatment, all the acetone evaporated despite the vessel being partially covered. An overnight treatment however does not encounter this problem.

The acetone pre-treatment appears to have been significantly beneficial. However, acetone pre-treatment did not increase the mass of sample which was disaggregated by the $(NaPO_3)_6$. Following overnight pre-treatment with acetone, 3.3 g of the 5.0 g subsample remained following the 20 minute treatment with $(NaPO_3)_6$ (Table 1). This means that acetone pre-treatment enabled 0.3 g less rock to be disaggregated compared with no pre-treatment (Table 1). Despite this, the palynomorph yield was increased by approximately 50% by the acetone pre-treatment. The pre-treated sample yielded 151,929 grains per gram, as opposed to 104,427 palynomorphs per gram with no pre-treatment (Table 1). The numbers of Carboniferous spores in the acetone pre-treated and control subsamples are 258,280 and 208,853 respectively (Table 1). Hence it appears that this pre-treatment softened the material, and began to deflocculate the clay fraction.

The acetone pre-treatment had no adverse effect on the $(NaPO_3)_6$ preparation. The residue was clean, the palynomorphs were abundant, and had not been bleached or damaged in any way (Fig. 4). Furthermore, no differential degradation or destruction of the pollen and spores was noted.

4.4. The subsample pre-prepared with detergent solutions

Two types of detergent were used in this study; these are a household carpet cleaner and an industrial grade detergent.

4.4.1 Domestic carpet cleaner solution

The first detergent is a domestic carpet cleaner especially formulated for use on heavily-used carpets. It is claimed to be an effective pre-treatment for soiled carpets, specifically breaking down oil and soil. The cleaner is a clear liquid which smells of the active ingredient, diethylene glycol monobutyl ether. It is not hazardous, but accidental spillage may cause eye and skin irritation, and it is moderately toxic if ingested.

It was thought that a 3% solution of this domestic carpet cleaner would partially disaggregate the sample prior to the main preparation procedure. According to Riding and Kyffin-Hughes (2004; 2006), samples to be prepared with $(NaPO_3)_6$ should be soaked overnight in a detergent solution. The overnight pre-treatment with the carpet cleaner solution increased the weight of sample which was then treated by the $(NaPO_3)_6$. Following the overnight pre-treatment with carpet cleaner solution, 1.7 g of the 5.0 g subsample remained undisaggregated following treatment with $(NaPO_3)_6$ (Table 1). This represents a significant improvement compared to no pre-treatment. The yield of palynomorphs, however, was enhanced by approximately 150%; this is assumed to be largely due to the pre-treatment with the carpet cleaner solution. The pre-treated subsample yielded 262,790 *in situ* palynomorphs per gram compared with 104,427 palynomorphs per gram with no pre-treatment (Table 1). This increase is also reflected in the absolute numbers of indigenous palynomorphs extracted; the numbers of Carboniferous spores in the control subsample and subsample pre-treated with carpet cleaner solution are 208,853 and 867,207 respectively (Table 1). Hence the pre-treatment with carpet cleaner solution apparently appears to be extremely effective. The pre-treatment apparently started the clay disaggregation process, thus allowing the $(NaPO_3)_6$ to act on partially softened clay and thereby extracting a higher proportion of Carboniferous palynomorphs. The pre-treatment with carpet cleaner solution does not appear to cause damage to either the *in situ* or the exotic palynomorphs (Fig. 5).

4.4.2 Industrial detergent solution

The second detergent used was Decon 90, an industrial grade concentrated liquid detergent suitable for use in ultrasonic baths. It is a biodegradable emulsion including anionic and non-ionic surface-active agents. Decon 90 is used for cleaning and decontaminating a wide variety of media, however, it is unsuitable for use on non-ferrous metals such as aluminium and zinc. A 2-5% solution is normally adequate. The toxicity level is low, but the high alkalinity (the concentrate has a pH of >13) means that it is potentially hazardous, especially at high concentrations.

It was anticipated that a 3% solution of Decon 90 may significantly soften the sample. Riding and Kyffin-Hughes (2004; 2006) recommended that samples to be prepared with $(NaPO_3)_6$ are soaked overnight in a dilute solution of a strong detergent. The overnight pre-treatment with Decon 90 did not increase the weight of sample which was then treated by the $(NaPO_3)_6$. Like with acetone, after the overnight pre-treatment with Decon 90, 3.3 g of the 5.0 g subsample remained undisaggregated

following the $(NaPO_3)_6$ treatment (Table 1). This therefore does not represent an improvement on no pre-treatment. However, the palynomorph yield was more than doubled, presumably by the Decon 90 pre-treatment. The Decon 90 pre-treated subsample yielded 216,599 grains per gram compared with 104,427 palynomorphs per gram with no pre-treatment (Table 1). The numbers of Carboniferous spores in the Decon 90 pre-treated and control subsamples are 368,219 and 208,853 respectively (Table 1). Hence the pre-treatment with Decon 90 also appears to have been highly effective. The pre-treatment initiated the deflocculation of the clay fraction, allowing the $(NaPO_3)_6$ to work on partially broken down clay and thus extracting a higher proportion of palynomorphs. The pre-treatment with Decon 90 did not apparently selectively degrade or destroy the palynomorphs (Fig. 6).

4.5. The subsample pre-prepared with formic acid

Formic acid (CH_2O_2) is a simple carboxylic acid, and occurs in the venom of ant and bee stings. It is miscible in water and most organic solvents, is partially soluble in hydrocarbons and may be dissociated by heat. Formic acid is not an oxidising agent, and has some reducing properties. This substance is used as an antibacterial agent and as a preservative. The principal hazards associated with formic acid are eye and respiratory tract damage, and skin burns. Thus full personal protective equipment should be worn when working with >10% formic acid. All use of this reagent should be done in a fume hood as carbon monoxide (CO) may be present in the vapours produced.

It was thought that 80% formic acid may be a potentially effective pre-treatment reagent and could effect some disaggregation before the $(NaPO_3)_6$ treatment is begun. The pre-treatment with formic acid initially appeared to have been effective because it increased the weight of sample which was available for treatment by $(NaPO_3)_6$. Following the overnight pre-treatment with formic acid, 1.8 g of the 5.0 g subsample remained following treatment for 20 minutes with $(NaPO_3)_6$ (Table 1). The subsample prepared simply using $(NaPO_3)_6$ with no pre-treatment left 3.0 g of rock undisaggregated (Table 1). However, this improved disaggregation did not translate to a higher palynomorph yield per gram. The yield was slightly reduced in comparison to the $(NaPO_3)_6$ control subsample. The sample pre-treated with formic acid yielded 93,889 grains per gram compared with 104,427 palynomorphs per gram for the control subsample (Table 1). The numbers of Carboniferous spores in the formic acid pre-treated and control subsamples are 300,444 and 208,853 respectively (Table 1). Therefore the pre-treatment with formic acid does not apparently give any advantage. However, the formic acid pre-treatment did not cause any discernible damage to the palynomorphs (Fig. 7).

4.6. The subsample pre-prepared with household bleach

Household bleach is a 3–6% aqueous solution of sodium hypochlorite (NaClO). The concentration gradually decreases during storage. A weak solution (i.e. ca. 1%) will sanitise kitchen surfaces; stronger solutions (12–15%) are used to chlorinate and disinfect water supplies. Sodium hypochlorite solution (0.5–5.25%) is also used in endodontics during root canal treatment to remove necrotic nerve tissue. Sodium hypochlorite is corrosive due to its alkaline nature, and concentrated solutions

448 can cause eye damage and burn skin. It is a strong oxidising agent, and it may release
449 chlorine if mixed with acids.
450 Sodium hypochlorite solution is an oxidant, and is used in the processing of
451 siliciclastic rocks for palynomorphs after the acid digestion stage (e.g. Lee, 1964;
452 Batten, 1999; Green, 2001; Traverse, 2007). It is one of the gentlest oxidising agents
453 used in palynological processing, being significantly less aggressive than any of the
454 nitric acid-based reagents such as Schultze's solution (Evitt, 1984; Eshet and Hoek,
455 1996). Sodium hypochlorite has been used to macerate coals (Hoffmeister, 1960;
456 Smith and Butterworth, 1967), to remove pyrite (Merrill, 1980), as a bleach for
457 darkened palynomorphs such as chitinozoa and megaspores (Jenkins, 1967) and to
458 remove organic matter from soils to allow clay mineral analysis (Siregar et al., 2005).
459 Because of its bleaching and oxidising properties, this reagent should be used
460 carefully to avoid the degradation or destruction of palynomorphs. Doher (1980, p.
461 21) stated that sodium hypochlorite solution corrodes pollen and spores over
462 prolonged periods, and can cause grain size changes. This was confirmed by Traverse
463 (1990), who warned how bleaching dark palynomorphs by oxidation may have
464 adverse effects. Traverse (1990) demonstrated that modern *Althea rosea* (hollyhock)
465 pollen is significantly altered by brief treatment with sodium hypochlorite bleach.
466 *Althea rosea* pollen grains that have simply been acetolysed are dark, spherical and
467 have numerous spines. However, if the pollen is acetolysed then bleached with a
468 dilute sodium hypochlorite solution for two minutes, the pollen morphology is
469 changed beyond recognition. The grains are lighter, the outermost layer including the
470 spines is destroyed, and the remaining exine shrinks producing a rounded square
471 outline. If these pre-and post-bleached forms were fossil pollen, they would be placed
472 in entirely different taxa.
473 It was anticipated that sodium hypochlorite solution will partially disaggregate
474 the sample material prior to the $(NaPO_3)_6$ treatment. The overnight pre-treatment with
475 5% sodium hypochlorite solution appears to have been highly effective. It greatly
476 increased the mass of sample which was broken down by the $(NaPO_3)_6$. Following the
477 overnight 5% sodium hypochlorite solution pre-treatment, only 1.0 g of the 5.0 g
478 subsample remained following 20 minutes treatment with $(NaPO_3)_6$ (Table 1). This
479 means that this sodium hypochlorite solution pre-treatment has enabled 2.0 g more
480 rock to be disaggregated, compared with no pre-treatment (Table 1). However, the
481 pre-treatment destroyed all the *Lycopodium* spores and noticeably bleached the
482 Carboniferous spores. The palynomorph residue comprises relatively light coloured
483 Carboniferous spores only (Fig. 8); no *Lycopodium* spores could be found, despite
484 scanning entire slides. This confirms the findings of Traverse (1990) that sodium
485 hypochlorite solution is highly destructive to modern pollen and spores. The
486 Carboniferous palynomorphs are markedly lighter in colour than with all the other
487 preparation strategies in this study (Figs. 2-7 and 10-11). However, the residue is still
488 extremely rich in palynomorphs and is devoid of AOM (Fig. 8). There does not
489 appear to have been any selective destruction of the Carboniferous palynomorphs.
490 A second test using sodium hypochlorite solution was undertaken to attempt to
491 establish if a gentler treatment would be less destructive to the *Lycopodium* spores. A
492 5.0 g subsample was pre-treated with 2.5% sodium hypochlorite solution for 6 hours.
493 This second sodium hypochlorite solution pre-treatment enabled 0.8 g more rock to be
494 disaggregated compared to the control with no pre-treatment (Table 1). The organic
495 material produced by this subsample was also noticeably lightened, and the
496 preservation of the *Lycopodium* spores was poor (Fig. 9). Only 29 of these poorly-
497 preserved *Lycopodium* spores were counted in an overall population of 617 grains

(Table 1). This ratio, as compared to the others in Table 1, means that significant numbers of *Lycopodium* spores were destroyed by this gentler treatment. Hence this count cannot be used to assess the concentration of the Carboniferous spores, which do not appear to have been destroyed by the bleach. This means that the calculation of 3,767,863 palynomorphs in the preparation and the two concentrations depicted in Table 1 are spurious due to the destruction of significant proportion of the *Lycopodium* spores.

It is therefore clear that sodium hypochlorite solution is extremely corrosive to modern and relatively young palynomorphs, and should be used with great care. This reagent can apparently be used with caution on material which contains old (i.e. Palaeozoic) and/or robust palynomorphs. By contrast, it should not be used to pre-treat Neogene and younger material because of its highly corrosive nature.

4.7. The subsample pre-prepared with methylated spirits ('meths')

Methylated spirits is ethanol (C_2H_5OH), which has been mixed with aniline dye in order to render it toxic and unpalatable. Methanol (CH_3OH) is also added to make the separation of pure ethanol via distillation difficult. Ethanol is a versatile fuel and solvent; it is miscible with light aliphatic hydrocarbons, other organic solvents and water. Methylated spirits is slightly basic (pH 7.33), and is volatile. This means that the level of the liquid should be monitored, if the pre-treatment with methylated spirits is prolonged (i.e. several days).

It was considered that methylated spirits may possibly help to render the sample material more susceptible to disaggregation using $(NaPO_3)_6$. Treatment with methylated spirits did not increase the weight of sample which was broken down by the $(NaPO_3)_6$. Following pre-treatment with methylated spirits, 3.5 g of the 5.0 g subsample remained after 20 minutes $(NaPO_3)_6$ treatment (Table 1). The methylated spirits pre-treatment thus enabled 0.5 g less rock to be disaggregated compared with no pre-treatment (Table 1). However, in terms of the palynomorph yield, the pre-treatment with methylated spirits appears to have been marginally beneficial. This pre-treatment yielded 142,449 grains per gram, compared with 104,427 palynomorphs per gram for the control subsample (Table 1). The absolute numbers of Carboniferous spores in the methylated spirit pre-treated subsample also show a marginal increase on the control subsample; these figures are 213,674 and 208,853 respectively (Table 1). The pre-treatment with methylated spirits apparently had no adverse effects on the palynomorphs. The organic concentrate proved generally free of extraneous materials, and the palynomorphs were abundant and undamaged (Fig. 10).

4.8. The subsample pre-prepared with white spirit

White spirit (also known as mineral spirits, Stoddard solvent and Varsol) is a petroleum-based distillate; it is a mixture of alicyclic, aliphatic and aromatic hydrocarbons. This clear liquid is used as an extraction solvent in degreasing and dry cleaning, a fuel additive, a viscosity-reducer and a general-purpose organic solvent (e.g. paint thinners). It is flammable, with a flash point of 39°C. Despite having a low acute toxicity, white spirit is an irritant and may cause contact dermatitis, various other skin complaints and lung damage. White spirit is a potential freshwater or marine pollutant, and hence should be disposed of responsibly.

In this study, technical grade white spirit was used. Its grade is determined by the nature of the crude oil used, and the conditions of distillation. It is highly volatile, and the level in the vessel should be checked if the pre-treatment lasts for a few days. Brown (1960; 2008, p. 76, 88, 89) described using white spirit (as Varsol) to disaggregate shale and to dissolve asphalt and other heavy hydrocarbons.

It was anticipated that white spirit will help to soften the sample material. It is well known as a disaggregating agent that can liberate microfossils *sensu lato* from partially indurated clay-rich lithotypes (Armstrong and Brasier, 2005, p. 275). This pre-treatment appears to have been markedly beneficial. It slightly increased the amount of sample material which was eventually disaggregated by the $(NaPO_3)_6$. Following pre-treatment with white spirit, 2.7 g of the 5.0 g subsample remained following the 20 minute treatment with $(NaPO_3)_6$ (Table 1). This means that the pre-treatment with white spirit enabled 0.3 g more rock to be dissaggregated than with no pre-treatment (Table 1). Regarding palynomorph yield, the pre-treatment with white spirit proved highly effective. The pre-treatment yielded 203,199 grains per gram of rock prepared, compared with 104,427 palynomorphs per gram for the control subsample (Table 1). The numbers of Carboniferous spores in the white spirit pre-treated and control subsamples are 467,357 and 208,853 respectively (Table 1). This marked enhancement of the palynomorph extraction process is comparable to that given by pre-treatment with Decon 90 (Table 1). The white spirit apparently started to deflocculate the clay, hence allowing the $(NaPO_3)_6$ to break down the partially disaggregated clay and explaining the higher palynomorph yield. The white spirit pre-treatment does not cause adverse preservational effects on the palynomorphs. The organic residue was extremely clean and pollen and spores were abundant and well-preserved (Fig. 11).

5. Summary

This study aimed to objectively assess the relative effectiveness of several pre-treatment regimes on a single sample of highly palynologically productive sedimentary rock. Another objective was to improve the effectiveness of the preparation method using $(NaPO_3)_6$ developed by Riding and Kyffin-Hughes (2004; 2006). Generally, pre-treatment increased the mass of sample that was eventually broken down by the $(NaPO_3)_6$ treatment. This is unsurprising because more soaking should soften lithified rocks. The pre-treatment aims at softening the sample material, thereby allowing the $(NaPO_3)_6$ to act on an increased surface area, and hence releasing more palynomorphs. Specifically, detergent one (carpet cleaner), formic acid, household bleach and white spirit allowed the disaggregation of more raw rock sample than without any pre-treatment. This also clearly demonstrates that the $(NaPO_3)_6$ preparation method is made more effective by pre-treatment. However, acetone, detergent two (Decon 90) and methylated spirits actually reduced the amount of rock broken down by $(NaPO_3)_6$. Unsurprisingly, the largest weight of rock prepared was with the control subsample prepared using HCl and HF digestion (Table 1).

The efficacy of pre-treatments is clear based on the absolute numbers of indigenous palynomorphs extracted from the subsamples. Only the pre-treatment using methylated spirits gave fewer specimens than the control subsample with no pre-treatment (Table 1). However, this comparison is somewhat misleading because the amounts of rock broken down were different.

The most significant data are the Carboniferous miospores per gram of rock which was disaggregated. Based on this, the $(NaPO_3)_6$ treatment with no pre-treatment produced 104,427 Carboniferous miospores per gram. Of the seven pre-treatment reagents tested, only formic acid was relatively ineffective; this reagent produced a yield of 93,889 Carboniferous miospores per gram. This represents a lower concentration of Carboniferous miospores than with no pre-treatment. Five of the others (i.e. acetone, both detergents, methylated spirits and white spirit) produced significantly higher concentrations of Carboniferous miospores from the sample studied than with no pre-treatment. Of these, the detergents and white spirit essentially doubled the palynomorph yield. Again, the subsample digested with HCl and HF produced the largest concentration of Carboniferous miospores (Table 1).

It is abundantly clear that household bleach is unsuitable for the pre-treatment of post-Neogene palynomorphs. It should be used with extreme caution as a pre-treatment reagent because it lightens (bleaches) and selectively destroys relatively young palynomorphs. All the *Lycopodium* spores were destroyed in the subsample which was soaked overnight in 5% sodium hypochlorite solution (Fig. 8). In the subsample treated with 2.5% sodium hypochlorite solution for six hours, a small proportion of the *Lycopodium* spores survived, however these are poorly-preserved. To summarise, bleach/sodium hypochlorite solution should be used only with extreme care on relatively old and robust palynomorphs.

This study confirms that the $(NaPO_3)_6$ method of Riding and Kyffin-Hughes (2004; 2006) is a highly effective technique for the extraction of palynomorphs from siliciclastic/clay-rich lithotypes, although the concentration of palynomorphs is generally lower than those obtained by HCl/HF digestions. Furthermore, the $(NaPO_3)_6$ treatment can help remove AOM from organic residues. Overnight pre-treatment with acetone, detergent, methylated spirits and white spirit makes the $(NaPO_3)_6$ preparation significantly more effective. These reagents all increase the concentration of the indigenous palynomorphs extracted. One detergent (the carpet cleaner) and white spirit increase the amount of rock that is disaggregated by the $(NaPO_3)_6$. Of the seven substances tested, detergent and white spirit are the most effective pre-treatment reagents. Consequently, these reagents are recommended as the best pre-treatment reagents in palynological preparation. It is interesting that one is essentially liquid soap and the other is an organic substance, hence they work in softening claystones in different ways.

It should be borne in mind that this study was only based on a single sample so these results should not be considered as being definitive; more research is needed. There is clearly scope for further investigations on non-acid palynological preparation. Tests for example using other reagents, different timings and different sample materials would enhance capability in this important area. It is also possible that pre-treating samples would make HCl-HF digestions faster, and enhance the final residue.

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792 **Fig. 1.** The location of BGS offshore borehole 74/13, offshore southeast Scotland,
793 United Kingdom.
794

Fig. 2. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared as a control with hydrochloric and hydrofluoric acids. Slide 'HF, 1 count, test B, #1', England Finder coordinate S65/1. The specimen of *Lycospora pusilla* in the centre-left is 24 μm in diameter. Note the presence of amorphous organic material at the top of the frame; for consistency with the non-acid preparations, this was not removed by oxidation.

Fig. 3. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ and no pre-treatment. Slide 'control, test B', England Finder coordinate O56. The specimen of *Lycospora pusilla* in the centre is 24 μm in diameter. Note the relatively clean nature of the residue, i.e the relative rarity of amorphous organic material.

Fig. 4. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using an overnight pre-treatment with acetone. Slide 'acetone, test B', England Finder coordinate M50/4. The saccate pollen grain in the centre-left is 84 μm long. Note the abundance of both Carboniferous miospores and *Lycopodium* spores.

Fig. 5. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using an overnight pre-treatment with 3% solution of domestic carpet cleaner. Slide 'R.D., overnight, 3 count', England Finder coordinate J51/2. The cracked specimen of *Lycospora pusilla* in the centre-left is 38 μm in diameter. Note the well-preserved miospores and the absence of amorphous organic material.

Fig. 6. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using an overnight pre-treatment with 3% Decon 90 detergent solution. Slide 'Decon 90, test B', England Finder coordinate M56. The specimen of *Densosporites* sp. near the bottom of the frame in the centre-left is 36 μm in maximum diameter. Note the 'clean' nature of the residue, i.e. the absence of amorphous organic material.

Fig. 7. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using an overnight pre-treatment with formic acid. Slide 'formic acid, test B', England Finder coordinate N50/4. The specimen of *Lycospora pusilla* in the centre is 33 μm in maximum diameter. Note the abundance of *Lycopodium* spores.

Fig. 8. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using an overnight pre-treatment with household bleach (a 5% solution of sodium hypochlorite). Slide 'NaOCl, test B', England Finder coordinate O40/2. The monolete spore in the upper-right is 45 μm in diameter. Note the light (bleached) Carboniferous miospores, and the complete absence of *Lycopodium* spores which have been destroyed by the bleach.

Fig. 9. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using a pre-treatment with household bleach (a 2.5% solution of sodium hypochlorite) for six

hours. Slide '2.5% NaOCl, 6 hours', England Finder coordinate O48. The prominent specimen of *Lycospora pusilla* in the centre-left is 31 μm in maximum diameter. Note the apparent absence of *Lycopodium* spores; these are present but in relatively low numbers (Table 1). This reflects partial destruction of the *Lycopodium* spores by the bleach. Note also the fact that the Carboniferous spores are only slightly bleached, as compared to the significantly lightened forms in Fig. 8.

Fig. 10. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using an overnight pre-treatment with methylated spirits. Slide 'meths, test B', England Finder coordinate N66/1. The prominent specimen of *Lycospora pusilla* in the upper-centre is 33 μm in maximum diameter. Note the well-preserved Carboniferous miospores and the absence of amorphous organic material.

Fig. 11. A representative low-magnification photomicrograph of the organic residue from the subsample of sample MPA 57940 which was prepared with $(NaPO_3)_6$ using an overnight pre-treatment with white spirit. Slide 'white spirit, test B', England Finder coordinate O69/3. The specimen of *Lycospora pusilla* in the upper-right is 31 μm in maximum diameter. Note the abundant, well-preserved Carboniferous miospores and the absence of amorphous organic material.

Table 1

The key data in this study. The numbers of Carboniferous miospores and marker *Lycopodium* spores which were counted, the dry weight of the rock sample that was broken down, the indigenous palynomorph concentrations (based on the actual weight prepared and 5.0 g) and the absolute numbers of indigenous palynomorphs based on 5.0 g are given for each of the subsamples prepared. It should be noted that the numbers and concentrations of palynomorphs in the row pertaining to the pre-treatment with 2.5% household bleach for six hours (italicised) are entirely spurious due to the destruction of large numbers of the exotic *Lycopodium* spores. The italicised abbreviations (e.g. m_c) refer to equation of Benninghoff (1962) where appropriate.