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An overview of techniques applied to the extraction of Non-Pollen Palynomorphs, their known taphonomic issues, and recommendations to maximise recovery

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Please accept my apologies for missing these minor corrections during the original revisions stage.

Line numbers refer to the tracked changes manuscript file

Consistency in use of glycerin and glycerine:

Corrections made in lines 328 and 340.

Inconsistent use of uppercase/lowercase in reference to panels in figure 1

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An overview of techniques applied to the extraction of Non-Pollen Palynomorphs, their known taphonomic issues, and recommendations to maximise recovery

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Abstract

This chapter synthesises the most common processing techniques applied to palynomorphs and their known issues. We primarily focus on NPPs, but include studies on pollen grains where the information might be relevant. An overview of recent (2017-2019) NPP publications is used to connect the most common techniques to known taphonomic issues. Finally, general recommendations are made to minimise processing bias and maximise NPP recovery.

1. Introduction

The techniques applied to the extraction and analysis of NPPs originate from the techniques applied to pollen analysis (Assarsson and Granlund, 1924) and are often unchanged in many recent studies (e.g.; Pound et al., 2019). However, as the process is described by Moore and

Webb (1978 pg. 22) "*The techniques involved in the process are aimed at the disintegration and dissolution or otherwise removal of the non-pollen matrix in the sediment*". Whether an intentional phrasing or otherwise, the emphasis placed on this quotation by the present authors should immediately raise issues with individuals seeking to study NPPs as well as, or instead of, pollen. It is possible to view NPPs in thin and polished rock sections (Munnecke and Servais, 1996; Hower et al., 2009; O'Keefe et al., 2011), but this is sometimes a chance encounter rather than a planned liaison. Whether the purpose of a study is taxonomical, morphological, ecological, or climatological, it is beneficial to maximise the quantity of specimens available for study, make them as identifiable as possible, and mount them in a manner appropriate to the observation techniques being applied.

In this overview chapter we detail the wide variety of processing techniques now in use, summarise concerns and criticisms raised against some treatments, and propose minimal processing as best practice for recovery of a wide array of NPPs. Some of this literature is focussed on pollen, but in this situation we speculate as to the likely comparability with the various groups of NPPs. Finally, we have surveyed articles reporting NPPs from 2017-2019 that were published in the Journal of Micropalaeontology; Palaeogeography, Palaeoclimatology, Palaeoecology; Palynology; and Review of Palaeobotany and Palynology to identify current consensus processing. We then link these consensus techniques to the published concerns on techniques to raise awareness of how as a community we might be influencing our results.

2. Liberation - Extraction and concentration

Whether working from rock (Wood et al., 1996; Batten, 1999; Traverse, 2007; Brown, 2008), sediment (Batten, 1999), amber (Brown, 2008; Halbwachs, in press), soil (Brown, 2008), faeces (Callen and Cameron, 1955; Callen, 1963; Martin and Sharrock, 1964; Bryant, 1974 a,b; Bryant and Williams-Dean, 1975; Reinhard and Bryant, 1992; Bryant and Holloway, 1996; Marshall, 1999; Marshall, 2008), forensic (Wiltshire, 2016; Horrocks, 2004; Bryant et al., 1996;), honey (Louveaux et al., 1978; Lieux, 1980; Jones and Bryant, 2004; Salonen et al., 2009; O'Keefe and Wymer, 2017; Sniderman et al., 2018) or modern (Erdtmann, 1943; Moore and Webb, 1978; Faegeri and Iverson, 1989) samples, a common aim is to remove the material of non-interest and concentrate as much interesting material of as many types as possible (Moore et al., 1991; Coil et al., 2003). This can be achieved through disaggregation, dissolving and digesting the

matrix, sieving and/or flotation. Numerous summaries, syntheses and reviews of palynological processing methods exist, including Bryant and Wrenn (1998), Riding and Kyffin-Hughes (2004) and Brown (2008), but none so far focuses on NPPs.

2.1. Acid digestion

Typical methodologies for the extraction of palynomorphs from sediments or sedimentary rocks involve the digestion of the mineral matter by hydrochloric (HCl) and hydrofluoric (HF) acids (e.g. Assarsson and Granlund, 1924; Traverse, 2008; Brown, 2008; O'Keefe and Eble, 2012; Pound et al., 2019). The concentration of acid typically depends on the sediment being digested, but 10-37% HCl and 40-60% HF are among the most frequent reported in the literature (O'Keefe and Eble, 2012; Pound et al., 2019). The former is primarily used to treat carbonates, whilst the latter is used to remove silicates (Moore et al., 1991). Sarmiento (1957) proposed a 50% solution of orthophoshoric acid as an alternative to HCl for the removal of carbonates (Staplin et al., 1960). This was proposed to be as efficient but gentler than the use of HCl (Sarmiento, 1957; Staplin et al., 1960). HCl is frequently used to prevent the formation of calcium fluoride following HF treatment, however, boric acid can also be used. It must be noted, however, that in neither case does the resultant solution become non-toxic or non-corrosive. Samples intended for HF treatment should always be pre-treated with HCl to ensure no carbonates are present to react with HF acid (Staplin et al., 1960). Clay rich sediments can take considerably longer/require more HF due to the clay particles aggregating around other particles (Bates et al., 1978). Warming and agitating the sample on a shaking table facilitates the digestion in samples rich in siliceous minerals (Herngreen 1983; Green, 2001). Both acids are hazardous, and in the case of HF, potentially life-threatening, which has led to techniques that avoid the use of acids for the removal of the mineral fraction.

While HF is generally considered to be non-damaging to palynomorphs, recent work has shown that this is not the case. Van Geel (2001) speculated that HF acid treatment might damage fungi and Clarke (1994) found that large buoyant fungal forms were lost during a treatment procedure involving HF. O'Keefe and Eble (2012) demonstrated that processing methods that use HF reduce the overall concentration values of palynomorphs obtained from clay-rich samples. Tintinnid and other cysts are known to be damaged by long-term immersion in HF and heated

HF treatment can destroy some dinoflagellate cysts (Reid and John, 1981; Mudie et al., 2010; Mertens et al., 2012).

Use of HCl is also problematic, especially use of hot HCl, which has been shown to adversely impact palynomorph preservation following HF treatment. Concentrated HCl is known to modify the colour of fresh pollen to yellow-green (Southworth, 1974). In their work on coprophilous fungi, van Asperen et al. (2016) showed better abundance recovery (closer to unprocessed) when both 10% HCl treatment and alkali digestion (see below) were omitted.

2.2. Non-acid techniques

Depending on the sample being processed there can be no need to use acids at all: unconsolidated or poorly consolidated sediments, forensic and honey samples can all be easily processed without acid digestion. Processing honey requires dissolving the sugars in water and then changing the specific gravity with ethyl alcohol (ETOH) or isopropyl alcohol (IPA) to ensure small pollen grains are not lost in the supernatant (Jones and Bryant, 2004). A similar procedure can also be achieved for sediments or sedimentary rocks using heavy liquid separation to adjust the specific gravity. Zinc bromide, zinc chloride and bromoform have been used for over 60 years (Frey, 1955; Staplin et al., 1960; Brown, 2008; Pound et al., 2012; Halbritter et al., 2018). Since the 1980s there has been a progressive shift to non-toxic and more easily recyclable heavy liquids such as sodium polytungstate (Munsterman and Kerstholt, 1996), sodium metatungstate (Krukowski, 1988) and lithium heteropolytungstate (O'Keefe and Eble, 2012; Caffrey and Horn, 2013; Van Ness et al., 2017; Leipe et al., 2019). As well as being non-toxic, heavy liquids based on inorganic tungsten have been reported to improve dinocyst recovery (Munsterman and Kerstholt, 1996). Methodological comparisons of heavy liquid separation and HF digestion produce comparable results for pollen analysis, with some studies reporting better pollen extraction with heavy liquids (Nakagawa et al., 1998; Lentfer and Boyd, 2000; Campbell et al., 2016; Leipe et al., 2019). Some studies also report a possible sediment specific reduction in pollen abundance when using heavy liquid separation over HF acid digestion (Leipe et al., 2019). This may be due to the presence of pyrite, which can result in palynomorphs with mineral growths/encrustations to sink in the heavy liquid separation (Barss and Williams, 1973; Leipe et al., 2019). Grey (1999) also found that the centrifuging during heavy liquid separation caused the fragmentation of large acanthomorph acritarchs. Gelsthorpe (2002) also investigated the

implications of centrifuging and heavy liquid separation. He found that Silurian samples required three rounds of centrifuging and extraction to ensure the relative proportions of genera was stable (Gelsthorpe, 2002).

Swirling a disaggregated sample can be an economical means of extracting the lighter fraction from the heavier mineral dominated fraction (Green, 2001; Riding and Kyffin-Hughes, 2004; 2006). Many methods of swirling exist. For samples small enough to fit in a watch glass, a simple rotary motion in one hand and a pipette in the other to capture the “plume” of finer particles, whilst the denser particles coalesce into the centre of the watch glass can be used (Green, 2001; Riding and Kyffin-Hughes, 2004; 2006; Traverse, 2008). For larger samples in a tri-cornered beaker, the beaker is swirled until the entire sample is entrained in the liquid, placed on a lab bench to permit the heavy materials to settle, then the supernatant is poured off and retained. This process is repeated until the supernatant is completely clear and no material is entrained when the beaker is swirled. In either case, swirling can clean a sample rich in sand, pyrites or other denser minerals that were retained following disaggregation and/or HF-treatment (Brown, 2008; Traverse, 2008). However, in all cases, the denser fraction should also be kept and examined, in case palynomorphs of interest have not separated or larger NPPs, especially those which may contain pyrite crystals, were retained (Green, 2001; Riding and Kyffin-Hughes, 2004; 2006).

Hydrogen peroxide is used to physically and chemically disaggregate rock samples, but should be kept to short durations to avoid oxidation of material (Hopkins and McCarthy, 2002; Williams et al., 2005). The resulting residue is then sieved to isolate the required size faction from unwanted material (Williams et al., 2005; Riding and Kyffin-Hughes, 2004; 2006).

Unconsolidated sediments can be disaggregated and sieved (selecting sieve mesh sizes appropriate for the target NPP). The sieving of clay rich sediments can be facilitated with a deflocculant, such as sodium hexametaphosphate, surfactants (such as Alconox®, Liquinox® or Teepol®) (Riding and Kyffin-Hughes, 2004; 2006), or sodium pyrophosphate (Brown, 2008; Heusser and Stock, 1984; Bates et al., 1978). Care must be taken with the use of sodium hexametaphosphate as in concentrated solutions it is mildly oxidizing (see below) and of high enough density that it can cause palynomorphs to remain in the float fraction following centrifugation, resulting in loss upon decanting. The choice of deflocculant used must be made in consultation with local regulations for phosphate in wastewater; in some areas both sodium

hexametaphosphate and sodium pyrophosphate must be collected and disposed of as hazardous waste.

2.3. Alkali digestion

Potassium hydroxide (KOH) or ammonium hydroxide (NH_4OH) (Batten, 1999) can be used to react depolymerized humic acids with their conjugate base pairs to produce soluble organic salts, which can be removed through water-washing (Green, 2001; Riding and Kyffin-Hughes, 2004; pers. comm. C.F. Eble to J. O'Keefe and M. Pound, 2019). A 5-10% solution of KOH (or NH_4OH) is added to the sample and either allowed to sit at room temperature or immersed in a water bath at 100°C for from 3-10 minutes to up to several days (especially in the case of peats and coals) (Green, 2001; Riding and Kyffin-Hughes, 2004), diluted with distilled water, centrifuged and decanted. This process is typically repeated until the supernatant is clear, especially in organic-rich samples. When the supernatant is close to clear, the reaction may be halted by adding a few drops of 10% HCl to neutralize any remaining alkali. Modern sporopollenin is somewhat soluble in KOH (Southworth, 1974), becoming less so with increasing thermal maturity. KOH has been linked with selective destruction of gonyaulacacean dinoflagellate cysts from the Cretaceous (Dodsworth, 1995). Boiling in KOH may cause a slight increase in pollen size (Reitsma, 1969), but a decrease in fungal spore size (van Asperen et al., 2016). KOH digestion damages testate amoebae cysts, reducing the identifiable specimens (Hendon and Charman, 1997). Both ancient and recent dinoflagellate cysts are also known to be susceptible to damage from KOH processing (Dodsworth, 1995; Hendon and Charman, 1997; Mertens et al., 2009). Indeed, Schrank (1988) noted that the periphragm separated from the endophragm in dinocysts treated with both nitric acid and KOH after treatment with 5% KOH and was unrelated to the length of treatment with nitric acid.

2.4. Sieving, filtering, sonication, and centrifugation

Sieving at a range of sieve sizes (from 750 to 100 micrometers, with base sieves of 63 and 35 micrometers to separate "fine", "coarse", and "very coarse" fractions in some studies of marine sediments) is used to aid in the extraction and concentration of palynomorphs. This is most frequently accomplished using metal or nylon mesh sieves. In all cases, the mesh size is based

on the width of the square openings in the sieve. The diagonal (corner to corner) distance in the sieve mesh is larger than the certified mesh size, which can lead to the loss of material that is, in one dimension, close to the size of the sieve mesh. Sieving at any size can result in loss of larger NPPs, such as large algae, testate amoebae, microthyriaceous fungi and fungal perithecia, as well as arthropod and tardigrade remains.

In addition to sieving, samples are often filtered to remove excess clay or fine zeolites, usually after acid treatments. Filtering at <10 µm is widely used, especially in non-acid preparations (Riding and Kyffin-Hughes, 2004; 2006). Many laboratories use fine nylon mesh sieves for filtering, often in the form of Nitex® or similar cloth or nylon mesh cell strainers (Urban et al., 2018). These fine sieves have the same problem as larger ones - NPPs that are close in size to the mesh size in one or more dimensions (including the diagonal) may slip through. A way around this problem is the use of Nylon filter membranes in a filtration apparatus, which have round, rather than square, openings with a maximum diameter of the listed gauge. Regardless of the type of filtration medium, the aperature size is known to impact NPP recovery. Lignum et al. (2008) found that a 15 µm nylon mesh lost an average of 5.8±1.2% of *Lycopodium* spores whilst a 15 µm polyester mesh lost 3.9±0.7%. No complete dinocyst was observed to pass through either of the 15 µm meshes, but fragments and operculae did (Lignum et al., 2008). For example, Silurian acritarchs may be lost when using a 10 micrometre mesh, but are retained by 7 µm mesh (Gelsthorpe, 2002). An even smaller mesh size, or not using fine sieves or filters at all is recommended for study of fungal spores, as palaeoecologically important small fungal spores may be lost through a 6 µm sieve (van Asperen et al., 2016).

Choice of a top sieve mesh size and bottom filter size really depends on the focus of the individual scientist. For example, are you limiting your study to microfossils only (by definition, those from about 5 micrometers to 1000 micrometers or one mm), or do you wish to co-analyze mesofossils (by definition, those from 1 to 100 mm), or nanofossils (those 5 micrometers and smaller), or limit the study to a fraction of the microfossil assemblage? Regardless, the choice of sieve and/or filter size should be reported and justified. Additionally, problems have been reported with palynomorphs adhering to the mesh (Bryant, 2017). Sieve sonication (see below) may circumvent this problem. Adherence to nylon membrane filters, frequently used in vacuum assisted filtration (Vidal, 1988; O'Keefe and Eble, 2012) is less problematic - the entire filter may be tucked into a polypropylene test tube which is then partially filled with distilled water, vortexed, and rinsed, thus removing any adhered grains.

Sonication has been applied to various NPP studies since the early days of the science. It is a simple and effective way to disaggregate siliciclastic samples and to shake palynomorphs free of fibrous peat. Great care must be taken when applying sonication, however, as it is known to damage NPPs of all types (Mertens et al., 2012; Jones, 2014; Perrotti et al., 2018). Sonication can be completed using an ultrasonic bath to treat whole samples in test tubes or an ultrasonic wand/horn to treat samples in sieves or filters (Perotti et al., 2018; Leipe et al., 2019). Sonic wands may be tunable or untunable. Both devices can cause damage to pollen and dinoflagellate cysts if applied at the strongest settings or for extensive time intervals (Mertens et al., 2009; 2012; Jones, 2014; Perrotti et al., 2018). Untuned ultrasonic, especially, has been shown to fragment bisaccate pollen grains (Leipe et al., 2019). Short applications of tuned ultrasound (20 seconds to 5 minutes), however, have been shown to be effective in facilitating disaggregation and sieving of palynological samples with minimal damage (Mertens et al., 2009; Price et al., 2016; Perrotti et al., 2018). Low amplitude sonication for long time periods (up to 4 min/sample) with a sonification horn has been shown to be especially effective in removing charcoal while not damaging palynomorphs (Perrotti et al., 2018).

Centrifuging is commonly used to separate the solid (including objects of interest) from the liquid during individual stages of processing. Centrifuging can cause damage to delicate palynomorphs (Grey, 1999; Green, 2001; Urban et al., 2018) or the loss of small/lighter specimens (through decanting) if insufficient spin time is used or the specific gravity is not modified to capture these specimens (Green, 2001; Jones and Bryant, 2004). Cellulose filters have been applied as an alternative to centrifuging in preparing honey for acetolysis (see section 3.1). Dissolved honey is poured through a 0.45-µm pore filter paper and the filter paper, with palynomorph content, is subjected to acetolysis (Lieux, 1980). This procedure eliminates a centrifuging stage, possibly limiting damage/loss of material, and could be applied to NPP studies where acetolysis will be applied. Likewise, use of nested cell sieves can be used to reduce centrifugation-caused damage to NPPs (Urban et al., 2018).

3. Spa treatment – beautifying and cleansing

Once liberated from their sediment shackles, palynomorphs may require some further treatment to be of value to the observer. This is particularly true of modern or Holocene pollen analysis where acetolysis is applied to clean and exaggerate surface textures. If samples contain

abundant woody material, or are thermally mature, they require oxidation to make the palynomorphs more recognisable and the wood more readily attacked by alkali (see below).

3.1. Acetolysis

Acetolysis is commonly used to remove organic remains of perceived limited interest as well asthe non-sporopollenin components of pollen grains, and to swell pollen grains, making the ornamentation easier to observe (Erdtman, 1960; Moore et al., 1991; Halbritter et al., 2018). The degradation of cellulose (and other polysaccharides) through the process of acetolysis, treatment with a solution of acetic anhydride and sulfuric acid, is routinely used on palynomorph samples from the Holocene and modern (Erdtman, 1960; Guthrie and McCarthy, 1967; Pound et al., 2018; Halbritter et al., 2018). Acetolysis solution is most frequently made using a 9:1 ratio of acetic anhydride and sulfuric acid, although other ratios may be used (Brown, 2008). The reaction of acetolysis solution with the palynomorph residue is most frequently quenched through the addition of glacial acetic acid, which is then centrifuged and decanted into a waste bottle for environmentally appropriate disposal. Some laboratories do not quench the reaction, rather simply centrifuge and decant the acetolysis solution. This method is risky, as acetolysis solution is explosive on contact with water and any remaining acetolysis solution may react adversely as processing transitions to water. Acetolysis may be completed either warm or cold. Cold acetolysis is performed at room temperature. Warm acetolysis typically takes place in a heating-block, sand-bath, or water-bath (not recommended, see above) at 80-100°C, with 90°C being considered optimal. Likewise the length of time the acetolysis solution is left on the sample varies from 3-10 minutes, depending upon fragility of the grains, relative amount of non-sporopollenin material present that must be removed, and whether the scientist wishes to darken the palynomorphs to a rich bourbon colour (9-10 minutes).The process is not without critique, especially in the study of NPPs.

Acetolysis of palynological samples destroys all but the sporopollenin, and this, too, can start to be damaged after 10 minutes (Hesse and Waha, 1989; Jardine et al., 2015; Ulrich et al., 2017). It is known to damage a wide range of NPPs including: dinoflagellate cysts (Marret, 1993; Mertens et al., 2009), *Pediastrum* (Komárek and Jankovská, 2001), coprophilous fungi (van Asperen et al., 2016), desmids (Riddick et al., 2017), and *Trachelomonas* (Shumilovskikh et al.,

2019). Acetolysis is also known to affect the size of pollen grains (Reitsma, 1969; Meltsov et al., 2008) and may cause similar swelling in NPPs (van Asperen et al., 2016).

3.2. Alternatives to acetolysis

With a growing body of evidence showing acetolysis to be unfavourable to an in-depth study of NPPs (see the above section), any alternative that can offer the sample cleaning benefits and provide better NPP recovery would be advantageous. Several alternatives to acetolysis have been explored (Schols et al., 2004; Brown, 2008). The use of the enzymes pectinase and cellulase (Schols et al., 2004; O'Keefe and Wymer, 2017) is gaining popularity, although care must be taken to use the appropriate number of units of enzyme per unit palynomorphs, maintain the pH of the buffer solution, and to use fresh enzymes. Too few units of enzyme will not effectively remove kitt and nuclear material; likewise older batches of enzymes may not retain enough efficacy to sufficiently clean the grains. Treatment with 10% KOH has also been found effective (Schols et al., 2004; Brown, 2008), however this may result in damage to NPPs as noted above.

3.3. Oxidation of palynomorphs

Although not commonly used on modern or Holocene samples, many deep-time samples occur co-mingled with woody material, amorphous organic matter and/or have a thermal maturity above the peat stage. To aid palynomorph visibility and identification potential, these samples may necessitate a degree of oxidation prior to alkali treatment to release palynomorphs and to depolymerize lignin. Following oxidation, molecules are then susceptible to attack by their conjugate alkalis, producing water-soluble salts, which can then be rinsed away (Green, 2001; Eble pers. comm. to J. O'Keefe and M. Pound, 2019). Additionally, many palynomorphs darken with thermal maturity, which can obscure accurate identification; oxidation can lighten these palynomorphs. Oxidation is known to damage palynomorphs of all types (Green, 2001). For this reason, the most gentle oxidant that will effectively depolymerize lignin for a sample's thermal maturity should be chosen (O'Keefe and Eble, 2012), and they should be applied for a minimum amount of time. Which oxidant and for how long will need to be determined on a site-by-site basis. In general, hypochlorous acid has been applied to samples with low thermal maturities

(equivalent to lignite), nitric acid to medium thermal maturities and Schulze's solution (concentrated nitric acid saturated with potassium chlorate) to higher thermal maturities (equivalent to bituminous coal) (O'Keefe and Eble, 2012). Sodium hypochlorite (NaOCl, common household bleach) has been applied to undeterminable dark-hued palynomorphs (Buratti and Cirilli, 2011; O'Keefe and Eble, 2012). Nitric acid is known to break down sporopollenin and modify its chemical structure, although limiting treatment to less than 10 minutes still preserves some of the sporopollenin chemistry (Jardine et al., 2015). Over-oxidation of any organic-walled NPPs can result in their loss (Schrank, 1988). Generally, some fungal NPPs are more refractory than pollen, plant spores, or algae, although they, too (particularly more delicate forms), can be lost (van Asperen et al., 2016).

4. Mounting

Residues are most frequently examined under light microscopy (LM), although scanning electron microscopy (SEM) is becoming more frequent. Glass slides with number 0 or number 1 coverslips are commonly used for mounting; number 0 coverslips are difficult to find in many countries but produce less light interference and superior image crisp-ness. It is well documented that the choice of mounting medium for LM can substantially impact visibility and size of the NPPs under observation (Coil et al., 2003; Meltsov et al., 2008). The refractive index of organic-walled NPPs is highly variable, and only well defined for siliceous, carbonate, and starch-based NPPs. In general, a mounting medium with a refractive index greater than 1.5 is sufficient for all but starch-based palynomorphs, which benefit from a medium with a slightly higher refractive index. Both solid (Permount®, PVA, Elvacite®, Glue4Glass®, MeltMount®, EuKitt®, canada balsam, glycerin jelly) and liquid (glycerin USP (glycerol), silicone oil, immersion oil) mounting media are commonly used. All are suitable for observation of multiple NPP types, however, silicone oil's refractive index of ~1.4 is too close to that of siliceous NPPs (Coil et al., 2003) and should be avoided when the goal is to co-analyze these with organic-walled NPPs. Acetolyzed organic-walled microfossils mounted in glycerin USP tend to swell somewhat and appear larger than those in silicone oil; this does appear to be less of a problem with un-acetolyzed grains (Meltsov et al., 2008).

Beyond caveats about refractive indices and swelling grains, type of mount should be chosen for purpose, i.e., if you suspect you may need to remove single grains to make type mounts or

for SEM work, you need a mounting medium that is easily reversible in your laboratory. MeltMount® and Canada Balsam can be reversed by heating to liquid then dissolving with a solvent (acetone for the former and warm detergent for the latter, although the latter is extremely difficult to reverse). Permount®, EuKitt®, can be dissolved in xylene, as can immersion oil. Elvacite® is soluble in Cellosolve® (ethylene glycol monoethyl ether). PVA from Elmer's School Glue® is soluble in soapy water. Glycerin jelly is reversed by re-heating and adding a minute drop of water, at which point specimens can be removed with a micropipette (Evitt, 1984). Both glycerin USP and silicon oil are soluble in 90% ethanol and water, although water takes longer to dissolve the latter. Mounting for SEM study should follow the "single grain" technique promoted by Daghlian (1982), developed by Zetter (1989) and detailed, most recently, by Halbritter et al. (2018)

Liquid mounts generally need to be sealed to prevent the mounting medium from drying out or escaping from beneath the coverslip. A variety of compounds can be used, including wax beads which are melted to produce a seal, clear acrylic resin (superglue), clear 2-part epoxy, and nail lacquer. Choosing the right nail lacquer is a perennial problem for palynologists; advice on choosing the most appropriate, i.e., one that will stick to the glass and not peel up or be subject to shrinkage problems can be found in Caffrey and Horn (2012).

5. Overview of techniques reported in select recent literature

So far we have provided an overview of techniques applied to the extraction of NPPs and known issues with these. To provide an insight into how widespread some of our fields processing biases might be, we have surveyed all publications in Journal of Micropalaeontology, Palaeogeograpy Palaeoclimatology Palaeoecology, Palynology and Review of Palaeobotany and Palynology released the past three years (2019, 2018 and 2017) for those reporting NPPs. From this targeted survey, 162 articles reported NPPs and here we present a summary of the processing techniques provided. This is not a thorough literature review and neither is it aimed at naming and shaming, but it is an attempt to highlight commonly used techniques and link these back to known processing issues in an anonymized way to demonstrate potential widespread processing biases..

From the 162 articles reporting NPPs published between 2017 and 2019 in the selected journals, 18.5% (n = 30) presented their methods as "standard processing techniques" without

364 further elaboration (Fig. 1A). These are excluded from the remainder of this section bringing the
365 total number to 132 articles. Over the last three years 53.8% (n = 71) of articles have used HCl
366 with 56/71 articles reporting the concentration of the acid. In general, the older the material the
367 stronger the acid used (Fig. 1C). If HCl is the cause of the observed processing bias van
368 Asperen et al. (2016) reported, then this could have affected over half of all studies in our small
369 survey of the literature. Whilst we are unaware of a systematic study of the impact of different
370 HCl concentration on NPPs, Dale (1976) reported that concentrated hot HCl destroyed recent
371 *Peridinium* cysts. HF use is even more widespread than HCl with 91.7% (n = 121) of articles
372 reporting NPPs having used this for the dissolution of silicates. However, only 52.4% (n = 55) of
373 articles report the concentration of HF used; with 10 - 72% HF reported (Fig. 1B). Although
374 commonly thought of as innocuous to palynomorphs, increasing numbers of studies are
375 showing, or suggesting, HF to be detrimental (Reid and John, 1981; Van Geel, 2001; Mudie et
376 al., 2010; Mertens et al., 2012; O'Keefe and Eble, 2012).

377 Alkali digestion is reported in 28.8% (n = 38) of articles. KOH is the more frequently used (n =
378 33) with NaOH only being reported in five articles that were focused on modern or Holocene
379 samples. Whereas KOH has been applied to samples covering the whole Phanerozoic. Of the
380 21 articles that report the concentration of KOH, 10% or less is the most frequent, with only one
381 article reporting the use of 100% KOH. Only six articles report the time that samples were
382 treated with KOH and these vary from 5 to 15 minutes. Despite widespread knowledge that
383 KOH damages and destroys dinoflagellate cysts (Schrank, 1988; Dodsworth, 1995; Hendon and
384 Charman, 1997; Mertens et al., 2009), four studies of dinoflagellate cysts in our survey of recent
385 literature used KOH in concentrations of 10% or more. The implications of size changes in
386 fungal spores treated with KOH or NaOH should also be carefully considered before further
387 processing (sieving) is undertaken and when morphological or abundance data is the target
388 outcome of the work (see results of van Asperen et al., 2016).

389 Heavy liquid separation use in surveyed NPP articles has most commonly been used in
390 combination with acid digestion. Only four of the 37 methodologies that report heavy liquid
391 separation have used it as an alternative to acid digestion, suggesting that it is being used as a
392 supplementary, rather than a non-acid alternative, treatment (Fig. 1D). Heavy liquid separation
393 alone has been shown to improve palynomorph recovery and could be of immense benefit to
394 future NPP studies (Nakagawa et al., 1998). However, concerns have been raised around
395 fragmentation and recovered abundance data in acritarchs and further systematic exploration of
396 these observations is needed for all NPP groups.

Acetolysis is reported in 24.2% (n = 32; Fig. 1E) of NPP articles published in the three years surveyed. Studies on the modern, Holocene, and Quaternary dominate the studies utilising acetolysis (n = 30). Although not commonly applied to “deep-time” samples, acetolysis use has been reported on two samples of pre-Quaternary age: dinoflagellate cyst samples from the Cretaceous and Paleocene; despite the known damage it can cause (Marret, 1993; Mertens et al., 2009). Despite the known damage to NPPs from acetolysis, nearly a quarter of studies surveyed continue to use it introducing potential taphonomic implications for reported data on: dinoflagellate cysts (Marret, 1993; Mertens et al., 2009), *Pediastrum* (Komárek and Jankovská, 2001), coprophilous fungi (van Asperen et al., 2016), desmids (Riddick et al., 2017), and *Trachelomonas* (Shumilovskikh et al., 2019). Oxidation is reported in 24.2% (n = 32) of the surveyed literature, mostly in pre-Quaternary studies on dinoflagellate cysts, acritarchs and other algae. Most commonly (n = 24), this is nitric acid >50% for <180 seconds. Only eleven studies report the use of Schulz Solution; the treatment time ranges from 45 to 1800 minutes. However, not all studies report the concentration of the nitric acid used and/or the time the oxidation process was applied for. Considering it is well established that it can have damaging and biasing impacts on any results (Mertens et al., 2009), these should always be reported..

6. General recommendations

The concept of taphonomic filtering is well established in palaeontological and archaeological research (Behrensmeyer et al., 2000). When you consider that the dispersal, death, decay, deposition, preservation and fossilisation of palynomorphs has enacted a substantial taphonomic filter on data before it is even generated (Ferguson, 2005), it becomes more pressing that the one taphonomic filter we can control – processing – is fully reported in detail. This permits others to recognise any further taphonomic filter applied to the data and the implications this has for interpretations (Coil et al., 2003). All processing techniques apply a taphonomic filter (Fig. 2), some we know to be especially detrimental to NPPs (e.g. acetolysis), others are speculated or under-investigated (e.g. HF; heavy liquids). In the past three years only 18.5% (n = 30; Fig. 1A) of articles reporting NPPs have failed to accurately detail their methods.

Among the most commonly used processing techniques in palynology, acetolysis has shown to be most risky. We recommend avoiding acetolysis in studies where multiple NPP types are to be analyzed. If acetolysis cannot be avoided, the duration and temperature of acetolysis must

be reported and commentary about potential NPP losses must be made. Oxidation is known to be damaging to most organic-walled NPP groups. Use of oxidation should be limited to the bare minimum necessary to liberate palynomorphs from rocks, and for modern material, ought to be completely avoided. Likewise, alkali treatment should be avoided where possible, especially for modern material, and of limited duration for organic-rich fossil material. As in the case of acetolysis, all chemical treatment should be detailed, as should its potential impacts on the NPP spectrum obtained. As a final consideration, the choice of mounting media for LM work should be documented, and if siliceous NPPs are to be co-analyzed, it should be of sufficiently high (or low) (<1.4<) refractive index to permit examination of these organisms' remains.

As an optimal standardization, especially for fossil material, we strongly recommend the simplest processing possible - disaggregation in a non-phosphatic deflocculant using gentle stirring, rather than sonication, sieving through a top mesh of 1-mm to remove very coarse material, and short-centrifugation or nylon membrane filtration followed by swirling to remove excessive clays and heavy minerals.

However, we also recognize that this may not be practical under site-specific circumstances. Peat samples may benefit from short alkali treatment while coal or NPPs from highly thermally altered sediments may benefit from oxidation followed by alkali treatment. Ultimately, we need more studies on the taphonomic filtering that these techniques have on NPPs. Whilst there have been important studies recently and in the past (not all have been cited in this chapter), we are still reliant on inferring likely impacts on NPPs from pollen and spore studies (although dinoflagellate cysts are nearly as well investigated). Whilst this provides useful indications, the myriad of NPP groups are biologically, structurally and chemically distinct from plant propagules and so their reaction under processing may be different (see the differing KOH-induced palynomorph size changes reported by Reitsma (1969) and van Asperen et al. (2016) as one example). While we continue to explore the taphonomic filter applied by processing on the various NPP groups, our take-home message has to be: the minimal processing needed to obtain NPPs relevant to your study is best and the standard procedure/method should be to fully document the entire process in detail.

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Figure 1. Visual summary of key findings from the survey of NPP literature published during 2017, 2018 and 2019 in Journal of Micropalaeontology; Palaeogeography, Palaeoclimatology, Palaeoecology; Palynology; and Review of Palaeobotany and Palynology. A. Level of detail provided in the methodology, pale = “standard procedure” and dark = details provided. B. Use of HF: dark = HF used, pale = no HF used. C. Concentration of HCl used grouped by age of material being studied. D. Heavy liquid use: pale = heavy liquid and acid digestion; dark = heavy liquid without acid digestion. E. Use of acetolysis: pale = acetolysis not used; dark = acetolysis used.

Figure 2. Summary of taphonomic filtering introduced during the three principal stages of processing. In the bottom right corner is an example method flow diagram showing the hypothetical taphonomic loss of NPP abundance. This is a diagrammatic summary of key taphonomic filters introduced in the text designed to give the reader an easy to refer to scheme and way to quickly evaluate how their intended methods may introduce bias. As more research is conducted on the processing of NPPs it is anticipated that this diagram can be expanded with detail and potentially introduced uncertainty for each process.

An overview of techniques applied to the extraction of Non-Pollen Palynomorphs, their known taphonomic issues, and recommendations to maximise recovery

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Abstract

This chapter synthesises the most common processing techniques applied to palynomorphs and their known issues. We primarily focus on NPPs, but include studies on pollen grains where the information might be relevant. An overview of recent (2017-2019) NPP publications is used to connect the most common techniques to known taphonomic issues. Finally, general recommendations are made to minimise processing bias and maximise NPP recovery.

1. Introduction

The techniques applied to the extraction and analysis of NPPs originate from the techniques applied to pollen analysis (Assarsson and Granlund, 1924) and are often unchanged in many recent studies (e.g.; Pound et al., 2019). However, as the process is described by Moore and

Webb (1978 pg. 22) "*The techniques involved in the process are aimed at the disintegration and dissolution or otherwise removal of the non-pollen matrix in the sediment*". Whether an intentional phrasing or otherwise, the emphasis placed on this quotation by the present authors should immediately raise issues with individuals seeking to study NPPs as well as, or instead of, pollen. It is possible to view NPPs in thin and polished rock sections (Munnecke and Servais, 1996; Hower et al., 2009; O'Keefe et al., 2011), but this is sometimes a chance encounter rather than a planned liaison. Whether the purpose of a study is taxonomical, morphological, ecological, or climatological, it is beneficial to maximise the quantity of specimens available for study, make them as identifiable as possible, and mount them in a manner appropriate to the observation techniques being applied.

In this overview chapter we detail the wide variety of processing techniques now in use, summarise concerns and criticisms raised against some treatments, and propose minimal processing as best practice for recovery of a wide array of NPPs. Some of this literature is focussed on pollen, but in this situation we speculate as to the likely comparability with the various groups of NPPs. Finally, we have surveyed articles reporting NPPs from 2017-2019 that were published in the Journal of Micropalaeontology; Palaeogeography, Palaeoclimatology, Palaeoecology; Palynology; and Review of Palaeobotany and Palynology to identify current consensus processing. We then link these consensus techniques to the published concerns on techniques to raise awareness of how as a community we might be influencing our results.

2. Liberation - Extraction and concentration

Whether working from rock (Wood et al., 1996; Batten, 1999; Traverse, 2007; Brown, 2008), sediment (Batten, 1999), amber (Brown, 2008; Halbwachs, in press), soil (Brown, 2008), faeces (Callen and Cameron, 1955; Callen, 1963; Martin and Sharrock, 1964; Bryant, 1974 a,b; Bryant and Williams-Dean, 1975; Reinhard and Bryant, 1992; Bryant and Holloway, 1996; Marshall, 1999; Marshall, 2008), forensic (Wiltshire, 2016; Horrocks, 2004; Bryant et al., 1996;), honey (Louveaux et al., 1978; Lieux, 1980; Jones and Bryant, 2004; Salonen et al., 2009; O'Keefe and Wymer, 2017; Sniderman et al., 2018) or modern (Erdtmann, 1943; Moore and Webb, 1978; Faegeri and Iverson, 1989) samples, a common aim is to remove the material of non-interest and concentrate as much interesting material of as many types as possible (Moore et al., 1991; Coil et al., 2003). This can be achieved through disaggregation, dissolving and digesting the

matrix, sieving and/or flotation. Numerous summaries, syntheses and reviews of palynological processing methods exist, including Bryant and Wrenn (1998), Riding and Kyffin-Hughes (2004) and Brown (2008), but none so far focuses on NPPs.

2.1. Acid digestion

Typical methodologies for the extraction of palynomorphs from sediments or sedimentary rocks involve the digestion of the mineral matter by hydrochloric (HCl) and hydrofluoric (HF) acids (e.g. Assarsson and Granlund, 1924; Traverse, 2008; Brown, 2008; O'Keefe and Eble, 2012; Pound et al., 2019). The concentration of acid typically depends on the sediment being digested, but 10-37% HCl and 40-60% HF are among the most frequent reported in the literature (O'Keefe and Eble, 2012; Pound et al., 2019). The former is primarily used to treat carbonates, whilst the latter is used to remove silicates (Moore et al., 1991). Sarmiento (1957) proposed a 50% solution of orthophoshoric acid as an alternative to HCl for the removal of carbonates (Staplin et al., 1960). This was proposed to be as efficient but gentler than the use of HCl (Sarmiento, 1957; Staplin et al., 1960). HCl is frequently used to prevent the formation of calcium fluoride following HF treatment, however, boric acid can also be used. It must be noted, however, that in neither case does the resultant solution become non-toxic or non-corrosive. Samples intended for HF treatment should always be pre-treated with HCl to ensure no carbonates are present to react with HF acid (Staplin et al., 1960). Clay rich sediments can take considerably longer/require more HF due to the clay particles aggregating around other particles (Bates et al., 1978). Warming and agitating the sample on a shaking table facilitates the digestion in samples rich in siliceous minerals (Herngreen 1983; Green, 2001). Both acids are hazardous, and in the case of HF, potentially life-threatening, which has led to techniques that avoid the use of acids for the removal of the mineral fraction.

While HF is generally considered to be non-damaging to palynomorphs, recent work has shown that this is not the case. Van Geel (2001) speculated that HF acid treatment might damage fungi and Clarke (1994) found that large buoyant fungal forms were lost during a treatment procedure involving HF. O'Keefe and Eble (2012) demonstrated that processing methods that use HF reduce the overall concentration values of palynomorphs obtained from clay-rich samples. Tintinnid and other cysts are known to be damaged by long-term immersion in HF and heated

HF treatment can destroy some dinoflagellate cysts (Reid and John, 1981; Mudie et al., 2010; Mertens et al., 2012).

Use of HCl is also problematic, especially use of hot HCl, which has been shown to adversely impact palynomorph preservation following HF treatment. Concentrated HCl is known to modify the colour of fresh pollen to yellow-green (Southworth, 1974). In their work on coprophilous fungi, van Asperen et al. (2016) showed better abundance recovery (closer to unprocessed) when both 10% HCl treatment and alkali digestion (see below) were omitted.

2.2. Non-acid techniques

Depending on the sample being processed there can be no need to use acids at all: unconsolidated or poorly consolidated sediments, forensic and honey samples can all be easily processed without acid digestion. Processing honey requires dissolving the sugars in water and then changing the specific gravity with ethyl alcohol (ETOH) or isopropyl alcohol (IPA) to ensure small pollen grains are not lost in the supernatant (Jones and Bryant, 2004). A similar procedure can also be achieved for sediments or sedimentary rocks using heavy liquid separation to adjust the specific gravity. Zinc bromide, zinc chloride and bromoform have been used for over 60 years (Frey, 1955; Staplin et al., 1960; Brown, 2008; Pound et al., 2012; Halbritter et al., 2018). Since the 1980s there has been a progressive shift to non-toxic and more easily recyclable heavy liquids such as sodium polytungstate (Munsterman and Kerstholt, 1996), sodium metatungstate (Krukowski, 1988) and lithium heteropolytungstate (O'Keefe and Eble, 2012; Caffrey and Horn, 2013; Van Ness et al., 2017; Leipe et al., 2019). As well as being non-toxic, heavy liquids based on inorganic tungsten have been reported to improve dinocyst recovery (Munsterman and Kerstholt, 1996). Methodological comparisons of heavy liquid separation and HF digestion produce comparable results for pollen analysis, with some studies reporting better pollen extraction with heavy liquids (Nakagawa et al., 1998; Lentfer and Boyd, 2000; Campbell et al., 2016; Leipe et al., 2019). Some studies also report a possible sediment specific reduction in pollen abundance when using heavy liquid separation over HF acid digestion (Leipe et al., 2019). This may be due to the presence of pyrite, which can result in palynomorphs with mineral growths/encrustations to sink in the heavy liquid separation (Barss and Williams, 1973; Leipe et al., 2019). Grey (1999) also found that the centrifuging during heavy liquid separation caused the fragmentation of large acanthomorph acritarchs. Gelsthorpe (2002) also investigated the

implications of centrifuging and heavy liquid separation. He found that Silurian samples required three rounds of centrifuging and extraction to ensure the relative proportions of genera was stable (Gelsthorpe, 2002).

Swirling a disaggregated sample can be an economical means of extracting the lighter fraction from the heavier mineral dominated fraction (Green, 2001; Riding and Kyffin-Hughes, 2004; 2006). Many methods of swirling exist. For samples small enough to fit in a watch glass, a simple rotary motion in one hand and a pipette in the other to capture the “plume” of finer particles, whilst the denser particles coalesce into the centre of the watch glass can be used (Green, 2001; Riding and Kyffin-Hughes, 2004; 2006; Traverse, 2008). For larger samples in a tri-cornered beaker, the beaker is swirled until the entire sample is entrained in the liquid, placed on a lab bench to permit the heavy materials to settle, then the supernatant is poured off and retained. This process is repeated until the supernatant is completely clear and no material is entrained when the beaker is swirled. In either case, swirling can clean a sample rich in sand, pyrites or other denser minerals that were retained following disaggregation and/or HF-treatment (Brown, 2008; Traverse, 2008). However, in all cases, the denser fraction should also be kept and examined, in case palynomorphs of interest have not separated or larger NPPs, especially those which may contain pyrite crystals, were retained (Green, 2001; Riding and Kyffin-Hughes, 2004; 2006).

Hydrogen peroxide is used to physically and chemically disaggregate rock samples, but should be kept to short durations to avoid oxidation of material (Hopkins and McCarthy, 2002; Williams et al., 2005). The resulting residue is then sieved to isolate the required size faction from unwanted material (Williams et al., 2005; Riding and Kyffin-Hughes, 2004; 2006).

Unconsolidated sediments can be disaggregated and sieved (selecting sieve mesh sizes appropriate for the target NPP). The sieving of clay rich sediments can be facilitated with a deflocculant, such as sodium hexametaphosphate, surfactants (such as Alconox®, Liquinox® or Teepol®) (Riding and Kyffin-Hughes, 2004; 2006), or sodium pyrophosphate (Brown, 2008; Heusser and Stock, 1984; Bates et al., 1978). Care must be taken with the use of sodium hexametaphosphate as in concentrated solutions it is mildly oxidizing (see below) and of high enough density that it can cause palynomorphs to remain in the float fraction following centrifugation, resulting in loss upon decanting. The choice of deflocculant used must be made in consultation with local regulations for phosphate in wastewater; in some areas both sodium

hexametaphosphate and sodium pyrophosphate must be collected and disposed of as hazardous waste.

2.3. Alkali digestion

Potassium hydroxide (KOH) or ammonium hydroxide (NH_4OH) (Batten, 1999) can be used to react depolymerized humic acids with their conjugate base pairs to produce soluble organic salts, which can be removed through water-washing (Green, 2001; Riding and Kyffin-Hughes, 2004; pers. comm. C.F. Eble to J. O'Keefe and M. Pound, 2019). A 5-10% solution of KOH (or NH_4OH) is added to the sample and either allowed to sit at room temperature or immersed in a water bath at 100°C for from 3-10 minutes to up to several days (especially in the case of peats and coals) (Green, 2001; Riding and Kyffin-Hughes, 2004), diluted with distilled water, centrifuged and decanted. This process is typically repeated until the supernatant is clear, especially in organic-rich samples. When the supernatant is close to clear, the reaction may be halted by adding a few drops of 10% HCl to neutralize any remaining alkali. Modern sporopollenin is somewhat soluble in KOH (Southworth, 1974), becoming less so with increasing thermal maturity. KOH has been linked with selective destruction of gonyaulacacean dinoflagellate cysts from the Cretaceous (Dodsworth, 1995). Boiling in KOH may cause a slight increase in pollen size (Reitsma, 1969), but a decrease in fungal spore size (van Asperen et al., 2016). KOH digestion damages testate amoebae cysts, reducing the identifiable specimens (Hendon and Charman, 1997). Both ancient and recent dinoflagellate cysts are also known to be susceptible to damage from KOH processing (Dodsworth, 1995; Hendon and Charman, 1997; Mertens et al., 2009). Indeed, Schrank (1988) noted that the periphragm separated from the endophragm in dinocysts treated with both nitric acid and KOH after treatment with 5% KOH and was unrelated to the length of treatment with nitric acid.

2.4. Sieving, filtering, sonication, and centrifugation

Sieving at a range of sieve sizes (from 750 to 100 micrometers, with base sieves of 63 and 35 micrometers to separate "fine", "coarse", and "very coarse" fractions in some studies of marine sediments) is used to aid in the extraction and concentration of palynomorphs. This is most frequently accomplished using metal or nylon mesh sieves. In all cases, the mesh size is based

on the width of the square openings in the sieve. The diagonal (corner to corner) distance in the sieve mesh is larger than the certified mesh size, which can lead to the loss of material that is, in one dimension, close to the size of the sieve mesh. Sieving at any size can result in loss of larger NPPs, such as large algae, testate amoebae, microthyriaceous fungi and fungal perithecia, as well as arthropod and tardigrade remains.

In addition to sieving, samples are often filtered to remove excess clay or fine zeolites, usually after acid treatments. Filtering at <10 µm is widely used, especially in non-acid preparations (Riding and Kyffin-Hughes, 2004; 2006). Many laboratories use fine nylon mesh sieves for filtering, often in the form of Nitex® or similar cloth or nylon mesh cell strainers (Urban et al., 2018). These fine sieves have the same problem as larger ones - NPPs that are close in size to the mesh size in one or more dimensions (including the diagonal) may slip through. A way around this problem is the use of Nylon filter membranes in a filtration apparatus, which have round, rather than square, openings with a maximum diameter of the listed gauge. Regardless of the type of filtration medium, the aperature size is known to impact NPP recovery. Lignum et al. (2008) found that a 15 µm nylon mesh lost an average of 5.8±1.2% of *Lycopodium* spores whilst a 15 µm polyester mesh lost 3.9±0.7%. No complete dinocyst was observed to pass through either of the 15 µm meshes, but fragments and operculae did (Lignum et al., 2008). For example, Silurian acritarchs may be lost when using a 10 micrometre mesh, but are retained by 7 µm mesh (Gelsthorpe, 2002). An even smaller mesh size, or not using fine sieves or filters at all is recommended for study of fungal spores, as palaeoecologically important small fungal spores may be lost through a 6 µm sieve (van Asperen et al., 2016).

Choice of a top sieve mesh size and bottom filter size really depends on the focus of the individual scientist. For example, are you limiting your study to microfossils only (by definition, those from about 5 micrometers to 1000 micrometers or one mm), or do you wish to co-analyze mesofossils (by definition, those from 1 to 100 mm), or nanofossils (those 5 micrometers and smaller), or limit the study to a fraction of the microfossil assemblage? Regardless, the choice of sieve and/or filter size should be reported and justified. Additionally, problems have been reported with palynomorphs adhering to the mesh (Bryant, 2017). Sieve sonication (see below) may circumvent this problem. Adherence to nylon membrane filters, frequently used in vacuum assisted filtration (Vidal, 1988; O'Keefe and Eble, 2012) is less problematic - the entire filter may be tucked into a polypropylene test tube which is then partially filled with distilled water, vortexed, and rinsed, thus removing any adhered grains.

Sonication has been applied to various NPP studies since the early days of the science. It is a simple and effective way to disaggregate siliciclastic samples and to shake palynomorphs free of fibrous peat. Great care must be taken when applying sonication, however, as it is known to damage NPPs of all types (Mertens et al., 2012; Jones, 2014; Perrotti et al., 2018). Sonication can be completed using an ultrasonic bath to treat whole samples in test tubes or an ultrasonic wand/horn to treat samples in sieves or filters (Perotti et al., 2018; Leipe et al., 2019). Sonic wands may be tunable or untunable. Both devices can cause damage to pollen and dinoflagellate cysts if applied at the strongest settings or for extensive time intervals (Mertens et al., 2009; 2012; Jones, 2014; Perrotti et al., 2018). Untuned ultrasonic, especially, has been shown to fragment bisaccate pollen grains (Leipe et al., 2019). Short applications of tuned ultrasound (20 seconds to 5 minutes), however, have been shown to be effective in facilitating disaggregation and sieving of palynological samples with minimal damage (Mertens et al., 2009; Price et al., 2016; Perrotti et al., 2018). Low amplitude sonication for long time periods (up to 4 min/sample) with a sonification horn has been shown to be especially effective in removing charcoal while not damaging palynomorphs (Perrotti et al., 2018).

Centrifuging is commonly used to separate the solid (including objects of interest) from the liquid during individual stages of processing. Centrifuging can cause damage to delicate palynomorphs (Grey, 1999; Green, 2001; Urban et al., 2018) or the loss of small/lighter specimens (through decanting) if insufficient spin time is used or the specific gravity is not modified to capture these specimens (Green, 2001; Jones and Bryant, 2004). Cellulose filters have been applied as an alternative to centrifuging in preparing honey for acetolysis (see section 3.1). Dissolved honey is poured through a 0.45-µm pore filter paper and the filter paper, with palynomorph content, is subjected to acetolysis (Lieux, 1980). This procedure eliminates a centrifuging stage, possibly limiting damage/loss of material, and could be applied to NPP studies where acetolysis will be applied. Likewise, use of nested cell sieves can be used to reduce centrifugation-caused damage to NPPs (Urban et al., 2018).

3. Spa treatment – beautifying and cleansing

Once liberated from their sediment shackles, palynomorphs may require some further treatment to be of value to the observer. This is particularly true of modern or Holocene pollen analysis where acetolysis is applied to clean and exaggerate surface textures. If samples contain

abundant woody material, or are thermally mature, they require oxidation to make the palynomorphs more recognisable and the wood more readily attacked by alkali (see below).

3.1. Acetolysis

Acetolysis is commonly used to remove organic remains of perceived limited interest as well asthe non-sporopollenin components of pollen grains, and to swell pollen grains, making the ornamentation easier to observe (Erdtman, 1960; Moore et al., 1991; Halbritter et al., 2018). The degradation of cellulose (and other polysaccharides) through the process of acetolysis, treatment with a solution of acetic anhydride and sulfuric acid, is routinely used on palynomorph samples from the Holocene and modern (Erdtman, 1960; Guthrie and McCarthy, 1967; Pound et al., 2018; Halbritter et al., 2018). Acetolysis solution is most frequently made using a 9:1 ratio of acetic anhydride and sulfuric acid, although other ratios may be used (Brown, 2008). The reaction of acetolysis solution with the palynomorph residue is most frequently quenched through the addition of glacial acetic acid, which is then centrifuged and decanted into a waste bottle for environmentally appropriate disposal. Some laboratories do not quench the reaction, rather simply centrifuge and decant the acetolysis solution. This method is risky, as acetolysis solution is explosive on contact with water and any remaining acetolysis solution may react adversely as processing transitions to water. Acetolysis may be completed either warm or cold. Cold acetolysis is performed at room temperature. Warm acetolysis typically takes place in a heating-block, sand-bath, or water-bath (not recommended, see above) at 80-100°C, with 90°C being considered optimal. Likewise the length of time the acetolysis solution is left on the sample varies from 3-10 minutes, depending upon fragility of the grains, relative amount of non-sporopollenin material present that must be removed, and whether the scientist wishes to darken the palynomorphs to a rich bourbon colour (9-10 minutes).The process is not without critique, especially in the study of NPPs.

Acetolysis of palynological samples destroys all but the sporopollenin, and this, too, can start to be damaged after 10 minutes (Hesse and Waha, 1989; Jardine et al., 2015; Ulrich et al., 2017). It is known to damage a wide range of NPPs including: dinoflagellate cysts (Marret, 1993; Mertens et al., 2009), *Pediastrum* (Komárek and Jankovská, 2001), coprophilous fungi (van Asperen et al., 2016), desmids (Riddick et al., 2017), and *Trachelomonas* (Shumilovskikh et al.,

2019). Acetolysis is also known to affect the size of pollen grains (Reitsma, 1969; Meltsov et al., 2008) and may cause similar swelling in NPPs (van Asperen et al., 2016).

3.2. Alternatives to acetolysis

With a growing body of evidence showing acetolysis to be unfavourable to an in-depth study of NPPs (see the above section), any alternative that can offer the sample cleaning benefits and provide better NPP recovery would be advantageous. Several alternatives to acetolysis have been explored (Schols et al., 2004; Brown, 2008). The use of the enzymes pectinase and cellulase (Schols et al., 2004; O'Keefe and Wymer, 2017) is gaining popularity, although care must be taken to use the appropriate number of units of enzyme per unit palynomorphs, maintain the pH of the buffer solution, and to use fresh enzymes. Too few units of enzyme will not effectively remove kitt and nuclear material; likewise older batches of enzymes may not retain enough efficacy to sufficiently clean the grains. Treatment with 10% KOH has also been found effective (Schols et al., 2004; Brown, 2008), however this may result in damage to NPPs as noted above.

3.3. Oxidation of palynomorphs

Although not commonly used on modern or Holocene samples, many deep-time samples occur co-mingled with woody material, amorphous organic matter and/or have a thermal maturity above the peat stage. To aid palynomorph visibility and identification potential, these samples may necessitate a degree of oxidation prior to alkali treatment to release palynomorphs and to depolymerize lignin. Following oxidation, molecules are then susceptible to attack by their conjugate alkalis, producing water-soluble salts, which can then be rinsed away (Green, 2001; Eble pers. comm. to J. O'Keefe and M. Pound, 2019). Additionally, many palynomorphs darken with thermal maturity, which can obscure accurate identification; oxidation can lighten these palynomorphs. Oxidation is known to damage palynomorphs of all types (Green, 2001). For this reason, the most gentle oxidant that will effectively depolymerize lignin for a sample's thermal maturity should be chosen (O'Keefe and Eble, 2012), and they should be applied for a minimum amount of time. Which oxidant and for how long will need to be determined on a site-by-site basis. In general, hypochlorous acid has been applied to samples with low thermal maturities

(equivalent to lignite), nitric acid to medium thermal maturities and Schulze's solution (concentrated nitric acid saturated with potassium chlorate) to higher thermal maturities (equivalent to bituminous coal) (O'Keefe and Eble, 2012). Sodium hypochlorite (NaOCl, common household bleach) has been applied to undeterminable dark-hued palynomorphs (Buratti and Cirilli, 2011; O'Keefe and Eble, 2012). Nitric acid is known to break down sporopollenin and modify its chemical structure, although limiting treatment to less than 10 minutes still preserves some of the sporopollenin chemistry (Jardine et al., 2015). Over-oxidation of any organic-walled NPPs can result in their loss (Schrank, 1988). Generally, some fungal NPPs are more refractory than pollen, plant spores, or algae, although they, too (particularly more delicate forms), can be lost (van Asperen et al., 2016).

4. Mounting

Residues are most frequently examined under light microscopy (LM), although scanning electron microscopy (SEM) is becoming more frequent. Glass slides with number 0 or number 1 coverslips are commonly used for mounting; number 0 coverslips are difficult to find in many countries but produce less light interference and superior image crisp-ness. It is well documented that the choice of mounting medium for LM can substantially impact visibility and size of the NPPs under observation (Coil et al., 2003; Meltsov et al., 2008). The refractive index of organic-walled NPPs is highly variable, and only well defined for siliceous, carbonate, and starch-based NPPs. In general, a mounting medium with a refractive index greater than 1.5 is sufficient for all but starch-based palynomorphs, which benefit from a medium with a slightly higher refractive index. Both solid (Permount®, PVA, Elvacite®, Glue4Glass®, MeltMount®, EuKitt®, canada balsam, glycerin jelly) and liquid (glycerin USP (glycerol), silicone oil, immersion oil) mounting media are commonly used. All are suitable for observation of multiple NPP types, however, silicone oil's refractive index of ~1.4 is too close to that of siliceous NPPs (Coil et al., 2003) and should be avoided when the goal is to co-analyze these with organic-walled NPPs. Acetolyzed organic-walled microfossils mounted in glycerine USP tend to swell somewhat and appear larger than those in silicone oil; this does appear to be less of a problem with un-acetolyzed grains (Meltsov et al., 2008).

Beyond caveats about refractive indices and swelling grains, type of mount should be chosen for purpose, i.e., if you suspect you may need to remove single grains to make type mounts or

for SEM work, you need a mounting medium that is easily reversible in your laboratory. MeltMount® and Canada Balsam can be reversed by heating to liquid then dissolving with a solvent (acetone for the former and warm detergent for the latter, although the latter is extremely difficult to reverse). Permount®, EuKitt®, can be dissolved in xylene, as can immersion oil. Elvacite® is soluble in Cellosolve® (ethylene glycol monoethyl ether). PVA from Elmer's School Glue® is soluble in soapy water. Glycerin jelly is reversed by re-heating and adding a minute drop of water, at which point specimens can be removed with a micropipette (Evitt, 1984). Both ~~Glycerin~~ glycerin USP and silicon oil are soluble in 90% ethanol and water, although water takes longer to dissolve the latter. Mounting for SEM study should follow the "single grain" technique promoted by Daghlian (1982), developed by Zetter (1989) and detailed, most recently, by Halbritter et al. (2018)

Liquid mounts generally need to be sealed to prevent the mounting medium from drying out or escaping from beneath the coverslip. A variety of compounds can be used, including wax beads which are melted to produce a seal, clear acrylic resin (superglue), clear 2-part epoxy, and nail lacquer. Choosing the right nail lacquer is a perennial problem for palynologists; advice on choosing the most appropriate, i.e., one that will stick to the glass and not peel up or be subject to shrinkage problems can be found in Caffrey and Horn (2012).

5. Overview of techniques reported in select recent literature

So far we have provided an overview of techniques applied to the extraction of NPPs and known issues with these. To provide an insight into how widespread some of our fields processing biases might be, we have surveyed all publications in Journal of Micropalaeontology, Palaeogeograpy Palaeoclimatology Palaeoecology, Palynology and Review of Palaeobotany and Palynology released the past three years (2019, 2018 and 2017) for those reporting NPPs. From this targeted survey, 162 articles reported NPPs and here we present a summary of the processing techniques provided. This is not a thorough literature review and neither is it aimed at naming and shaming, but it is an attempt to highlight commonly used techniques and link these back to known processing issues in an anonymized way to demonstrate potential widespread processing biases..

From the 162 articles reporting NPPs published between 2017 and 2019 in the selected journals, 18.5% (n = 30) presented their methods as "standard processing techniques" without

further elaboration (Fig. 1A~~a~~). These are excluded from the remainder of this section bringing the total number to 132 articles. Over the last three years 53.8% (n = 71) of articles have used HCl with 56/71 articles reporting the concentration of the acid. In general, the older the material the stronger the acid used (Fig. ~~1c~~1C). If HCl is the cause of the observed processing bias van Asperen et al. (2016) reported, then this could have affected over half of all studies in our small survey of the literature. Whilst we are unaware of a systematic study of the impact of different HCl concentration on NPPs, Dale (1976) reported that concentrated hot HCl destroyed recent *Peridinium* cysts. HF use is even more widespread than HCl with 91.7% (n = 121) of articles reporting NPPs having used this for the dissolution of silicates. However, only 52.4% (n = 55) of articles report the concentration of HF used; with 10 - 72% HF reported (Fig. ~~1b~~1B). Although commonly thought of as innocuous to palynomorphs, increasing numbers of studies are showing, or suggesting, HF to be detrimental (Reid and John, 1981; Van Geel, 2001; Mudie et al., 2010; Mertens et al., 2012; O'Keefe and Eble, 2012).

Alkali digestion is reported in 28.8% (n = 38) of articles. KOH is the more frequently used (n = 33) with NaOH only being reported in five articles that were focused on modern or Holocene samples. Whereas KOH has been applied to samples covering the whole Phanerozoic. Of the 21 articles that report the concentration of KOH, 10% or less is the most frequent, with only one article reporting the use of 100% KOH. Only six articles report the time that samples were treated with KOH and these vary from 5 to 15 minutes. Despite widespread knowledge that KOH damages and destroys dinoflagellate cysts (Schrank, 1988; Dodsworth, 1995; Hendon and Charman, 1997; Mertens et al., 2009), four studies of dinoflagellate cysts in our survey of recent literature used KOH in concentrations of 10% or more. The implications of size changes in fungal spores treated with KOH or NaOH should also be carefully considered before further processing (sieving) is undertaken and when morphological or abundance data is the target outcome of the work (see results of van Asperen et al., 2016).

Heavy liquid separation use in surveyed NPP articles has most commonly been used in combination with acid digestion. Only four of the 37 methodologies that report heavy liquid separation have used it as an alternative to acid digestion, suggesting that it is being used as a supplementary, rather than a non-acid alternative, treatment (Fig. ~~1d~~1D). Heavy liquid separation alone has been shown to improve palynomorph recovery and could be of immense benefit to future NPP studies (Nakagawa et al., 1998). However, concerns have been raised around fragmentation and recovered abundance data in acritarchs and further systematic exploration of these observations is needed for all NPP groups.

Acetolysis is reported in 24.2% (n = 32; Fig. ~~1e~~1E) of NPP articles published in the three years surveyed. Studies on the modern, Holocene, and Quaternary dominate the studies utilising acetolysis (n = 30). Although not commonly applied to “deep-time” samples, acetolysis use has been reported on two samples of pre-Quaternary age: dinoflagellate cyst samples from the Cretaceous and Paleocene; despite the known damage it can cause (Marret, 1993; Mertens et al., 2009). Despite the known damage to NPPs from acetolysis, nearly a quarter of studies surveyed continue to use it introducing potential taphonomic implications for reported data on: dinoflagellate cysts (Marret, 1993; Mertens et al., 2009), *Pediastrum* (Komárek and Jankovská, 2001), coprophilous fungi (van Asperen et al., 2016), desmids (Riddick et al., 2017), and *Trachelomonas* (Shumilovskikh et al., 2019). Oxidation is reported in 24.2% (n = 32) of the surveyed literature, mostly in pre-Quaternary studies on dinoflagellate cysts, acritarchs and other algae. Most commonly (n = 24), this is nitric acid >50% for <180 seconds. Only eleven studies report the use of Schulz Solution; the treatment time ranges from 45 to 1800 minutes. However, not all studies report the concentration of the nitric acid used and/or the time the oxidation process was applied for. Considering it is well established that it can have damaging and biasing impacts on any results (Mertens et al., 2009), these should always be reported..

6. General recommendations

The concept of taphonomic filtering is well established in palaeontological and archaeological research (Behrensmeyer et al., 2000). When you consider that the dispersal, death, decay, deposition, preservation and fossilisation of palynomorphs has enacted a substantial taphonomic filter on data before it is even generated (Ferguson, 2005), it becomes more pressing that the one taphonomic filter we can control – processing – is fully reported in detail. This permits others to recognise any further taphonomic filter applied to the data and the implications this has for interpretations (Coil et al., 2003). All processing techniques apply a taphonomic filter (Fig. 2), some we know to be especially detrimental to NPPs (e.g. acetolysis), others are speculated or under-investigated (e.g. HF; heavy liquids). In the past three years only 18.5% (n = 30; Fig. ~~1a~~1A) of articles reporting NPPs have failed to accurately detail their methods.

Among the most commonly used processing techniques in palynology, acetolysis has shown to be most risky. We recommend avoiding acetolysis in studies where multiple NPP types are to

be analyzed. If acetolysis cannot be avoided, the duration and temperature of acetolysis must be reported and commentary about potential NPP losses must be made. Oxidation is known to be damaging to most organic-walled NPP groups. Use of oxidation should be limited to the bare minimum necessary to liberate palynomorphs from rocks, and for modern material, ought to be completely avoided. Likewise, alkali treatment should be avoided where possible, especially for modern material, and of limited duration for organic-rich fossil material. As in the case of acetolysis, all chemical treatment should be detailed, as should its potential impacts on the NPP spectrum obtained. As a final consideration, the choice of mounting media for LM work should be documented, and if siliceous NPPs are to be co-analyzed, it should be of sufficiently high (or low) (<1.4<) refractive index to permit examination of these organisms' remains.

As an optimal standardization, especially for fossil material, we strongly recommend the simplest processing possible - disaggregation in a non-phosphatic deflocculant using gentle stirring, rather than sonication, sieving through a top mesh of 1-mm to remove very coarse material, and short-centrifugation or nylon membrane filtration followed by swirling to remove excessive clays and heavy minerals.

However, we also recognize that this may not be practical under site-specific circumstances. Peat samples may benefit from short alkali treatment while coal or NPPs from highly thermally altered sediments may benefit from oxidation followed by alkali treatment. Ultimately, we need more studies on the taphonomic filtering that these techniques have on NPPs. Whilst there have been important studies recently and in the past (not all have been cited in this chapter), we are still reliant on inferring likely impacts on NPPs from pollen and spore studies (although dinoflagellate cysts are nearly as well investigated). Whilst this provides useful indications, the myriad of NPP groups are biologically, structurally and chemically distinct from plant propagules and so their reaction under processing may be different (see the differing KOH-induced palynomorph size changes reported by Reitsma (1969) and van Asperen et al. (2016) as one example). While we continue to explore the taphonomic filter applied by processing on the various NPP groups, our take-home message has to be: the minimal processing needed to obtain NPPs relevant to your study is best and the standard procedure/method should be to fully document the entire process in detail.

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Figure 1. Visual summary of key findings from the survey of NPP literature published during 2017, 2018 and 2019 in Journal of Micropalaeontology; Palaeogeography, Palaeoclimatology, Palaeoecology; Palynology; and Review of Palaeobotany and Palynology. A. Level of detail provided in the methodology, pale = “standard procedure” and dark = details provided. B. Use of HF: dark = HF used, pale = no HF used. C. Concentration of HCl used grouped by age of material being studied. D. Heavy liquid use: pale = heavy liquid and acid digestion; dark = heavy liquid without acid digestion. E. Use of acetolysis: pale = acetolysis not used; dark = acetolysis used.

Figure 2. Summary of taphonomic filtering introduced during the three principal stages of processing. In the bottom right corner is an example method flow diagram showing the hypothetical taphonomic loss of NPP abundance. This is a diagrammatic summary of key taphonomic filters introduced in the text designed to give the reader an easy to refer to scheme and way to quickly evaluate how their intended methods may introduce bias. As more research is conducted on the processing of NPPs it is anticipated that this diagram can be expanded with detail and potentially introduced uncertainty for each process.

Figure 1

Click here to access/download;figure;Fig. 1_revised.tif

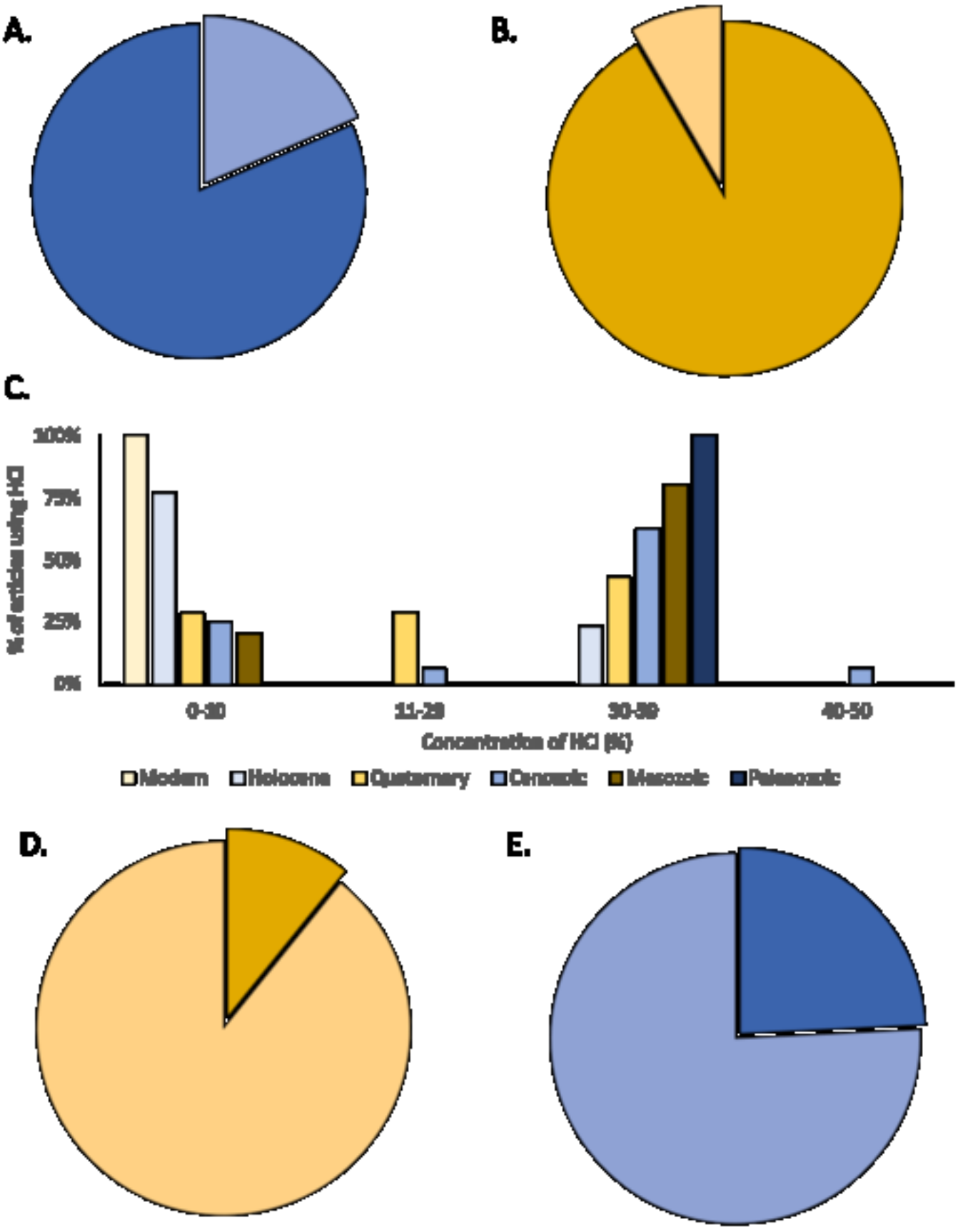

Figure 2