The effect of preparation methods on dung fungal spores: Implications for recognition of megafaunal populations

Eline N. van Asperen[a,*], Jason R. Kirby[a] and Chris O. Hunt[a]

[a] School of Natural Sciences and Psychology, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, United Kingdom

[*] Corresponding author, envanasperen@palaeo.eu

Abstract

Spores from coprophilous fungi are emerging as an important palaeoecological indicator of the presence of large herbivores, but the methods by which they are recovered may have a significant impact on their preservation and recognition. Here, we test a number of chemical and mechanical techniques used in palynology. Spore occurrence, size and shape is affected by preparation technique and is particularly affected by acetolysis. This has important implications for recognition of past megafaunal populations.

Highlights:

- We investigate the impact of preparation techniques on coprophilous fungal spores

- Taxonomically significant change in size and shape with most preparation techniques

- Acetolysis impacts on most taxa and is particularly damaging to hyaline spores

- We outline implications for using fungal spores to study ancient megafauna

1. Introduction

The past decades have seen an increase in the use of spores of coprophilous fungi from sedimentary sequences as a proxy for large herbivore presence and abundance (e.g. Davis, 1987; Davis and Shafer, 2006; Cugny et al., 2010; Jeffers et al., 2011; Baker et al., 2013). Although they have great potential for advancing our understanding of past herbivore

population dynamics, extinction events and the impact of husbandry practices on the natural environment, there remains significant uncertainty regarding preservation and recovery of these spores (Feranec et al., 2011; Baker et al., 2013). The validity of this proxy method depends heavily on a robust understanding of the relationships between large herbivore presence and abundance, spore presence, variety and abundance in the natural/modern environment, spore preservation in the soil, and spore recovery. Whilst some of these relationships are increasingly under study (e.g. Blackford and Innes, 2006; Raper and Bush, 2009; Dietre et al., 2012; Parker and Williams, 2012; Etienne et al., 2013; Gill et al., 2013), the most basic level of recovery techniques has yet to be addressed in a substantive fashion.

In most cases, fungal spores are extracted from palaeoenvironmental samples along with other relevant taxa, especially pollen, commonly using the 'standard' pollen preparation method (Faegri and Iversen, 1989; Moore et al., 1991). This is at least in part because palaeoecologists have tended to identify and count fungal material as part of pollen-based studies and because both microfossil groups are found in pollen slides. Knowledge of the effect of these chemical preparation methods on fungal spore representation in pollen slides is limited. Thus, Van Geel (2001) cautions against including treatment with HF in the standard procedure since its effect on fungal spores is unknown and thought to be deleterious. Clarke (1994) processed samples from a variety of substrates with three different techniques:

A. boiling in KOH, HF, acetolysis, mounting using tertiary butyl alcohol (TBA);

B. boiling in NH_4OH, sieving, swirling, mounting using TBA;

C. boiling in KOH, sieving, heavy liquid separation with $ZnCl_2$, mounting using TBA.

She found that small round to oval fungal spores behaved in a similar manner to pollen. Large, buoyant forms were lost in treatment A, whereas only these forms were consistently recovered in treatment B. Thick-walled forms were lost in treatment C. Although she did not notice any deterioration of the spores, Clarke's (1994) results suggest that the preparation method chosen can affect spore representation.

The most common method used by pollen analysts today involves alkali treatment followed by acetolysis. Alkali treatment is intended for the initial disaggregation of the sample and goes back to the earliest days of pollen analysis, when a small piece of peat was typically boiled in a drop of water with a grain of KOH or NaOH on a microscope slide, using a candle as a heat source. Later a more standardised technique used a known weight or volume of peat boiled in 10% KOH or NaOH solution, followed by centrifugation, then repeated rinsing and re-centrifuging (e.g. Godwin, 1934).

The acetolysis part of the standard pollen preparation method was devised by Gunnar Erdtman, who introduced several related techniques in the 1930s (Erdtman and Erdtman, 1933; Erdtman, 1934, 1935, 1936). This involves heating the sample in a mixture of acetic anhydride and sulphuric acid (9:1), with the intention of removing the protoplasm of the pollen grain. Acetolysis is also known to degrade or destroy cellulose (Brown, 1960, p. 144). The acetolysation process destroys all pollen material apart from sporopollenin which forms the outer wall (exine) of the pollen grain, which is visually inspected to enable identification based on morphological characteristics.

Alkali treatment has been implicated in the destruction of pollen grains (Brown, 1960, p. 139). Trials by Faegri and Deuse (1960) showed, however, that prolonged boiling in KOH solution led to shrinkage of pollen rather than destruction. Shrinkage of pollen following alkali treatment was also noted by Christensen (1946). It has also been argued that

acetolysation destroys some important pollen characteristics including the intine structure and aperture morphology, and can also degrade pollen grains with more fragile exines (Hesse and Waha, 1989). The use of acetolysis can lead to damage or destroy pollen of Cannaceae, Juncaceae, Lauraceae and Musaceae (Erdtman, 1952, p. 9) and causes the loss of fern perispores (Brown, 1960, p. 140), of up to 50% of *Sphagnum* spores in peat samples (Wenner, 1947, p. 101) and of most protoperidinioid dinoflagellate cysts in marine sequences (Mertens et al., 2009). In studies of the palynology of sediments known to be less than optimal for pollen preservation, including arid-zone and cave sequences, acetolysis is known to degrade pollen assemblages seriously (Coles, 1987; Horowitz, 1992). Furthermore, acetolysis affects the size and shape of pollen grains, causing swelling of up to one half (Christensen, 1946, p. 17; Faegri and Deuse, 1960, pp. 296-7). These issues have significant implications for the recovery and identification of not just pollen but also a variety of microfossil groups which are likely to be similarly affected by chemical treatment.

Mounting media have also been shown to impact pollen size and shape. Glycerol gel was argued to cause swelling of grains (Christensen, 1946; Anderson, 1960), and although a series of experiments by Faegri and Deuse (1960) did not show the same phenomenon consistently, a series of later papers also showed size changes in various mounting media (e.g. Reitsma, 1969; Praglowski, 1970; Large and Braggins, 1970).

In studies of fungal spores, species identification provides crucial information, e.g. when certain species within a genus are not exclusively coprophilous, or when certain species preferentially grow on the dung of particular animal species. Spore size is an important characteristic for species determination. Published spore sizes, however, refer to unprocessed spores, usually mounted in water (e.g. Ahmed and Cain, 1972; Lundqvist, 1972; Bell, 2005; Doveri, 2007; Guarro et al., 2012). Spore sizes vary slightly in other mounting

media, such as lactic acid, lactophenol and alcohol, or mounting agents such as Indian ink, Congo red or Melzer's reagent that highlight certain features of particular fungal taxa (Lundqvist, 1972). So far, no studies have investigated the effect of pollen preparation procedures on spore size.

Building on Clarke's (1994) study, we present here an investigation into the effect of the various chemicals used in standard pollen preparation procedures, as well as several mechanical preparation techniques, on dung fungal spore representation, preservation state and size. We then consider the potential broader implications of using traditional chemical extraction procedures in palaeoecological studies.

2. Materials and methods

Fresh dung was collected of African elephant (*Loxodonta africana*, n=2) and white rhinoceros (*Ceratotherium simum*, n=2) from Knowsley Safari Park (Prescot, UK) and wild cattle (*Bos taurus*, n=4) from Chillingham Wild Cattle Park (Chillingham, UK) and stored for 2 days in the dark at 4°C. 100-150 g of each sample was placed on moist paper towels in a sterilised glass dish with a glass lid and incubated under laboratory conditions (20°C, ~12 hours of daylight per day; Krug, 2004). The samples were kept moist by periodically wetting the paper towel with distilled water.

During the incubation period, the dung samples were examined regularly using a stereomicroscope. Spore-producing fruit bodies growing on the dung were isolated, mounted in alcohol and lactophenol cotton blue, and identified under a light microscope. Measurements were taken on the fungal spores present in these preparations.

After an incubation period of 9-13 weeks, four 1 cm^3 subsamples were taken from the surface of each dung type (Table 1). One dung subsample was subjected to standard pollen preparation methods (method A; see Table 2); a second subsample was prepared using the same method, but excluding acetolysis (methods B1 and B2); the third subsample followed method B2 but in addition was subjected to density separation by swirling (method C); and the fourth subsample was sieved only (method D). Fungal spores present in the samples were, where possible, identified and measured. The species richness, relative abundance, preservation state and size of spores observed *in vivo* were compared with those observed after pollen preparation. Spore counts and measurements are included in the Supplementary data.

3. Results

The incubated dung samples produced fruit bodies of 25 fungal taxa (Table 3): the Zygomycete genera *Pilobolus*, *Mucor* and *Mortierella*; the Ascomycete genera *Ascobolus* (4 species), *Saccobolus*, *Cheilymenia* (2 species), *Sordaria*, *Cercophora/Podospora* (10 species), *Sporormiella* and *Peziza*; and the Basidiomycete genera *Coprinus* and *Conocybe*. Some taxa, such as the Zygomycete genera, *Sordaria*, *Podospora* and *Coprinus*, occurred on all or nearly all samples, whereas other taxa, such as *Saccobolus*, *Sporormiella, Peziza* and *Conocybe*, occurred on only one or a few samples. The ubiquitous taxa also tend to be more abundant when present than the less common taxa.

3.1. Zygomycota and Basidiomycota

Whilst Zygomycete genera were abundantly present on the dung, they were absent from most of the prepared samples. Where they were encountered, they were present in the form of collapsed sporangia still containing some sporangiospores.

Basidiomycete genera, in particular *Coprinus* species, were also abundant on the dung. Although they are mostly absent from the prepared samples, occasionally an abundance of spores is encountered, regardless of the preparation method employed.

3.2. Ascomycota

3.2.1. *Cheilymenia* and *Peziza*

When present on dung, *Cheilymenia* species tend to be abundant (Plate I, 1a). Their spores, however, are entirely absent from samples prepared with methods A and B and sparsely present in samples prepared with method C (Plate I, 1c-d). They are abundant in samples which were sieved only (method D), although spores of the species with the larger-sized spores were rare. The less common genus *Peziza* was only encountered on one dung sample, which was prepared using methods C and D (Plate I, 1b-d). A small number of *Peziza* spores was found in these preparations. There are no obvious size changes in the spores of these two genera compared with spores mounted in alcohol and lactophenol.

3.2.2. *Ascobolus*

Heating with NaOH and/or acetolysis has a strong adverse effect on *Ascobolus* spores: they were absent or sparsely present in samples prepared with methods A and B1. Samples

treated with KOH and HCl (methods B2 and C) preserve a similar number of spores to samples that were sieved only (method D). The spores found in these samples have a crumpled appearance (Plate I, 2d-g). When the sizes of the *Ascobolus* spores treated with these various methods are compared with the sizes of spores mounted directly from the dung (Plate I, 2a-c), it becomes clear that the large spores (50-65 x 25-38 µm) of the species *Ascobolus immersus* are entirely absent from all treated samples. In comparison with the spores mounted in alcohol and lactophenol, spores treated with methods B1 and C appear smaller, whereas those treated with method A are larger, especially in width. The sieving-only (method D) spores are most similar in size to the spores mounted in alcohol and lactophenol (Plate I, 2h).

3.2.3. *Saccobolus*

The spores of the related genus *Saccobolus* tend to form clusters of 4 or 8 spores, sticking together by the pigment in their epispore. In some species these clusters fall apart easily, while in other species the clusters persist for longer (Bell, 2005). When species with 'sticky' clusters are present on the dung, these clusters are commonly encountered regardless of the preparation method used (Plate I, 3a-e). Single spores are rarely encountered due to their small size. Spores treated with methods B1, C and D are smaller in size than spores mounted in alcohol and lactophenol, whereas spores treated with method A are more variable than spores mounted in alcohol and lactophenol (Figure 1).

3.2.4. *Sporormiella*

A long-standing debate concerning the relationship between the genera *Sporormiella* and *Preussia* leads some authors to include *Sporormiella* in *Preussia* as a later synonym (e.g. Kruys and Wedin, 2009; Guarro et al., 2012), while others regard the two genera as sufficiently different to retain both generic names (e.g. Lumbsch and Huhndorf, 2007; Kirk et al., 2008; Doveri, 2011). The differences between the two genera pertain primarily to their fruit bodies and their preferred substrate (dung for *Sporormiella*; plant debris, wood or soil for *Preussia*) rather than their spores (Kruys and Wedin, 2009). Since pollen analysts have so far used the generic name *Sporormiella* for 4-celled spores with germ slits, this name is used here.

Sporormiella species were not common on the dung, but where present, spores were encountered in prepared samples regardless of preparation technique used (Plate I, 4a-d). In samples prepared with method A, no complete spores were found. Instead, the four-celled spores had broken up into their constituent cells (Plate I, 4c). Samples prepared with the other methods did contain complete spores (Plate I, 4d), as well as single cells. Treatment with method A in general led to an increase in length and width of the spores, although a wide range of variation was documented (Figure 1). Spores treated with method B1 are shorter but wider than spores mounted directly from the dung. Spores prepared with methods C and D also increased in width.

3.2.5. *Sordaria*

Sordaria was one of the most common taxa growing on the dung samples. *Sordaria* spores survive all preparation treatments tested here, and are common to abundant in the prepared samples (Plate I, 5a-f). However, spores treated with method A are often less well-

preserved, frequently appearing wrinkled and deformed (Plate I, 5c-e). In addition, the pore complex tends to be more prominent, protruding clearly from the spore, when method A is used.

Sordaria ascospores are produced in 8-spored asci, and tend to stick together in small groups as they are discharged from the fruit body (Ingold, 1933; Ingold and Hadland, 1959). About 39% of spores in samples prepared with methods B1, C and D preserved these groupings, forming groups of 2-8 spores even after preparation (Plate I, 5b). In contrast, in samples prepared with method A only 9% of spores were found in groups, and no groups of more than 3 spores were found. Spores prepared with methods A and B1 are similar in length to spores mounted in alcohol and lactophenol, while spores prepared with methods C and D are shorter (Figure 1). However, a number of spores are clearly larger, particularly in width. It is unclear whether this increased size variation is due to the chemical preparation or whether this represents another species which remained unnoticed on the dung samples.

3.2.5. *Cercophora* and *Podospora*

Although usually less abundant than *Sordaria*, one or more species of *Cercophora* or *Podospora* were present on each dung sample (Plate I, 6a-d). Their spores were present regardless of the preparation technique used (Plate I, 6e-o), but often in very low numbers. As in *Sordaria*, but less frequently, spores stick together by their appendages when discharged (Ingold, 1933; Yafetto et al., 2008) and are found in small groups in samples prepared with methods B1, B2, C and D, but not in samples prepared with method A. Sizes are similar between the different preparation techniques. Only a single spore falls within the size range of the spores of the largest species measured when mounted in alcohol and

lactophenol, and a large number of spores fall below the size range of the spores mounted directly from the dung. This could indicate a significant size difference in these spores when prepared and mounted in different fluids, although a small number of *Podospora* species has such small spores, and it is therefore possible that the small spores represent another species which remained unnoticed on the dung samples. Spore size is also more variable in the treated samples, particularly in width. The preparation treatment thus seems to have some effect on spore size and shape, although it is difficult to quantify this effect from the current experiment.

4. Discussion

4.1. Zygomycota and Basidiomycota

Some taxa found on the dung are lost altogether or almost entirely. Zygomycete spores are too small to be retained in the 6 μm sieve. However, since these spores tend to have few distinguishing characteristics that would enable identification to genus or species level, they are not regarded as having significant palaeoecological value. Although some taxa, such as *Pilobolus*, are obligate coprophiles (Krug et al., 2004), many Zygomycete taxa can grow on a wide range of substrates (Webster and Weber, 2007). They therefore cannot be used as dung indicators.

Similarly, Basidiomycete spores are also small and mostly lost through the 6 μm sieve. When they are sufficiently large, their pigmented spore walls are tough enough to be preserved through all preparation treatments. However, the spores are often difficult to distinguish

and many taxa grow on a wide range of substrates (Webster and Weber, 2007), limiting their usefulness as dung indicators.

4.2. Ascomycota

4.2.1. Genera with hyaline, thin-walled spores

Cheilymenia species characteristically grow on the dung of cattle and other ruminants (Lundqvist, 1972; Richardson, 1972; Bell, 2005), although not exclusively so. Indeed, in the experiment presented here, the genus was encountered on one sample of elephant dung as well as on all cattle samples (see also Ebersohn and Eicker, 1992). The spores of this genus, as well as those of *Peziza* species, are hyaline and thin-walled. *Peziza* species are common on a wide range of substrates, although they are not commonly isolated from soil (Bell, 2005; Doveri, 2007; Guarro et al., 2012). Spores of these and other genera with hyaline spores (e.g. *Coprotus, Iodophanus* and *Thelebolus*) are too fragile to withstand preparation methods using corrosive chemical methods such as acetolysis and boiling with NaOH or KOH. It is doubtful whether these spores could survive in soil or other substrates for extended periods. Furthermore, these spores are difficult to distinguish and would not be identifiable to genus when encountered in a fossil sample. However, the absence of such spores in pollen preparations, whether because they are not preserved in fossil samples or because they are destroyed in the preparation procedure, could result in a lack of recognition of the presence of those dung types which are dominated by *Cheilymenia* species.

4.2.2. Genera with pigmented epispores

The predominantly coprophilous genus *Ascobolus* is readily recognisable by its pinkish epispores with striated, dotted or warted ornamentation (Bell, 2005; Webster and Weber, 2007; Guarro et al., 2012). These epispores often disintegrate or peel off in ageing spores (Bell, 2005). Spores in prepared samples tend to have collapsed epispores, making it difficult to ascertain the original size and ornamentation of the spore. Spore size also seems to be affected by the preparation method used, with boiling with NaOH or KOH leading to shrinking, and acetolysis leading to swelling.

Underneath the epispore, *Ascobolus* spores are similar to the hyaline spores of *Cheilymenia* and *Peziza*, and are therefore vulnerable when more corrosive chemicals are used, although the presence of the epispore provides limited protection. Given the tendency of the epispore to disintegrate in older spores, however, these spores may not survive in soils or sediments for extended periods.

Almost all species of *Saccobolus* are coprophilous (Doveri, 2007; Guarro et al., 2012). Their spores have pinkish pigmented epispores, which enables spore clusters to survive through all preparation treatments. However, these clusters often fall apart with time, and it is unlikely that clusters persist in substrate for longer periods. Single spores are often so small that they are lost through the 6 μ sieve.

4.2.3. Genera with thick-walled, pigmented spores

The spores of *Sporormiella, Sordaria* and *Cercophora/Podospora* are more resistant to chemical degradation due to their thicker, pigmented spore walls. These three genera are

generally regarded as being among the strongest indicators of dung in palaeoecological studies (e.g. Baker et al., 2013).

Most species of *Sporormiella* grow on dung (Doveri, 2007; Guarro et al., 2012). The *Sporormiella* species present in the samples analysed here were not common and developed late in the succession. The fruit bodies, asci and 4-celled spores observed are most similar in size to those of *Sporormiella australis, S. lageniformis* and *S. tetramera*, but the range of variation is relatively large, so that more than one of these species may be present. In some species, the spores frequently break up into their constituent cells, while in other species, the integrity of the spore persists longer (Ahmed and Cain, 1972). The spore morphotypes present here seem to break up when subjected to acetolysis, whilst complete spores persist in larger numbers under other treatments. However, such complete spores will be rare in sedimentary samples, whereas single cells are commonly encountered. It should be kept in mind that the single cells of some *Sporormiella* species are so small that they would not be retained in the 6 μm sieve, leading to a potential loss of information. Most preparation methods seem to lead to swelling of *Sporormiella* spores, particularly in width.

Sordaria is almost exclusively coprophilous (Bell, 2005; Doveri, 2007), although some species are frequently isolated from soil (Guarro et al., 2012). Its spores are abundant and remain largely unchanged regardless of the preparation method, except when acetolysis is used, which causes deterioration of the spores.

Species in the genus *Podospora* are mostly coprophilous, while only some *Cercophora* species are coprophilous (Bell, 2005; Doveri, 2007). The pigmented cell of the spores of these genera survives chemical preparation, whilst the hyaline cell usually collapses or is destroyed. The other hyaline appendages that some of these spores bear are fragile and do

not survive even sieving, and they are hard to observe without the use of Indian ink, cotton blue or Congo red (Bell, 2005).

The pigmented cells of the spores of coprophilous species of *Cercophora* tend to be relatively small (<25 x 15 µm) compared with the mostly larger pigmented cells of *Podospora* spores (Bell, 2005; Doveri, 2007), but there is overlap in size and shape between the two genera (Guarro et al., 2012). However, *Cercophora* spores tend to remain hyaline until after maturation and discharge (Lundqvist, 1972). Such hyaline spores are likely to be equally vulnerable to destruction in chemical preparation as those of *Cheilymenia* and *Peziza*, although they were sporadically present in method D samples. Furthermore, *Cercophora* species are not commonly isolated from soils, whereas *Podospora* species are (Guarro et al., 2012). It can tentatively be assumed, therefore, that most *Podospora*-like spores encountered in sedimentary samples represent coprophilous species.

4.3. Taphonomic considerations

Our results have wider implications for our understanding of the preservation potential of fungal spores in soils and sediments. It is probable that fungal spores are not preserved uniformly. In biologically active soils, in particular, it is likely that there would be fairly rapid degradation of those fungal spore types which are particularly vulnerable to acetolysis and other chemical treatment, since similar chemical processes occur in biologically active systems. This vulnerability will likely be especially marked in genera with hyaline spores, such as *Peziza*, *Cheilymenia,* unpigmented *Cercophora* upper cells, and to a lesser degree *Ascobolus*. If such spores do survive in favourable conditions, they will be vulnerable to chemical degradation during the preparation procedure.

Overall, recovery is biased towards genera with thick-walled, pigmented spores, which do not represent a comprehensive sample of the diversity of the dung fungal community. Furthermore, small single cells of *Sporormiella* spores may be lost when a sieving mesh with an aperture size larger than the cell diameter is used. It is therefore important to use a sieving mesh with as small an aperture size as is practicable.

4.4. Wider implications

This study provides experimental evidence for alteration of size, shape and morphological characteristics (or complete destruction) of some fungal spore taxa depending on the nature of the preparatory treatment of samples. Other studies (e.g. Wenner, 1947; Erdtman, 1952; Coles, 1987; Mertens et al., 2009) have shown the impact of chemical pre-treatment, particularly acetolysis, on the preservation and recovery of a range of microfossil types including pollen, spores and dinoflagellate cysts.

Use of acetolysis is confined to Quaternary pollen analysts (Brown, 1960; Wood et al., 1986), but is not used more widely in palaeopalynology. Many palaeopalynologists regard the technique as a dangerous aberration (K.J. Dorning, pers. comm. to COH, 1983), because it is unnecessary for recognition of pollen from stratigraphic samples and because of the health and safety risks to the palynologist preparing the sample. Efficient palynological preparation methods which avoid the use of acetolysis and most strong acids have been available for several decades (Hunt, 1985; Lentfer and Boyd, 2000; Mudie and Lalièvre, 2013). Although it has a place in the preparation of modern type material, other uses of acetolysis in palynology should be reconsidered.

The recovery bias towards thick-walled, pigmented genera also has implications for their use as proxies for large herbivore abundance. The Late Quaternary extinction event is one of the key events that has been studied using dung fungal spores as a proxy for megaherbivore presence (Davis, 1987; Feranec et al., 2011). The Late Quaternary extinctions disproportionately affected the largest species, including many hind-gut fermenters such as elephants, rhinoceroses and horses (e.g. Alroy, 1999; Lyons et al., 2004), whose dung is richer in thick-walled, pigmented taxa. Genera with thin-walled, hyaline spores (esp. *Cheilymenia*) often dominate the dung of ruminants (Lundqvist, 1972; Richardson, 1972; Bell, 2005). The observed drop in dung fungal spore abundance may be influenced by the fact that the spore types which are more characteristic of the surviving ruminants tend to be underrepresented in pollen slides. The use of thick-walled, pigmented dung fungal taxa as proxies for large herbivore abundance therefore needs to be revisited, especially since some large herbivore dung types are dominated by dung fungal genera that are not normally preserved in and recovered from soil samples. Absence of thick-walled, pigmented taxa cannot necessarily be taken as absence of animal dung.

5. Conclusions

Different protocols for the preparation of palaeoenvironmental samples have a range of different impacts on the preservation and recovery of dung fungal spores. Deleterious effects vary depending on the type of fungal spore and the chemical and mechanical treatments used. Spores of some taxa disappear from the samples entirely, either due to their small size (Zygomycota, Basidiomycota, individual spores / cells of *Saccobolus* and *Sporormiella*), or due to the fragile nature of their hyaline and/or thin-walled cells

(*Cheilymenia, Peziza,* unpigmented *Cercophora* upper cells, and to a lesser extent

Ascobolus), which impedes both their long-term preservation in substrates as well as their

survival through preparation procedures. This is a potentially serious issue, especially where

such species dominate particular dung types, as is the case for *Cheilymenia* on cattle dung.

Loss of these spores may bias reconstructions of past herbivore abundance.

Pigmented, thick-walled spores are differentially impacted by different methods. Acetolysis

leads to the deterioration of the preservation state of certain types of spores. *Sordaria*

spores obtain a crumpled appearance, making it more difficult to observe salient

identification features. Groups of *Sordaria* and *Podospora* spores, which tend to stick

together when they are discharged from the fruit body, fall apart, and *Sporormiella* spores

break up into their constituent cells when subjected to acetolysis. Where mineral-rich

samples are subjected to treatment with HF, the effect on spore preservation is likely to be

even more deleterious, although this remains to be shown experimentally. However, the

relative abundance of these spores appears not to be changed significantly by the use of

acetolysis, treatment with HCl, boiling in NaOH or KOH, or swirling. This parallels experience

with other palynomorph groups including pollen, spores and dinoflagellate cysts.

Spore size and shape are also affected. Spores treated by boiling in NaOH or KOH frequently

shrink in size, or increase in width. Acetolysis often produces swelling. Due to these size

changes, measurements to determine species on spores mounted in silicon oil or other

mounting media commonly used in pollen analysis cannot be compared directly with

measurements from the fungal literature, which are usually made on spores mounted

directly in water. Although a large variation in spore size may tentatively be taken to

indicate more than one species is present (Johnson et al., 2015), the results of this study

caution against trying to pinpoint which species are present in the absence of morphological characteristics other than size.

This study shows that the method that most closely preserves the diversity and relative abundance of dung fungal spores is sieving only. As it stands, however, this method is likely to be unsuitable for most other palaeoenvironmental materials (e.g. pollen) where additional concentration of samples is often necessary. We recommend using a method that is the least corrosive as is possible given sample characteristics, starting with sieving and swirling, then boiling in KOH and treatment with HCl, then heavy liquids, then boiling in NaOH. There appear to be no advantages in using acetolysis in the preparation of fungal spore samples, and fungal spore data obtained from pollen slides prepared using acetolysis may lead to the under and over-representation of taxa.

The results presented here of course are not exhaustive, and other methods could be used (but see Clarke, 1994 for an assessment of two further methods). These results show, however, that it is necessary to investigate the effect of the preparation method used on spore representation and preservation before drawing any conclusions regarding past herbivore presence and abundance based on spore counts.

Acknowledgements

References

Ahmed, S.I., Cain, R.F., 1972. Revision of the genera *Sporormia* and *Sporormiella*. Can. J. Bot. 50, 419-477.

Alroy, J., 1999. Putting North America's end-Pleistocene megafaunal extinction in context. Large-scale analyses of spatial patterns, extinction rates, and size distributions. In: MacPhee, R.D.E. (Ed.), Extinctions in near time. Causes, contexts, and consequences. Kluwer Academic, New York, pp. 105-143.

Anderson, S.T., 1960. Silicone oil as a mounting medium for pollen grains. Danmarks Geologiske Undersøgelse 4, 1-24.

Baker, A.G., Bhagwat, S.A., Willis, K.J., 2013. Do dung fungal spores make a good proxy for past distribution of large herbivores? Quat. Sci. Rev. 62, 21-31.

Bell, A., 2005. An illustrated guide to the coprophilous Ascomycetes of Australia. CBS Biodiversity Series No. 3, Fungal Biodiversity Centre, Utrecht.

Blackford, J.J., Innes, J.B., 2006. Linking current environments and processes to fungal spore assemblages: Surface NMP data from woodland environments. Rev. Palaeobot. Palynol. 141, 179-187.

Brown, C.A., 1960. Palynological Techniques. C.A. Brown, Baton Rouge.

Christenson, B.B., 1946. Measurement as a means of identifying fossil pollen. Danmarks Geologiske Undersøgelse 3, 1-22.

Clarke, C.M., 1994. Differential recovery of fungal and algal palynomorphs versus embryophyte pollen and spores by three processing techniques. In: Davis, O.K. (Ed.), Aspects of archaeological palynology: methodology and applications. American Association of Stratigraphic Palynologists, College Station, pp. 53-62.

Coles, G.M., 1987. Aspects of application of palynology to cave deposits in the Magnesian Limestone region of North Nottinghamshire. PhD Thesis, University of Sheffield.

Cugny, C., Mazier, F., Galop, D., 2010. Modern and fossil non-pollen palynomorphs from the Basque mountains (western Pyrenees, France): the use of coprophilous fungi to reconstruct pastoral activity. Vegetation History and Archaeobotany 19, 391-408.

Davis, O.K., 1987. Spores of the dung fungus *Sporormiella*: increased abundance in historic sediments and before Pleistocene megafaunal extinction. Quat. Res. 28, 290-294.

Davis, O.K., Shafer, D.S., 2006. *Sporormiella* fungal spores, a palynological means of detecting herbivore density. Palaeogeogr., Palaeoclimatol., Palaeoecol. 237, 40-50.

Dietre, B., Gauthier, É., Gillet, F., 2012. Modern pollen rain and fungal spore assemblages from pasture woodlands around Lake Saint-Point (France). Rev. Palaeobot. Palynol. 186, 69-89.

Doveri, F., 2007. Fungi fimicoli Italici. Associazione Micologica Bresadola / Fondazione Centro Studio Micologici Dell'A.M.B, Trento.

Doveri, F., 2011. Additions to "Fungi Fimicoli Italici": An update on the occurrence of coprophilous Basidiomycetes and Ascomycetes in Italy with new records and descriptions. Mycosphere 2, 331-427.

Ebersohn, C., Eicker, A., 1992. Coprophilous fungal species composition and species diversity on various dung substrates of African game animals. Botanical Bulletin of Academia Sinica 33, 85-95.

Erdtman, G., 1934. Über die Verwendung von Essigsäurenhydrid bei Pollenuntersuchungen. Svensk Botanisk Tidskrift 28, 354-358.

Erdtman, G., 1935. Investigation of honey pollen. Svensk Botanisk Tidskrift 29, 79-80.

Erdtman, G., 1936. New methods in pollen analysis. Svensk Botanisk Tidskrift 30, 154-164.

Erdtman, G., 1952. Pollen morphology and plant taxonomy. Angiosperms: an introduction to palynology. Alqvist and Wiksell, Stockholm.

Erdtman, G., Erdtman, H., 1933. The improvement of pollen analysis technique. Svensk Botanisk Tidskrift 27, 347-357.

Etienne, D., Wilhelm, B., Sabatier, P., Reyss, J.L., Arnaud, F., 2013. Influence of sample location and livestock numbers on *Sporormiella* concentrations and accumulations rates in surface sediments of Lake Allos, French Alps. J. Paleolimnol. 49, 117-127.

Faegri, K., Deuse, P., 1960. Size variations in pollen grains with different treatments. Pollen et Spores, 11, 293-298.

Faegri, K., Iversen, J., 1989. Textbook of pollen analysis, fourth ed. John Wiley and Sons, Chichester.

Feranec, R.S., Miller, N.G., Lothrop, J.C., Graham, R.W., 2011. The *Sporormiella* proxy and end-Pleistocene megafaunal extinction: A perspective. Quat. Int. 245: 333-338.

Gill, J.L., McLauchlan, K.K., Skibbe, A.M., Goring, S., Zirbel, C.R., Williams, J.W., 2013. Linking abundances of the dung fungus *Sporormiella* to the density of bison: implications for assessing grazing by megaherbivores in palaeorecords. J. Ecol. 101, 1125-1136.

Godwin, H., 1934. Pollen analysis. An outline of the problems and potentialities of the method. Part 1. Technique and interpretation. New Phytol. 33, 278-305.

Guarro, J., Gené, J., Stchigel, A.M., Figueras, M.J., 2012. Atlas of soil ascomycetes. CBS Biodiversity Series 10, CBS-KNAW Fungal Biodiversity Centre, Utrecht.

Hesse, M., Waha, M., 1989. A new look at the acetolysis method. Plant Syst. and Evol., 163, 147-152.

Horowitz, A., 1992. Palynology of arid lands. Elsevier, Amsterdam.

Hunt, C.O., 1985. Recent advances in pollen extraction techniques: a brief review. In: Fieller, N.R.J., Gilbertson, D.D., Ralph, N.G.A. (Eds.), Palaeobiological Investigations. British Archaeological Reports, International Series, Oxford, 266, pp. 181-187.

Ingold, C.T., 1933. Spore discharge in the Ascomycetes, I. Pyrenomycetes. New Phytol. 32, 175-196.

Ingold, C.T., Hadland, S.A., 1959. The ballistics of *Sordaria*. New Phytol. 58, 46-57.

Jeffers, E.S., Bonsall, M.B., Brooks, S.J., Willis, K.J., 2011. Abrupt environmental changes drive shifts in tree-grass interaction outcomes. J. Ecol. 99, 1063-1070.

Johnson, C.N., Rule, S., Haberle, S.G., Turney, C.S.M., Kershaw, A.P., Brook, B.W., 2015. Using dung fungi to interpret decline and extinction of megaherbivores: problems and solutions. Quat. Sci. Rev. 110, 107-113.

Kirk, P.M., Cannon, P.F., Minter, D.W., Stalpers, J.A. (Eds.), 2008. Dictionary of the fungi, tenth ed. CSIRO Publishing, Oxon.

Krug, J.C., 2004. Moist chambers for the development of fungi. In: Mueller, G.M., Bills, G.F., Foster, M.S. (Eds.), Biodiversity of fungi, Inventory and monitoring methods. Elsevier Academic Press, San Diego, pp. 589-593.

Krug, J.C., Benny, G.L., Keller, H.W., 2004. Coprophilous fungi. In: Mueller, G.M., Bills, G.F., Foster, M.S. (Eds.), Biodiversity of fungi, Inventory and monitoring methods. Elsevier Academic Press, San Diego, pp. 467-499.

Kruys, Å., Wedin, M., 2009. Phylogenetic relationships and an assessment of traditionally used taxonomic characters in the Sporormiaceae (Pleosporales, Dothideomycetes, Ascomycota), utilising multi-gene phylogenies. Systematics and Biodiversity 7, 465-478.

Large, M.F., Braggins, J.E., 1990. Effect of different treatments on the morphology and size of fern spores. Rev. Palaeobot. Palynol. 64, 213-221.

Lentfer, C.J., Boyd, W.E., 2000. Simultaneous extraction of phytoliths, pollen and spores from sediments. J. Archaeol. Sci. 27, 363-372.

Lumbsch, H.T., Huhndorf, S.M., 2007. Outline of Ascomycota – 2007. Myconet 13, 1-58.

Lundqvist, N., 1972. Nordic Sordariaceae s. lat. Acta Universitas Upsaliensis, *Symbolae Botanicae Upsaliensis* XX: 1.

Lyons, S.K., Smith, F.A., Brown, J.H., 2004. Of mice, mastodons and men: human-mediated extinctions on four continents. Evol. Ecol. Res. 6, 339-358.

Mertens, K.N., Verhoeven, K., Verleye, T., Louwye, S., Amorim, A., Ribeiro, S., Deaf, A.S., Harding, I.C., De Schepper, S., Kodrans-Nsiah, M., de Vernal, A., Henry, M., Radi, T., Dybkjaer, K., Poulsen, N.E., Feist-Burkhardt, S., Chitolie, J., González Arango, C.M., Heilmann-Clausen, C., Londeix, L., Turon, J.-J., Marret, F., Matthiessen, J., McCarthy, F.M.G., Prasad, V., Pospelova, V., Kyffin Hughes, J.E., Riding, J.B., Rochon, A., Sangiorgi, F., Welters, N., Sinclair, N., Thun, C., Soliman, A., Van Nieuwenhove, N., Vink, A., Young, M., 2009. Determining the absolute abundance of dinoflagellate cysts in recent marine sediments: the *Lycopodium* marker-grain method put to the test. Rev. Palaeobot. Palynol. 157, 238–252.

Moore, P.D., Webb, J.A., Collinson, M.E., 1991. Pollen analysis, second ed. Blackwell Scientific, London.

Mudie, P.J, Lalièvre, M.E., 2013. Palynological study of a Mi'kmaw shell midden, Northeast Nova Scotia, Canada. J. Archaeol. Sci. 40, 2161-2175.

Parker, N.E., Williams, J.W., 2012. Influences of climate, cattle density, and lake morphology on *Sporormiella* abundances in modern lake sediments in the US Great Plains. The Holocene 22, 475-483.

Praglowski, J., 1970. The effects of pre-treatment and mounting media on the shape of pollen grains. Rev. Palaeobot. Palynol. 10, 203-208.

Raper, D., Bush, M., 2009. A test of *Sporormiella* representation as a predictor of megaherbivore presence and abundance. Quat. Res. 71, 490-496.

Reitsma, T., 1969. Size modification of recent pollen grains under different treatments. Rev. Palaobot. Palynol. 9, 175-202.

Richardson, M.J., 1972. Coprophilous ascomycetes on different dung types. Transactions of the British Mycological Society 58, 37-48.

Van Geel, B., 2001. Non-pollen palynomorphs. In: Smol, J.P., Birks, H.J.B., Last, W.M. (Eds.), Tracking environmental change using lake sediments, Volume 3: Terrestrial, algal and silicaceous indicators. Kluwer, Dordrecht, pp. 99-119.

Webster, J., Weber, R.W.S., 2007. Introduction to fungi, third ed. Cambridge University Press, Cambridge.

Wenner, C.-G., 1947. Pollen diagrams from Labrador. Geog. Ann. 29, 3-4, 137-373.

Wood, G.D., Gabriel, A.M., Lawson, J.C. 1996. Palynological Techniques – Processing and Microscopy. In: Jansonius, J., McGregor, D.C. (Eds.), Palynology: Principles and Applications.

Vol. 1. American Association of Stratigraphic Palynologists Foundation, College Station, pp. 29-50.

Yafetto, L., Carroll, L., Cui, Y., Davis, D.J., Fischer, M.W.F., Henterly, A.C., Kessler, J.D., Kilroy, H.A., Shidler, J.B., Stolze-Rybczynski, J.L., Sugawara, Z., Money, N.P., 2008. The fastest flights in nature: high-speed spore discharge mechanisms among fungi. PLoS One 3, e3237.

Table 1. Dung samples

Sample no.	Species	Preparation methods
KDLA1	*Loxodonta africana*	A & B1
KDLA2	*Loxodonta africana*	C & D
KDCS1	*Ceratotherium simum*	A & B1
KDCS2	*Ceratotherium simum*	C & D
CDBT1	*Bos taurus*	A & B1
CDBT2	*Bos taurus*	A & B1
CDBT3	*Bos taurus*	A & B1 & B2
CDBT4	*Bos taurus*	C & D

Table 2. Description of sample preparation methods

Method A[1]	Method B	Method C	Method D
Addition of *Lycopodium tracer tablets*	Addition of *Lycopodium tracer tablets*	Addition of *Lycopodium tracer tablets*	Addition of *Lycopodium tracer tablets*
Volumetric sampling	Volumetric sampling	Volumetric sampling	Volumetric sampling
Heating with 10% sodium hydroxide	Heating with 10% sodium hydroxide (method B1) or 10% potassium hydroxide (method B2)	Heating with 10% potassium hydroxide	
Sieving out the fraction >125 μm	Sieving out the fraction >125 μm	Sieving out the fraction >125 μm	Sieving out the fraction >125 μm
		Density separation using a swirling dish	
Sieving out the fraction <6 μm	Sieving out the fraction <6 μm	Sieving out the fraction <6 μm	Sieving out the fraction <6 μm
Treatment with 10% hydrochloric acid	Treatment with 10% hydrochloric acid	Treatment with 10% hydrochloric acid	
Acetolysis by washing with glacial acetic acid and heating with sulphuric acid and acetic anhydride			
Staining with safranine and mounting in silicon oil using tertiary butyl alcohol	Staining with safranine and mounting in silicon oil using tertiary butyl alcohol	Staining with safranine and mounting in silicon oil using tertiary butyl alcohol	Staining with safranine and mounting in silicon oil using tertiary butyl alcohol

[1] Although the standard pollen preparation method generally includes treatment with HF, this was not included here due to laboratory restrictions

Table 3. Taxa present on dung samples and in preparations

Taxon	Dung[1]	Method A	Method B	Method C	Method D
Zygomycetes	+++[2]	-	-	+	-
Ascobolus	+++	+	+	++	++
Saccobolus	++	++	++	++	++
Cheilymenia	+++	-	-	+	+++
Sordaria	++	++	++	++	++
Cercophora / Podospora	++	+	+	+	+
Sporormiella	+	+	+	+	+
Peziza	+	na[3]	na	+	+
Basidiomycetes	+++	+	+	+	+

[1] This refers to abundance when present, not to how commonly the genus is encountered across the different dung samples
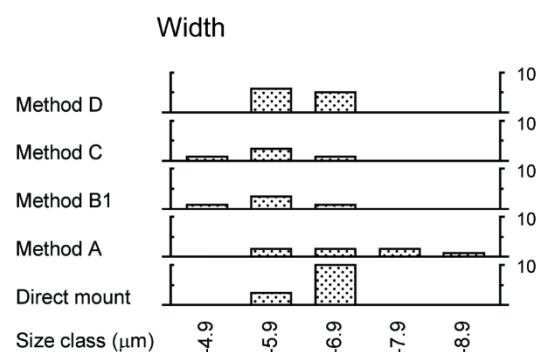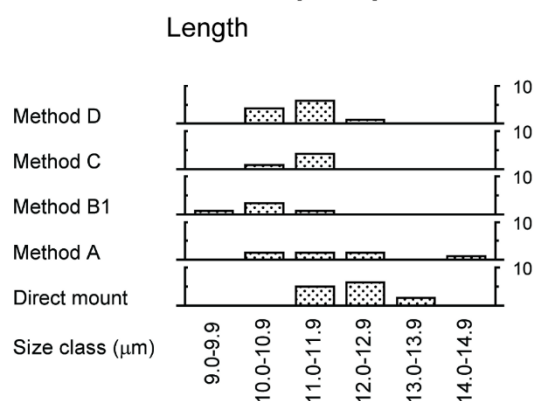
[2] +++ abundant; ++ common; + present but rare; - absent

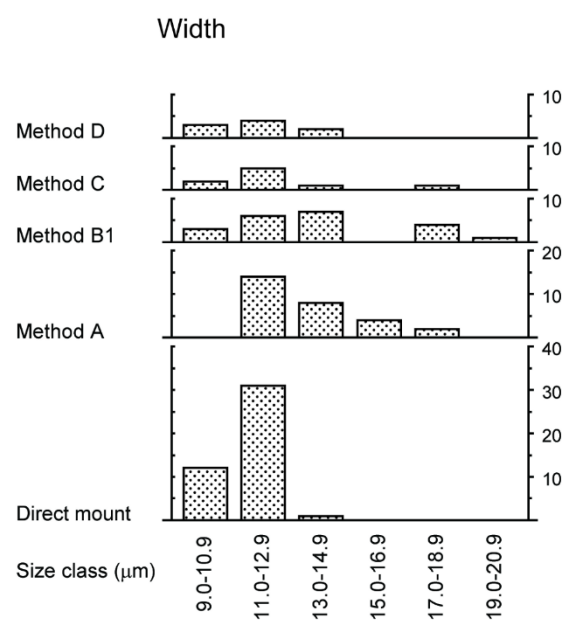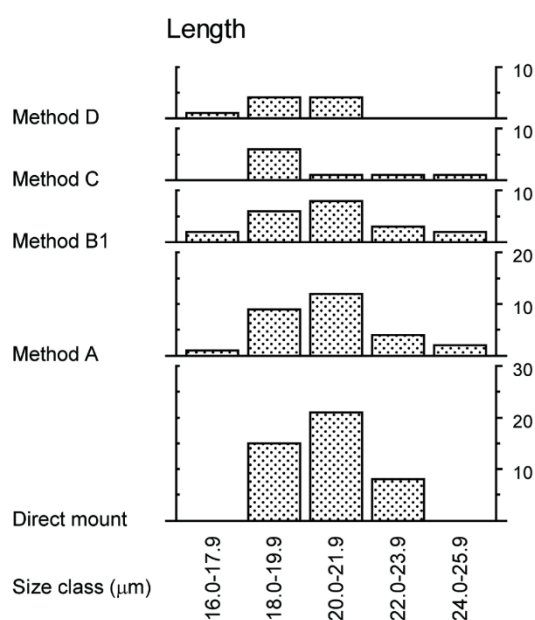[3] This genus was not present on the dung samples that were prepared using methods A and B

Figure 1. Histograms of length and width of spores of *Saccobolus depauperatus, Sordaria fimicola* and *Sporormiella* spp. when mounted directly from dung in alcohol and lactophenol and after treatment with a range of pollen preparation methods

Saccobolus depauperatus

Length

Method D · Method C · Method B1 · Method A · Direct mount

Size class (µm): 9.0-9.9, 10.0-10.9, 11.0-11.9, 12.0-12.9, 13.0-13.9, 14.0-14.9

Width

Method D · Method C · Method B1 · Method A · Direct mount

Size class (µm): 4.0-4.9, 5.0-5.9, 6.0-6.9, 7.0-7.9, 8.0-8.9

Sordaria fimicola

Length

Method D · Method C · Method B1 · Method A · Direct mount

Size class (µm): 16.0-17.9, 18.0-19.9, 20.0-21.9, 22.0-23.9, 24.0-25.9

Width

Method D · Method C · Method B1 · Method A · Direct mount

Size class (µm): 9.0-10.9, 11.0-12.9, 13.0-14.9, 15.0-16.9, 17.0-18.9, 19.0-20.9

Sporormiella spp.

Length

Method D · Method C · Method B1 · Method A · Direct mount

Size class (µm): 7.0-7.9, 8.0-8.9, 9.0-9.9, 10.0-10.9, 11.0-11.9, 12.0-12.9

Width

Method D · Method C · Method B1 · Method A · Direct mount

Size class (µm): 4.0-4.9, 5.0-5.9, 6.0-6.9, 7.0-7.9, 8.0-8.9, 9.0-9.9
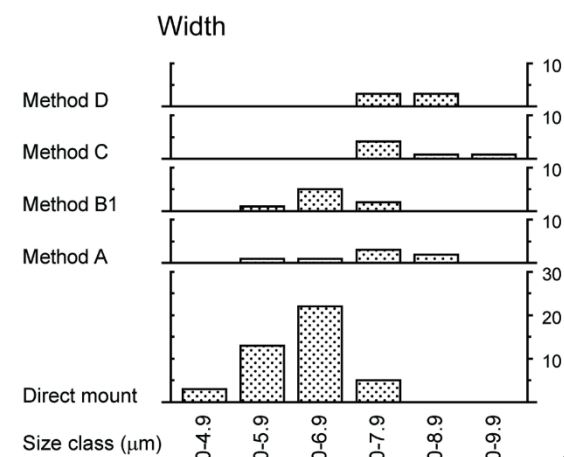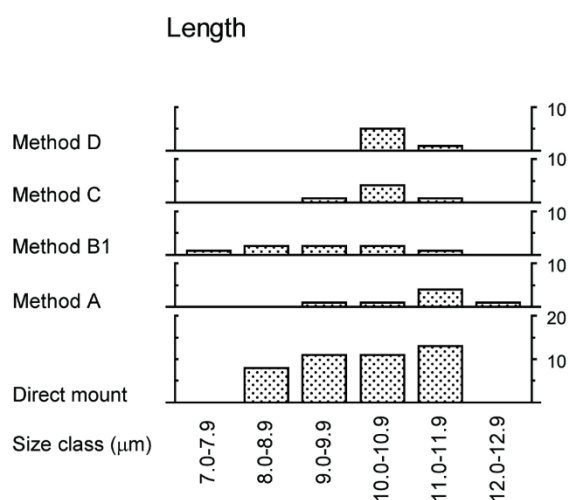
Plate I. Size and shape of spores after treatment with a range of pollen preparation methods or mounted directly from dung in alcohol and lactophenol.

1a. Spores of *Cheilymenia granulata* mounted in alcohol and lactophenol; 1b. Spores of *Peziza vesiculosa* mounted in alcohol and lactophenol; 1c. Spore of *Cheilymenia/Peziza* after treatment with method D; 1d. Spores of *Cheilymenia* after treatment with method D.

2. Spores of *Ascobolus*; a-c. mounted in alcohol and lactophenol; a. *A. immersus*; b. *A. albidus*; c. *A.* cf. *michaudii*; 2d. after treatment with method A; 2e-f. after treatment with method B1; g. after treatment with method C; h. after treatment with method D.

3. Spores of *Saccobolus*; a. *S. depauperatus* mounted in alcohol and lactophenol; b. after treatment with method A; c. after treatment with method B1; d. after treatment with method C; e. after treatment with method D.

4. Spores of *Sporormiella*; a-b. mounted in alcohol and lactophenol; c. after treatment with method A; d. after treatment with method D.

5. Spores of *Sordaria*; a. *S. fimicola* mounted in alcohol and lactophenol; b, f. after treatment with method B1; c-e. after treatment with method A.

6. Spores of *Cercophora/Podospora*; a-d. mounted in alcohol and lactophenol; a. *C. mirabilis*; b. *P. pauciseta*; c. *P. fimiseda*; d. *P. conica*; e-h. after treatment with method A; i-j. after treatment with method B1; k-m. after treatment with method C; n-o. after treatment with method D.