




Review

# On the Use of Spores of Coprophilous Fungi Preserved in Sediments to Indicate Past Herbivore Presence

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**Abstract:** Fungal spores that grew on the faeces of herbivores in the past can be extracted from sediments and used to identify the presence of herbivores in former ecosystems. This review: (i) examines the factors that should be considered when interpreting these fungal spores, (ii) assesses the degree to which they can be used to estimate past herbivore populations and biomass density change, and (iii) identifies gaps in our current understanding that limit, or confound, the information that can be extracted from the fungal spore record. We focus on the life cycles of coprophilous fungi and highlight the importance of understanding spore dispersal mechanisms to ensure robust palaeoecological interpretation. We then discuss how variation in methodological approaches across studies and modifications can influence comparability between studies. The key recommendations that emerge relate to: (i) improving our understanding of the relationship between spores of coprophilous fungi (SCF) and herbivores through the study of the coprophilous fungi succession; (ii) refining our understanding of how climate and environment parameters effect fungal spore abundance, with particular reference to estimating past herbivore biomass density; and (iii) enhancing sedimentary DNA (SedaDNA) analysis to identify SCF that do not allow preservation in a way that allows visual identification. To further this field of study and provide more robust insights into herbivores in the past, we suggest that additional research is required to help to reduce bias during the preparation process, that concertation metrics are used for the quantification of SCF, and that multiple cores should be taken in each site and multiproxy analysis should be utilised.

**Keywords:** faeces; non-pollen palynomorphs; palaeoecology; mycology; herbivore biomass; life cycle; ecosystems



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## 1. Introduction

Palynology is the study of ancient and modern pollen and non-pollen palynomorphs (NPPs) [1,2]. Ancient NPPs can preserve, due to their resistant organic composition [2], and provide information regarding the ecosystem in which they were deposited. NPP is an umbrella term for all microfossil material in a sample that are not pollen or fern spores, including fungal spores, cyanobacteria, rhizopods, and other invertebrates [2–5]. They can provide additional (palaeo)ecological information depending on which microfossil is found and quantified [4].

Spores of coprophilous fungi (SCF) are NPPs that are frequently used in conjunction with other palaeoenvironmental proxies, such as pollen, macrofossils, and charcoal [6,7]. When used together in this way, it is possible to reconstruct the vegetation, climate, fire regime, and herbivore activity to reveal more clearly past ecosystem functions. These palaeoenvironmental reconstructions can provide information on current and future ecosystems and help to guide management policies [8]. Although the potential for SCF to play an important role is high, their application has, however, been hindered because no standardised methodology for recording fungal spores exists [9–11]. The lack of standard methodology has impeded cross site comparisons [12,13], but, regardless of this, the practice of using SCF as indicators for herbivore activity continues to increase [13,14]. It is,

therefore, timely to review the literature, evaluate the success of different methodologies, and summarise implications for future SCF research.

The principle underpinning the use of SCF in past ecosystem reconstructions is that coprophilous fungi grow most frequently on the substrate of herbivore faeces [13,15–17]. Consequently, when herbivores are taken out of an ecosystem, the numbers of SCF are much reduced [18,19]. Based on this principle, SCF found in sedimentary samples have been used to indicate the presence vs. absence, or trends of density changes, of herbivores in the past [13,15,20,21]. In particular, SCF have been extensively used to trace back the presence of large herbivores during the Pleistocene and Holocene; for example, in North America, researchers have used SCF to create records of mass megaherbivore extinctions at the end of the last ice age [13,14,18,19,22–24].

In this review, we provide an overview of the state of knowledge and gaps that remain related to the use of SCF. The key questions we address are: Are spores of copro-philous fungi an accurate indicator of herbivore presence, and can they be used to make herbivore biomass estimations? Firstly, we discuss the development of SCF within palynology and detail the often-overlooked role of the life cycles of different fungal classes. Secondly, we examine the development of the SCF record with insight into preparation, identification, quantification, and interpretation and specifically address: (i) the popular use of the *Sporormiella* type with comparisons to other SCF, (ii) new research avenues and further interdisciplinary inclusion, and (iii) the abiotic and biotic influences on spores, emphasising the role of transportation of spores and the effects of specific sample environments. The review ends with a series of recommendations of how this subdiscipline can develop and improve.

## 2. A Short History of Coprophilous Fungi in Palynology

Coprophilous fungi have been studied for many decades [25,26]. Initially, they were studied as essential biomass decomposers [25,27] and were considered to be too fragile to preserve in the sedimentary record [28]; however, in the 1970s and 1980s, they were recognized within sediments and proposed as indicators of herbivores in the past. The first observation relating SCF abundances (*Sporormiella*) in sedimentary deposits to past changes in grazing pressure came from Davis et al. [29] based in a study from Wildcat Lake (USA).

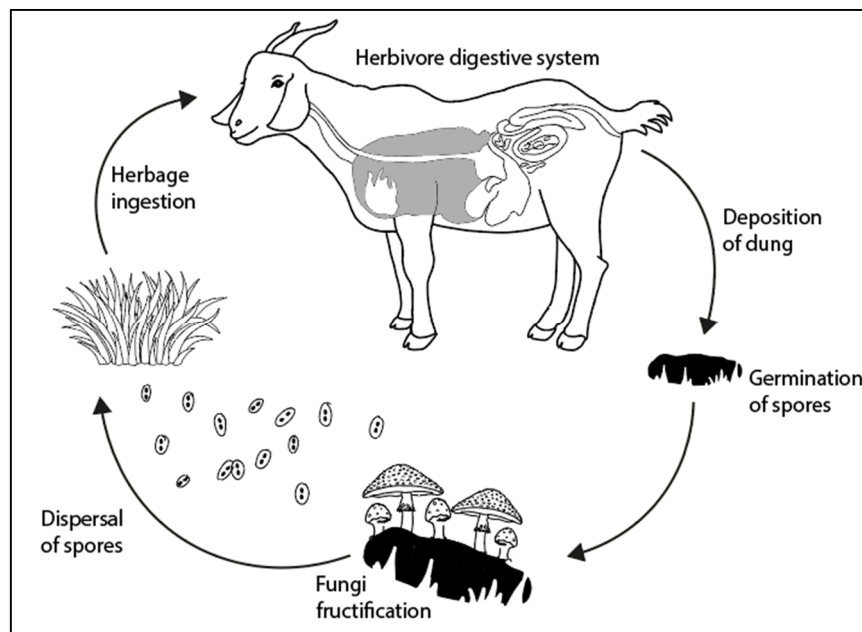
The pioneering work of B. van Geel and co-workers at the University of Amsterdam led to the incorporation of NPPs in traditional (pollen and fern spore) palynological studies [4]. The microscopic analysis of many sub-samples extracted from many sedimentary records resulted in hundreds of fungal types being identified. This led to a paradigm shift in palaeoecological research as the huge increase in indicators allowed previously unseen aspects of past ecosystems to be reconstructed [4]. The process of categorizing and establishing NPPs as palaeoecological proxies was, and remains, necessarily interdisciplinary, gaining contributions from zoology, mycology, phycology, and plant anatomy [4]. In some cases, fungal spores extracted from sediments can be given genera names that are associated with their modern-day equivalent [30]; however, other fungal spores cannot be reliably attributed to modern equivalents, and these unidentified types are therefore given a type number that follows the location where they were first identified [31].

Over the past decades, our understanding of complex fungal successions has developed [32]. Initially, it was postulated that fungal succession was a function of nutrition [13,16]; however, this was disproven in 1964 by Harper and Webster [16,33], and it is now believed that various life cycles of fungi control the succession of coprophilous fungi [25].

## 3. Fungal Spore Life Cycle

In addition to fungi-class-specific life cycles, some SCF also experience the cycle of being consumed and ejected by herbivores (this cycle is termed endocoprophilous) (Figure 1). There are two types of SCF: obligate spores, which must pass through an animal's digestion system in order to germinate, and facultative spores, which germinate

without having to pass through a digestion system [27,34]. Recent experimental work has confirmed the long-held belief that the majority of SCF fall into the obligate category [13,32]. This strong link between animals and spore germination strengthens the reliability of SCF as a proxy for past animals in landscapes.

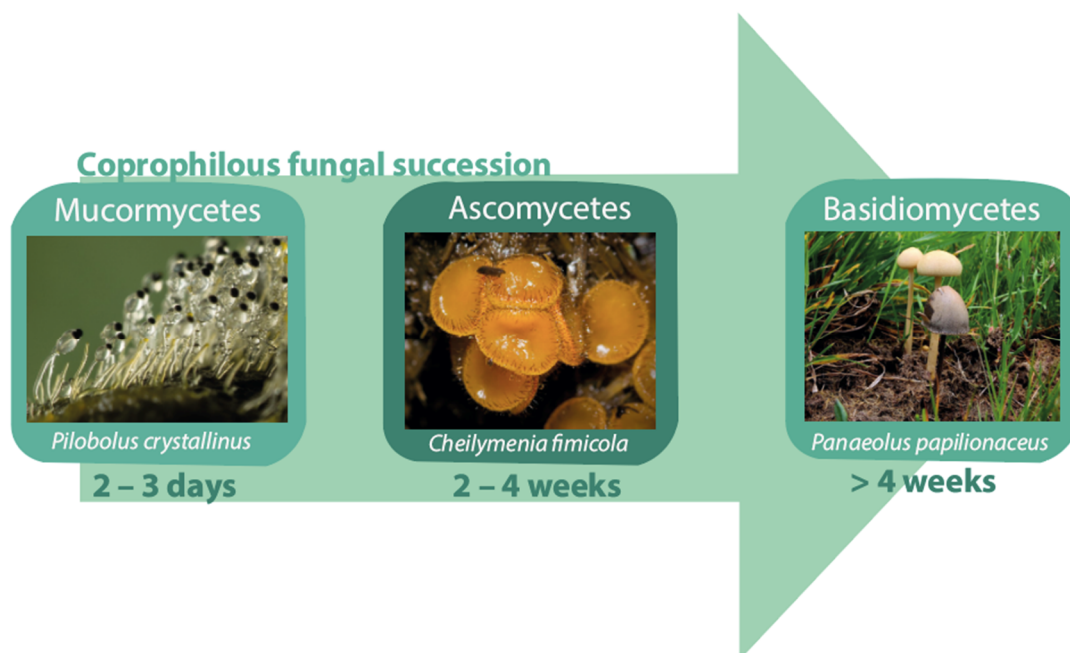


**Figure 1.** The endocoprophilous life cycle of coprophilous fungi.

The endocoprophilous life cycle is well researched [32,34]. When herbivores engage in coprophagy, they simultaneously consume the fungal fruitbodies (with spores inside) that grow on the surface of faeces, and plant material with attached spores [14,20]. The spores have thick melanised walls that act as protective layers, allowing them to travel through the digestive system without being harmed [16,32]; the thick wall also improves the likelihood of SCF being preserved in sedimentary records [35]. Having passed through the digestive track of an animal, ultimately dung and the incorporated spores will then be ejected and the spores are left to germinate and grow on the dung substrate [16,19,32,35]. At a mature stage of fructification, the fungi eject spores away from the dung onto the surrounding herbage for the cycle to repeat itself [13,14,16]. Consequently, a top-down process takes place [36] as increased production of spores is then available to be incorporated into the sedimentary record that will mainly reflect changes in the population density of herbivores [20].

Faeces provide the perfect substrate for fungi to grow, being high in minerals, vitamins, and nitrogen, with a relatively high temperature and moisture, which reduces over time [25,34]. However, spatially, the mycelium is limited to the size of the faeces [32,37]. Consequently, coprophilous fungi studies are likely to be related to megaherbivores rather than smaller herbivores due to their faeces commonly being larger. The struggle for substrate space also affects the fungal succession. Coprophilous fungi include four classes, the three main ones being Mucormycetes, Ascomycetes, and Basidiomycetes (Figure 2) [13,38]. Life cycles vary between the different fungal classes, which creates different timescales for fruiting. These different fruiting timescales create a succession of coprophilous fungi (Figure 2) [25]. The fruiting succession commences rapidly after deposition, which is likely due to microbial and invertebrate competition [32]. Despite this, the detailed understanding of coprophilous fungal succession, mycology, and the biology of spores is rarely introduced in palaeoecological SCF studies. Therefore, the following section aims to provide a comprehensive overview of the succession of coprophilous fungi for each of the fungal classes, so that the important role of reproduction in spore dispersal can be more easily considered in future studies. This fusion of palaeoecological and mycological information of fungal

spores has been previously highlighted as necessary for the further expansion of the field by Gauthier and Jouffroy-Bapicot [38].



**Figure 2.** Coprophilous fungal class succession. Left to right: Mucormycetes image of *Pilobolus crystallinus* (image from [39]), Ascomycetes image of *Cheilymenia fimicola* (image from [40]), and Basidiomycetes image of *Panaeolus papilionaceus* (image from [41]).

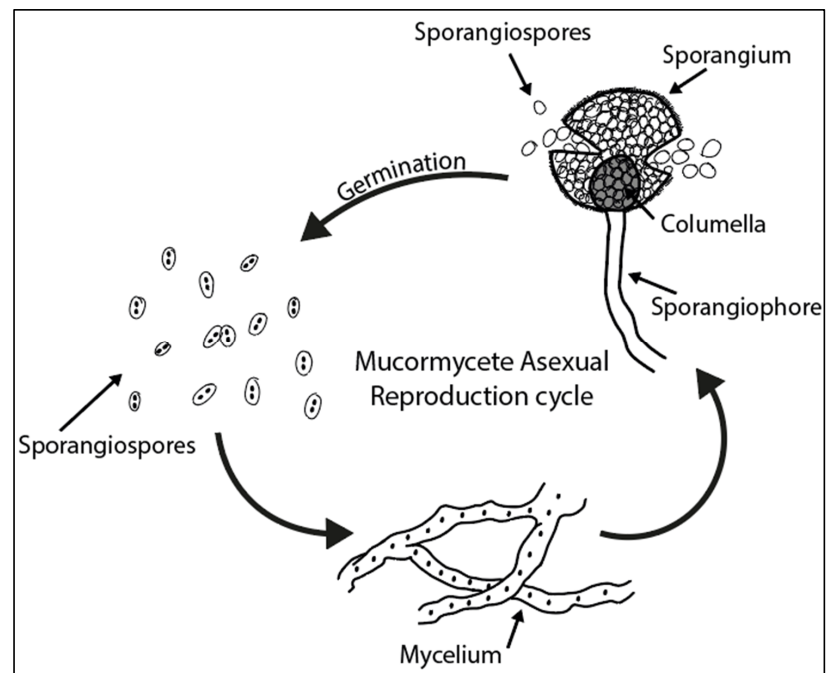
### 3.1. Mucormycetes

After the deposition of dung, Mucormycete fungi are the first to grow, producing between 100–100,000 spores within the first 2–3 days after deposition [16,42]. The Mucormycete or Zygomycete class are not exclusively coprophilous; however, the species *Pilobolus* and *Pilaira* are [16]. The optimum growing and sporulation temperature for Mucormycete fungi is 27 °C with high humidity, making them ideal primary colonizers [42]. Their mycelium is typically aseptate, i.e., not divided by cells [16] and their reproduction process is heterothallic [43]. Nevertheless, they can reproduce sexually or asexually, depending on the mating type present on the substrate [16,43].

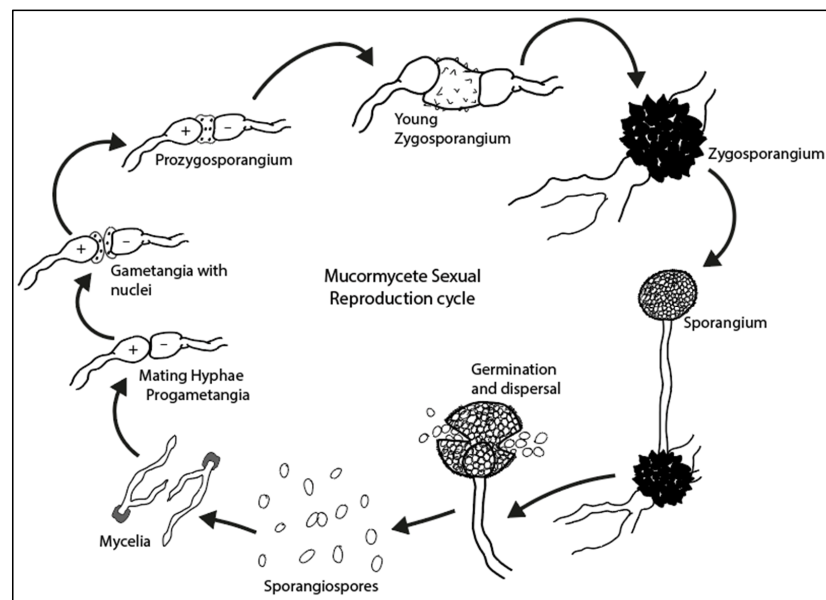
Mucormycetes' asexual reproduction (Figure 3) is possible through spores produced in a sporangium [28]. This is the most common reproduction method for mucormycetes on dung as usually only one mating types is present on the substrate [43]. When sporangiospores germinate on dung, a hyphal thallus is formed, which produces sporangia or merosporangia after a few days [43]. The sporangia then form sporangiospores, which are ejected once mature (Figure 3) [42,43]. Mucormycete fungi, which are homothallic, meaning they have both male and female reproductive structures, produce zygospores simultaneously with sporangia [43]. This is a classic Mucormycete characteristic [28]. Unfortunately, zygospore germination is infrequently mentioned in studies [43] and lacks research.

Heterothallic Mucormycetes species have two sexual hyphae, which grow together [43]. The hyphae apex is fused through the formation of progametangia once trisporic acid, a pheromone compound, is produced [43]. Subsequently, the progametangia become the gametangia when the apex fuse; this is followed by the septum separating the apex [43]. After the septum disintegrates, the nuclei in the cell are fused creating the prozygosporangium, which enlarges into zygosporangia [43]. These then form hyphae, and after germination, produce germ sporangia [43]. Germ sporangia often contain both mating types of sporangiospores [43], thus completing the negative feedback loop (Figure 4). The

sporangiospores are then ejected through various mechanisms that are dependent on the species and the environment [16,26], but are likely dispersed through the air [42].



**Figure 3.** Generalised mucormycetes’ asexual reproduction cycle (adapted after Bell [16]). Sporangiospores are the spores produced inside the sporangium which is a cell of a fungus which contains spores. The sporangiochore is the stalk of the sporangium and the columella is a column of sterile tissue which bears spores.

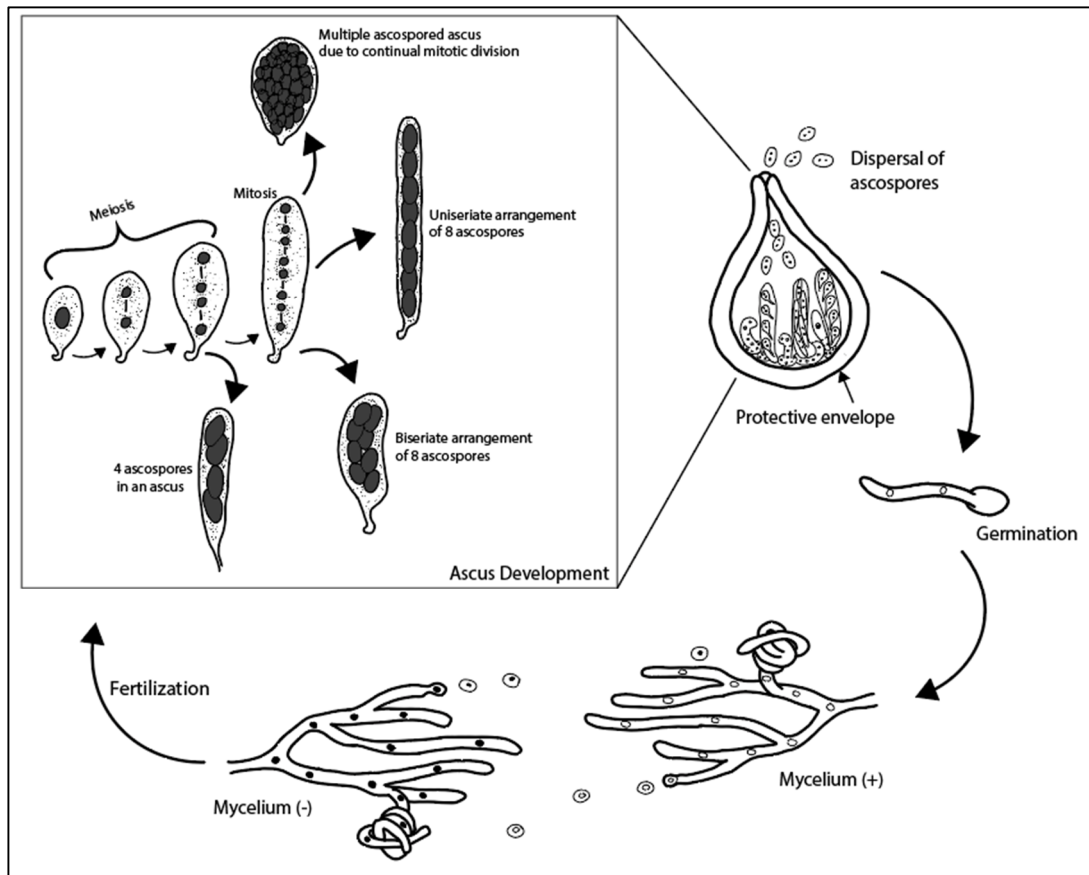


**Figure 4.** Generalised mucormycetes’ sexual reproduction cycle (adapted after Nwe et al. [44]). Mycelia is the plural form of mycelium. Progametangia is the hyphal threads on the tip of which the gametangia form. The gametangia are the sex organs of fungus. The fusion of + and – nuclei is referred to as the prozygosporangium. The young zygosporangium forms from the prozygosporangium secreting a heavy wall. This then matures to become the zygosporangium. Once this cracks, the up-right hypha called promycelium form, which has the sporangium at its top. The sporangium is the enclosure in which spores, in this case known as sporangiospores, from [45].



### 3.2. Ascomycetes

Once the Mucormycetes population on the substrate has depleted, the second stage of succession takes place [16]. Ascomycetes appear for 2–4 weeks, as their complex fruit-bodies [16] require more time to complete their life cycle. Palaeoecological studies most commonly use spores which belong to the class Ascomycetes as they are usually most abundantly preserved and identified [30,38]. They all have a distinguishing characteristic called the ‘ascus’ in which multiple ascospores are formed [26] (Figure 5). These hyaline asci do not preserve in sediments, while the ascospores do.



**Figure 5.** Development of ascospores (adapted after Peraza Reyes and Berteaux-Lecellier [46]). Top right corner displayed the formation of fruitbodies with asci (adapted after Bell [16]). An ascospore is a spore contained, or produced from, an ascus. The ascogonium is the female mating type that contains the gametic nuclei. The process of meiosis is cell division, reducing chromosomes in gametes. The process of this takes place inside a protective envelope through which the ascospores are discharged from the ostiole.

The life cycle of Ascomycetes species can vary; however, the general pattern is shown in Figure 5. Once an ascospore germinates, a mycelium forms [26]. The nuclei in the spore divide and the hypha grow, branching off in some places [26]. Fertilization is then possible if there are two opposing mating types; the male nucleus is retrieved by the female nuclei or ascogonium [46]. From this, the hymenium is created in which ascospores are produced [46] (Figure 5).

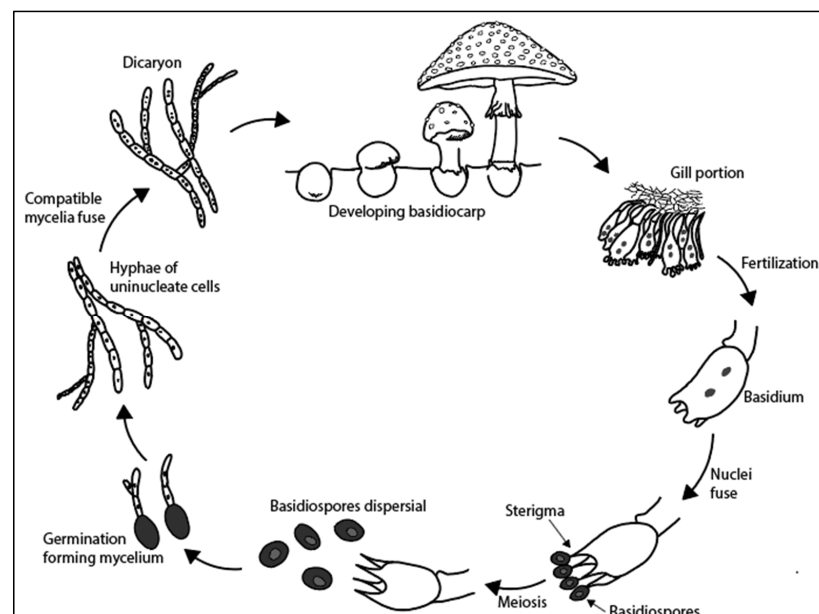
Ascospores form through the fusion of two nuclei after which the process of meiosis, i.e., the division of cells, takes place and four nuclei are produced inside the ascus [16,28]. This division is then repeated, sometimes numerous, and can result in the production of 64, or more, ascospores in the ascus [16,26,28]. Once mature after mitosis, the ascospores are then ejected from the ascus, allowing the formation of mycelium post-germination [16,43].

The arrangement of the ascus varies depending on the Ascomycete genus, which could provide a useful tool for genus identification [16]; however, they are rarely, if ever, preserved. Many coprophilous Ascomycetes have phototropic asci, allowing them to move towards light [16]. This characteristic guarantees that the ascospores are ejected and discharged in the direction of herbage, so the spores can be digested [16]. Many species are primarily transported aerially or through water [43]. Van Geel et al. [47,48] studied macrofossils in intestinal faecal samples of mammoths preserved in permafrost. Fruitbodies—still filled with spores—of coprophilous Ascomycetes were found. Such fruitbodies do not grow inside intestines and, thus, the conclusion was made that mammoths consumed faeces (coprophagy).

### 3.3. Basidiomycetes

Basidiomycetes are a sister group to Ascomycetes [28] and are usually the last to appear in the coprophilous fungi succession [16]. Only few genera of this class are coprophilous [16,43] and their small spore size results in little representation in SCF studies [26]. The order most often preserved in palaeoecological studies of SCF are the *Ustilaginales*, which is a parasitic order generally with bigger spores [26].

A characteristic that makes Basidiomycete fungi so different to other classes is their production of basidiospores on the outside of the basidium (the body that produces the spores) (see Figure 6) [26]. The formation of basidiospores is similar to that of ascospores [26]. The basidiospores are powerfully exerted from the basidium, where they are then able to germinate and form mycelium with hyphae of uninucleate cells [43]. Next, the cytoplasm within the cells, from two compatible mycelia, fuse to form a dicaryon (cell with two nuclei), which results in a binucleate cell [26,28,43]. This then divides, forming a binucleate mycelium that fruits from the hyphae [43]. The fusion and meiosis of the hyphae creates the basidia, on which there are sterigmata where the basidiospores form on the outside (Figure 6) [28,43]. Once spores are released, if they find themselves on suitable substratum, they will germinate [43] and the cycle continues (Figure 6).



**Figure 6.** Generalised basidiomycetes' sexual reproduction cycle (adapted after Taylor et al. [28]). Basidiospores are reproductive spores produced by Basidiomycetes. The dicaryon is mycelium that contains cells with two nuclei. Basidiocarp is a multicellular structure from which the hymenium and subsequently spores are produced. The gill portion is the hymenophore rib found under the cap of the mushroom. The basidium is where basidiospores are produced and the sterigma is the supporting structure.

### 3.4. Fungi Imperfecti

Fungi Imperfecti, also known as Deuteromycetes, are not strictly coprophilous, but do play an important role in substrate decomposition [16,49] and, therefore, fungal succession. The Fungi Imperfecti comprises fungi whose sexual characteristics are unknown, but are postulated to be asexual forms of Ascomycetes or Basidiomycetes [49,50]. Therefore, they follow a similar life cycle, but asexual multiplication takes place through mitosis after the formation of conidia, asexual immobile spores, from the conidiophore [49]. The structures of spore formation, i.e., conidio-genesis, varies across species [49].

## 4. Constructing a Record of SCF from Sedimentary Samples

The preparation of sub-samples of sediment for NPPs generally follows one or two protocols: (i) the 'standard' pollen preparation techniques for NPPs outlined by Faegri and Iversen [51] and (ii) the University of Amsterdam (UvA) method as detailed by van Geel [4]. These protocols differ in the way they use the size of the material and chemical treatment to clean the samples before inspection under the microscope.

### 4.1. Preparation

The morphology of a fungal spore determines the likelihood of SCF 'surviving' sample preparation [11]. Spores with thicker walls will be more resistant to deterioration and larger spores are more likely to be caught in sieves, while smaller spores may be damaged [14]. In standard procedures, circular SCF will likely perform similarly to pollen grains, but other morphological configurations can potentially behave differently [11]. Morphological configurations among SCF in general can lead to a potential bias when using the 'standard' pollen preparation of Faegri and Iversen [51]. This is because the sieving technique early in the procedure is intended for the removal of clay size particles, yet it will inevitably also remove some smaller spores. In particular, many Basidiomycetes spores are smaller than 6–10µm and are consequently unlikely to be retained [14]. This bias causes an unbalanced source of information because Basidiomycetes fruit later in the coprophilous fungal succession. The alternate (UvA) method requires sieving (mesh size 215 µm) at the beginning of the preparation with the intention to remove unnecessary larger plant remains. The recommended width of the mesh ensures that all, even very small, NPPs and pollen grains are preserved. Another consideration is fruitbodies that are likely to break up during the preparation process [14] and release the previously withheld ascospores, affecting the overall spore count. However, fruitbodies are usually only observed in macrofossil samples. Similarly, fungal types such as *Cheilymenia* are abundant in cattle dung, but are often destroyed in preparation techniques due to their fragility [11].

The chemical treatment of sub-samples during the preparation process has also been shown to impact the recovery of SCF and NPPs [11]. Clarke's experiments in 1994 revealed that, when spores are boiled with potassium or sodium hydroxide (KOH and NaOH) or treated with acetolysis, their morphology can change, i.e., swell or shrink. This change in morphology could lead to the misidentification of spores and so should be avoided in laboratory preparation techniques if possible. Interestingly, van Asperen et al. [11] found that sieving alone produces the best fungal spore diversity and abundance in samples. Ideally, to obtain a full picture of SCF within samples, a separate, sieve-only procedure should be followed. However, it should be noted that this procedure is time-consuming.

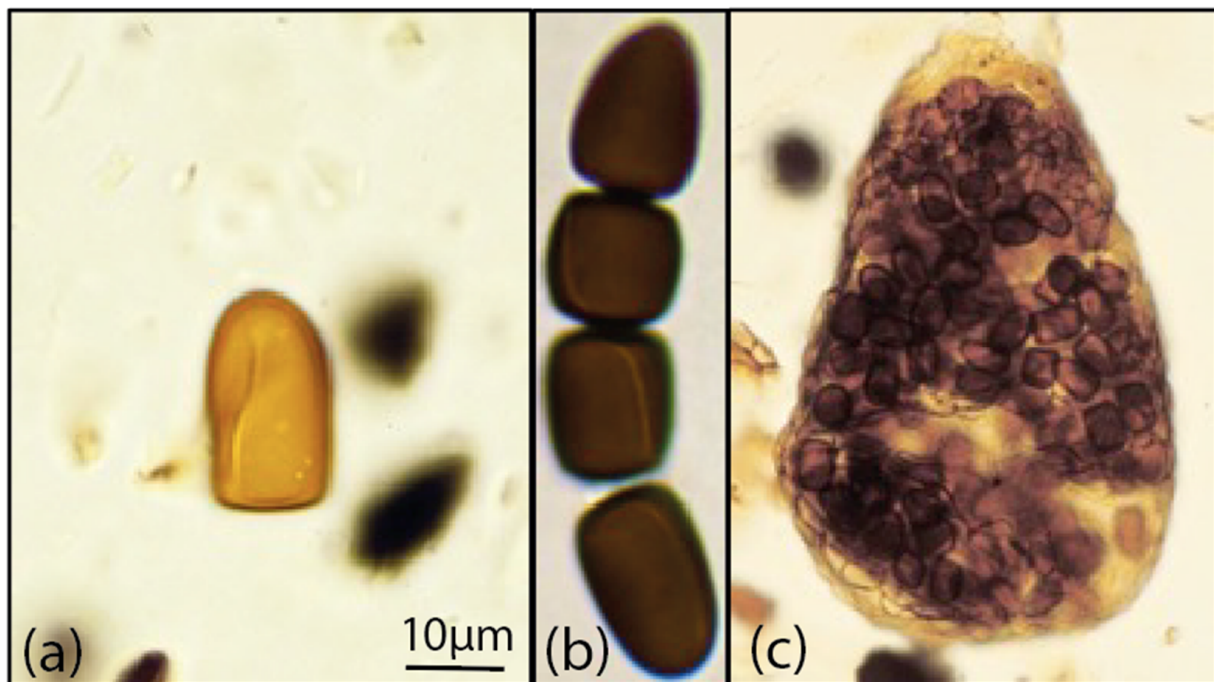
### 4.2. Morphological Identification

The *Sporormiella* type has dominated palaeoecological studies after first being introduced by Davis et al. [29]. The spores of this genus became a popular tool due to *Sporormiella* species being easily morphologically identifiable in fossil samples [11] and they were found to be a sensitive indicator of herbivore presence [22,38,52]. In many studies, *Sporormiella* was the only SCF identified, counted, and used to indicate past herbivore presence [14,18,22]. This repeated use has resulted in the use of *Sporormiella* becoming well embedded in the research community with Étienne and Jouffroy-Bapicot stating that the



“genus *Sporormiella* . . . [has] demonstrated to be the most valuable proxy for the presence of wild and domestic herbivores” [12] (p. 1).

The best method for the *Sporormiella* type is counting individual spore cells, such as those in Figure 7a, and not complete spores (that are of rare occurrence) (see Figure 7b) [53]. This method is commonly applied in studies and is attributed to the fragility of the connections between spore cells [4], although it remains unknown whether the breakdown of the complete spores is predominantly due to breakdown in the natural environment or during processing. Despite the success of *Sporormiella*, it is recommended that researchers identify and count multiple SCF taxa [13,14,38] in order to reduce biases. To obtain a more complete picture of fungal activity, other SCF have been proven to be just as successful as *Sporormiella* at indicating the presence of herbivores in a landscape [14,35,36] (Figure 8). Generally, the best other SCF indicators are: the *Cercophora* type [4,6,54,55], the *Podospora*-type [9,35,38,54,56], and the *Sordaria* type [4,35,38,56,57]. However, it should be noted that this is attributed to their ability to withstand the ‘standard’ pollen preparation procedure. The indicative value of other spores preserved in the sediment may simply be unused because they do not ‘survive’ standard preparation procedures.



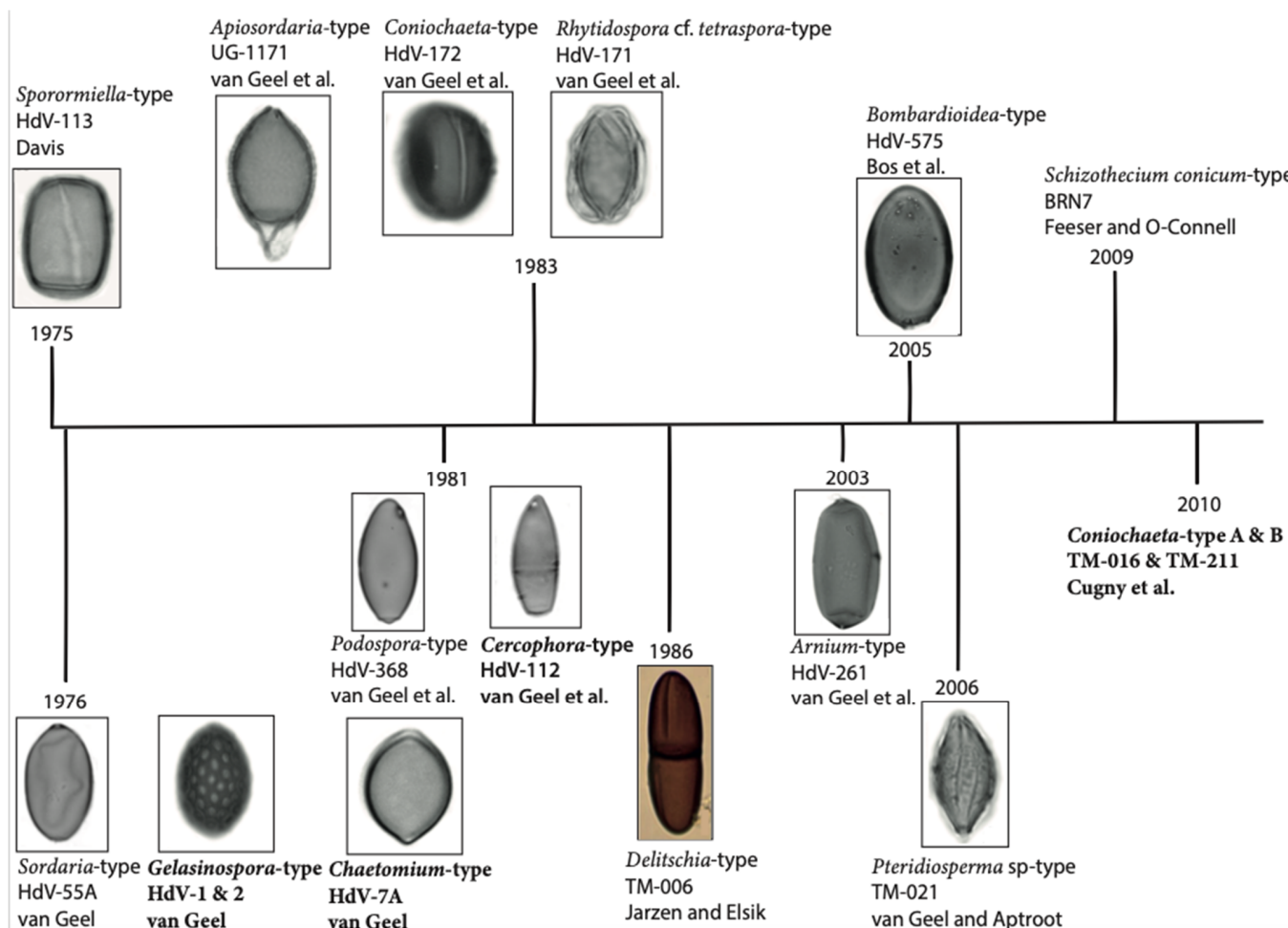
**Figure 7.** Microscope images of: (a) *Sporormiella* separate cell of ascospore. (b) *Sporormiella* ascospore, and (c) *Sporormiella* fruit body holding ascospore cells.

#### 4.3. Genetic Identification

Analysing sedimentary ancient DNA (SedaDNA) [59] involves extracting genetic information from ancient organic tissues [60]. There are two approaches to SedaDNA: (i) metabarcoding, and (ii) shotgun sequencing [61,62]. Metabarcoding is the process of identifying single organisms through the isolation of DNA [63], while shotgun sequencing looks at the taxonomic community as a whole and provides DNA diversity data [13,64]. The potential for contamination with modern genetic material means sub-samples for SedaDNA analysis must be collected and prepared separately and differently from sub-samples extracted for microscopic analysis. Sub-sampling for SedaDNA will commonly be carried out by a specialist in more strictly controlled environmental conditions [62].

SedaDNA can be used as an additional proxy for palaeoecological reconstructions, but it also has a taxonomic benefit. Through the metabarcoding of palaeo-environmental DNA (PalEnDNA) [13,63] and phylogenetic markers it is possible to identify species

assemblages [62,65]. This allows the systematic advancement of molecular taxonomic units (MOTUs) [61] and archives, such as GenBank. However, without large reference and image databases, the misidentification of spores using this approach remains possible [65]. Consequently, the construction of databases related to SCF are required for it to become a powerful palaeoecological tool [61].



**Figure 8.** Timeline of when SCF were discovered and who identified them. From oldest to youngest: Sporormiella-type ascospore [30]. Sordaria-type ascospore [30]. Gelasinospora-type ascospore [30]. Chaetomium-type ascospore [30]. Podospora-type ascospore [30]. Cercophora-type ascospore [30]. Apiosordaria-type [30]. Coniochaeta-type [30]. Rhytidospora cf. tetraspora-type [30]. Coniochaeta ligniaria-type [30]. Delitschia-type [13]. Arnium-type [30]. Bombardioidea-type [30]. Pteridiosperma-type [30]. Schizothecium conicum-type. Coniochaeta-type A and B. (Adapted after Baker et al. [58]). Citations in the caption refer to the source of the image.

The largest limitation to the widespread use of SedaDNA is the preservation of tissue in samples. Leaching damages DNA over time and chemical build up can skew results [60,61]. Samples that come from arid and cool environments tend to have the best-preserved DNA tissue [60,61]. However, studies from waterlogged environments have also proven successful [60]. Epp et al. [62] have developed a suitable standard protocol for SedaDNA sampling. Yet, our understanding of why DNA preservation varies between samples is still under investigation [61].

#### 4.4. Quantification

The relative abundances of SCF are typically calculated with reference to the pollen abundance in the same sample. The range of methods used to quantify SCF in samples

is shown in Table 1. Spores %Total Pollen Assemblage (TPA) is a popular approach as it allows the counting of SCF and pollen simultaneously. However, it can lead to under or overrepresentation of spores in samples as changes in pollen automatically cause changes to the spore count [13,19]. The distinction between %TPA and %Total Land Pollen (TLP) + Total Non-Pollen Palynomorphs (TNPP) is that %TPA represents spores as a percentage within the total pollen assemblage, while %TLP+TNPP adds the percentage of spores onto the total pollen percentage. Therefore, many studies opt for %TLP+TNPP as this method counts SCF separately.

**Table 1.** Quantification method, abbreviations used, units, and references for each quantification method currently used in SCF studies. Abbreviations: total pollen (TLP), total non-pollen palynomorphs (TNPP), total pollen assemblage (TPA), and pollen influx (PI).

| Quantification                                      | Abbreviations | Units                                   |
|---|---------------|---|
| Percentage of the sum of total pollen and total NPP | % TLP + TNPP  | % [4,19,66]                             |
| Percentage of total pollen assemblage               | % TPA or % TP | % [13,14]                               |
| Total spore concentration                           | -             | Spores/cm <sup>3</sup> [9,13,67]        |
| Pollen influx or spore accumulation rate            | PI            | Spores/cm <sup>2</sup> /year [10,13,36] |

The abundance of SCF extracted from sedimentary samples can be quantified either as relative or absolute abundance. Each method has its limitations [9,11] (Table 1). The most widely used approach for calculating SCF abundances is through comparison with an exotic marker (typically *Lycopodium* spores) [67], which has been added in a known amount to the sub-sample during preparation [12]. Common practice is for SCF to be identified and counted under the microscope until a predetermined number of *Lycopodium* spores are counted [67]. However, the accuracy of this measure can vary depending on the volume of the sample and number of exotic markers added [13]. It is therefore important when calculating absolute abundances of SCF that uncertainties are calculated [68].

Additionally, total pollen and spore concentration are sensitive to changes in the sediment accumulation rate [13,58]. Therefore, there is the potential for a high degree of variability between study sites and even within a single record if sedimentation rates vary. Calculating influx rates (spores/cm<sup>2</sup>/year) is generally accepted as the most desirable and accurate method of quantification [19]. However, to reliably calculate influx rates, a comprehensive well-constrained age-depth model is required, which is only sometimes available [35]. If available, such a model will include information on changes in sediment accumulation and facilitate the calculation of influx rates; however, the calculation of influx rates against a poorly constrained age-depth model is not advised as this can lead to spurious results [14].

## 5. Interpretation

The abundance of megaherbivores in a landscape is not the only factor that determines the SCF abundance in a sedimentary record [53]. The climate, environment conditions, and herbivore type also have differential effects [18,35,37,38,53,69,70] and must be considered when estimating herbivore abundance/biomass or density. Despite the numerous studies of SCF, our understanding of these biotic and abiotic effects on the nature of the SCF record is still limited [13]. However, such information is crucial for improving palaeoecological reconstructions and providing insight regarding the management of modern-day ecosystems [35].

### 5.1. Herbivore Density Influences

Coprophilous fungi are able to grow on a wide range of faeces; however, some fungal types are more likely to grow on the dung of specific herbivore species [15,32,70]. For

example, *Coprinus stercoreus* is commonly found on sheep, cattle, deer, and rabbit dung, while *Ascobolus carletonii* has been reported to grow exclusively on grouse dung [15]. Consequently, certain herbivores will facilitate the growth of coprophilous fungi more than others, which could cause an inaccurate reconstruction of the abundance of megaherbivores present at that time [35]. For example, a small population of deer could contribute more to the fungal record than a larger population of cattle [35,70] or sheep, as seen in Figure 9c. Reasons for this variation in growth have often been neglected in the interpretation of SCF datasets. Even though the factor of some spores being more abundant in specific animal dung should be considered, its overall influence on reconstructions is likely to be little. This is because it is not possible to identify SCF to the species level and many species within the same genus grow on different herbivore dung. Thus, the different contribution of SCF growing on the dung of different herbivore species cannot be recognised in the sedimentary records, although other types of SCF, such as the *Coprinus* type (class Basidiomycetes), do not favour a specific dung type [15]. Therefore, these fungal types have the potential to be unbiased indicators of historic megaherbivore abundance. However, it should be noted that Basidiomycetes are rarely found during analysis [53,70], either because they do not preserve well in sediment or are lost during sample preparation (see Section 4.1). Our understanding of the relationship between herbivores, their surrounding landscape, and how this is reflected in the fossil fungal record needs further study [36].

Additionally, studies that only count *Sporormiella* are vulnerable to inaccurate results. The absence of *Sporormiella* spores should not on its own be interpreted as the absence of herbivores [14,70]. Secondly, the misidentification of spores is possible based on its morphology, which is due to some *Sporormiella* spores that have a similar size and shape to the genus *Preussia* [13,14,35,71]. This could cause inconsistencies if not identified correctly. The possibility of false negative and positive results from the use of *Sporormiella* alone highlights the importance of identifying multiple spore types within the samples. Furthermore, SedaDNA could be especially beneficial here by accurately identifying spore types and expanding our catalogue of SCF.

## 5.2. Vegetation and Landscape Influences

Due to the endocoprophilous nature of SCF, there will necessarily be a relationship between the vegetation at a site and the animals that frequent it, i.e., vegetation and water resources need to be sufficient to sustain an animal population. The vegetation available to be consumed by herbivores is determined by multiple factors, including biosphere, geology, and climate, potentially modulated by positive or negative feedback effects from the herbivore populations themselves (Figure 9) [72]. SCF are consequently very valuable for developing an understanding of vegetation—animal dynamics, such as the effects of overgrazing, changes in animal husbandry, or human colonization [17,38]. The SCF signal related to human activity can be modulated by factors other than the simple addition, or removal, of animals in a landscape. Goethals and Verschuren [55] found that the farming style (e.g., mixed subsistence) to which livestock are subject also has an effect on the fungal spore record. Furthermore, the growth of fungi, and thus the coprophilous fungal succession, will be influenced by local environments [13], seasonal changes, and geographical change. This in turn will affect the timing of sporulation [38,73]. Therefore, it is important to consider the SCF record in the context of the vegetation record, i.e., pollen and macrofossil records may provide additional insights [13]. For example, in a European context when *Asteroideae*, *Cichorioideae*, *Cirsium* type, *Galium* type, Ranunculaceae, *Stellaria*-type, and *Potentilla* type are found together, they can be interpreted to suggest local grazing [74].



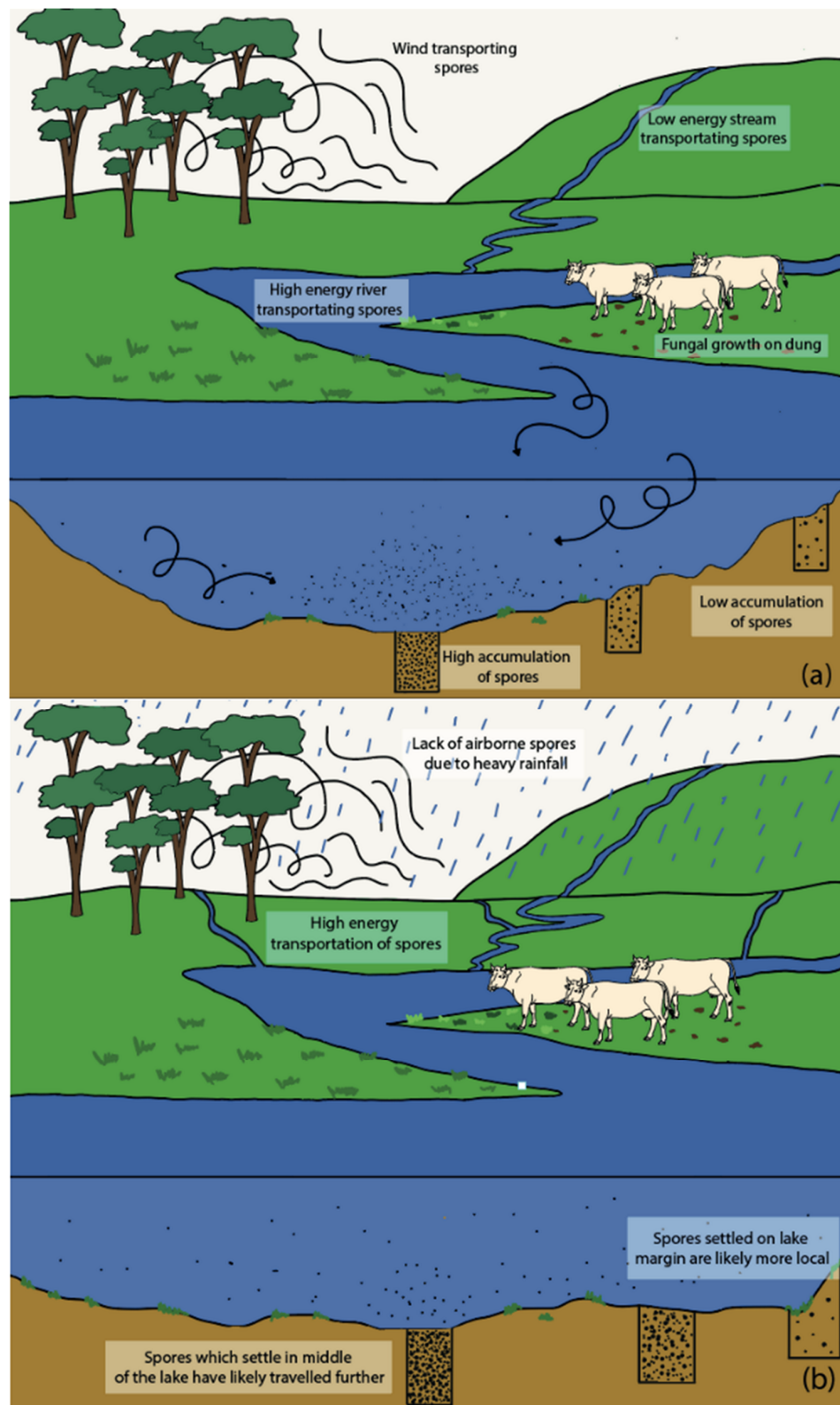
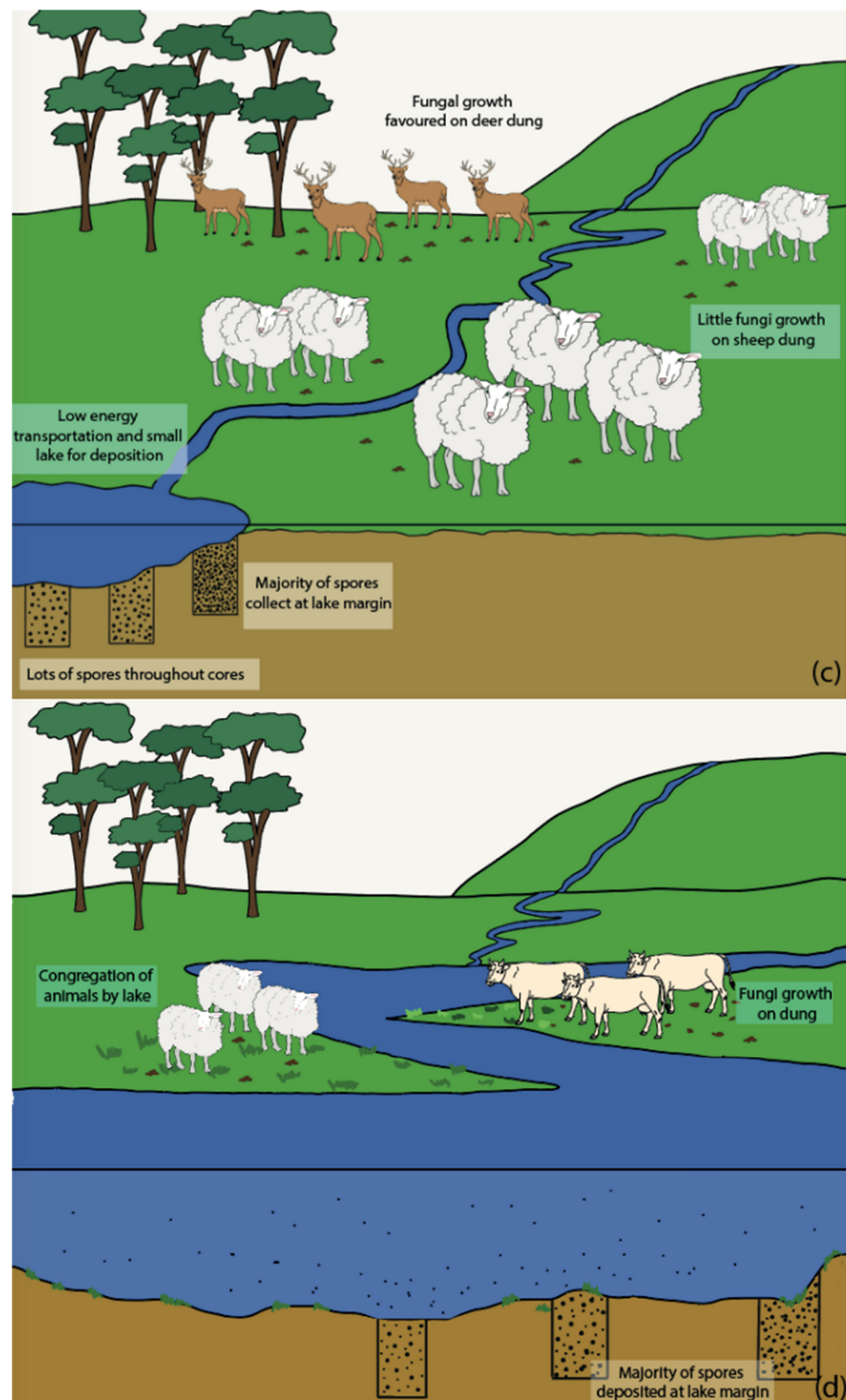


Figure 9. Cont.





**Figure 9.** Schematic diagram of the possible external influences on spores at the time of dispersal and deposition. (a) Cows in a lacustrine environment with high energy river influx, resulting in the large deposition of spores in the middle of the lake, despite cow dung deposits being far away from lake. (b) Heavy rain and wind in a lacustrine environment with cows congregating and multiple river influx into the lake. (c) Low energy stream with small lake and multiple sheep and deer resulting in large amounts of spores throughout the lake. (d) Sheep and cows in a lacustrine environment with no rain, no wind, and one low energy river influx resulting in small amount of spore deposition that congregates on the edge of the lake.

A study by Etienne et al. [9] assessed the relationship between SCF concentration and influx rates in relation to herbivore grazing pressure and sediment accumulation. Multiple cores were taken from two lakes in the French Alps. This area was perfect for such a study as grazing pressure had been historically catalogued for the last 200 years and lake deposits contain spores that are especially influenced by transportation, livestock type, and environment. Through exploration of the lake deposits, the authors could recognise the high amount of soil erosion, indicating overgrazing. Furthermore, evidence of thick layers from flooding events were not analysed for SCF as these likely contained high number of spores transported from a non-local range. Etienne et al. [9] compared the historic data with the coprophilous spore influx and showed an agreement in the reduction in the sheep population between ca. 1894–1895. Overall, the pattern of SCF influx over time correlated well with historic data, thus validating the use of SCF for reconstructing fluctuations of the population density of sheep.

### 5.3. Influence of Lake Depositional Environments

SCF tend to be well preserved in cores from lake sediments [9] and sometimes even undamaged *Sporormiella* asci with multiple spores are found [53]. The excellent preservation of pollen and spores make lakes attractive study sites. However, there are biases that must be considered when reconstructing environmental conditions based on lake deposits. Fortunately, SCF can be found in large and small water bodies, although lake area, depth and type of basin, and changes in lake dynamics are independent factors in modulating the SCF signal [52,53].

Although lake deposits can show a clearer change in SCF over time, the potential effects of the catchment area must be considered. The catchment area will be much larger for lake sediments than for peatbogs, as waterholes attract more and non-local herbivores (Figure 9b–d) [13]. Once there, visiting herbivores will eat vegetation and eject dung with spores that will germinate and form fruitbodies, thus creating a condensed and potentially inaccurate record of the local herbivore density [14,35]. Furthermore, the position in a lake from which cores are extracted can influence the SCF record (Figure 9) [9,52]. The combination of water bodies attracting animals from the wider region and likely incorporation of spores over a larger distance (water or airborne) suggests that lake sediments are more likely to contain a regional, or landscape, scale signal of herbivores, rather than specific local conditions [10,14,75]. Parker and Williams [53] tested the variation by taking cores from the centre and margin of 24 lakes in South Dakota, Minnesota, and Wisconsin. Depending on the mechanism of spore transportation, some lakes have an accumulation of SCF in the centre rather than the margin, or vice versa [9] (see difference in spore deposition between Figure 9b,d). This illustrates the necessity for many more than just one core from a lake basin [10].

### 5.4. Transportation of Spores

Although the majority of fungal spores will be deposited and germinate in the local surroundings of their source [4,13,30,76], some may travel further [14,35]. Spores can be transported through three means: the air (Figure 9a), hydrological mechanisms (Figure 9), or animal migration [13,77]. The most likely mode of transportation for SCF typically considered in palaeoecology studies is water, through rivers and surface runoff [13,36]. Van Geel [78] has shown *Kretzschmaria deusta* to be a useful indicator of runoff events [38]. Other SCF types, such as Basidiomycete fungi, which mainly travel through air [43], are often not preserved or recovered from sedimentary samples. The means of transportation of SCF is usually dictated by the species morphology [77], with round and oval spores dispersing in a similar fashion to pollen [11].

The energy of hydrological transportation mechanisms tends to control where spores will accumulate in a lake. High-energy situations, such as transportation through rivers, streams or extreme surface runoff, cause a concentration and settling of spores in the centre of the lake [9] (see difference between Figure 9a,b). This can be referred to as “degree

of storminess" [14]; however, deposition across a lake is also dependent on the size of the lake [10]. Figure 9c has a higher quantity of spores throughout the cores, while the difference between spore abundance at the margin and middle of the lake in Figure 9d is larger. Therefore, it is important that studies take multiple cores from lake sediments, such as the French Alps study by Etienne et al. [9]. Using a multi-core approach can provide additional insights into whether the SCF data reflecting a more local or regional signal of herbivory [14,75].

On occasions, high amounts of rainfall may inhibit SCF transportation by restricting airborne spores [53] (Figure 9). A recent study found that the time of day in which sporulation takes place can have a large effect on the distance spores will travel [73]. If spores are released during daylight, they could be airborne for multiple days, but if sporulation takes place at night, then spores will likely only travel for a few hours [73]. This is down to differences in air turbulence through the day [73] and greatly influences the fungus' natural dispersal distance [13].

### 5.5. Climatic Influences

Climatic influences, notably moisture and temperature, affect fungal growth on dung, which is, in turn, reflected in the number of spores produced and ultimately preserved in the sedimentary record. The influence is mainly attributed to differences in the fungal species' adaptability and structure [15]. In temperate zones, more species will be found in samples from winter or spring than those from summer or autumn [15,70]. This is due to the relatively higher moisture availability in winter, which improves fruiting potential [13], while in summer, dung is prone to drying out [79]. In addition, temperature can also modulate fungal spore production; when temperatures are too low, they produce less fruitbodies [13] and consequently, in temperate zones, spore production is at its highest in spring when it is both moist and warm.

In general, the main climatic influence on SCF is humidity [35]. Cool but moist climates are favourable for fungal community diversity [37,53]. However, the quality of the water can also have an influence; in particular, salt concentrations can hinder sporulation [14]. The initial water content of the dung substrate will be determined by the climate and weather at the time of consumption, while the salt content varies depending on the deposition of animal urine [13]. When temperature is too high, and moisture is low the dung will experience desiccation. This drying out of the substrate means that only a few species are able to grow. Notably, *Sporormiella* has adapted to somewhat dried out substrates [13,15], partly, but not fully, explaining reason for its regular occurrence in sedimentary records. Furthermore, larger faeces are subject to less desiccation than the faeces of smaller herbivores [14,15]; therefore, larger faeces are likely to produce a relatively greater number of spores than smaller faeces of the same weight or volume. Supporting the inference in the majority of SCF studies that the SCF signal relates mainly to megaherbivores, rather than all herbivores, in the landscape.

Our understanding of how SCF production, transport and preservation can be altered by climatic variations remains incomplete [36,38]. For example, high rainfall events suppress fungal growth [13] and could disrupt spore dispersal or facilitate transportation away from the site through runoff [35]. Therefore, caution must be taken when there is an absence of SCF as this could reflect a change in weather conditions (either drying or wetting) rather than a change in herbivore abundance [35].

## 6. Outlook

The main purpose of this review is to synthesise the current literature in order to analyse the accuracy of using SCF to indicate past herbivore presence and reconstructing herbivore biomass density. In the following section, we present suggestions on possible future directions for the subdiscipline of SCF herbivore studies related to knowledge gaps within the previously highlighted literature.

### 6.1. Improve Understanding of SCF—Herbivore Relationships through the Study of Successions of Coprophilous Fungi

Current information on the succession of coprophilous fungi is something that, to date, has been neglected in palaeoecological studies [27]. SCF as indicators of herbivore populations are increasingly popular, but the knowledge of many palynologists about the full fungal life cycle is limited. This is despite Faegri and Iversen stressing the necessity of the inclusion of botanical and ecological knowledge in 1950 [80]. An increased consideration of this will improve our understanding of the SCF–herbivore relationship and indicate adaptations to environment and climate, fundamentally, contributing to improved estimations of herbivore biomass.

A recent review by Perrotti and van Asperen [14] aimed to analyse the mycological literature and the effects spore production has on palaeoecological studies yet overlooked their life cycles. Alternatively, Richardson [15] explored the Ascomycetes fungal communities found on specific dung types (identified on an animal species level). Future studies should consider investigating Basidiomycetes fungal community dung specificities [32]. By including the biology of spores into palaeoecological studies, we can grasp a further understanding on why certain communities are found in specific dung types and how spores arrived in samples. Not only will this reduce discrepancies between studies, but will also provide insight into herbivore biomass estimations.

van Asperen [13] detailed the succession of coprophilous fungi and its temporal evolution [28]; similar future SCF studies could benefit from the utilization of SedaDNA, which could be utilised for detecting different fungal taxa and species identification [60]. Additionally, the research of samples older than Quaternary would improve our understanding of the origination and development of the coprophilous fungi and herbivore relationship.

### 6.2. Improve Understanding of SCF—Climate Relationship

Currently, little is known about the effects of different compositions of faeces [15], yet further research could provide insight into the relationship between SCF and the diets of herbivore species. To this effect, we must further expand our knowledge on domesticated and wild herbivore grazing in the past [36,81]. A study by van Asperen [70] was aimed at comparing the fungal community found on the faeces of exotic and (semi-)native herbivores. The results confirmed the validity of using megaherbivores to signal extinct herbivore presence; however, this is less so when comparing with wild extinct and non-extinct relative herbivores [70]. Furthermore, Richardson [15] recommends researchers consider the animal type studied, especially when comparing dung or SCF in different geographic regions. Therefore, we believe that influences of livestock versus wild herbivores needs to be explicitly considered in future SCF and herbivore palaeoecological studies, especially if we wish to develop biomass estimations. Such field studies are highly anticipated [20].

Lake studies benefit from multiple cores as this provides information on the method of transportation spores took, but also decreases uncertainty in biomass estimations [20]. Studies similar to Etienne et al. [9] would be beneficial. Therefore, we recommend taking multiple cores from the same basin as good practice in SCF–herbivore studies.

Our understanding of how external factors may influence the SCF signal, which is preserved in the sedimentary record, needs improvement. Focus should be on the mechanisms of spore transportation and how/when spores are dispersed [9]. In addition, controlled modern-day experiments could provide insight into the factors that influence fungal spore counts in dung (e.g., desiccation or urine concentrations) when the herbivore species and biomass are kept the same. Quantifying the relationship between herbivore biomass and fungal spore count will be a great advancement in this subdiscipline, which is in concordance with Johnson et al. [20].

Biomass estimations can be improved by utilising statistical software. For example, Mottl et al. [82] have produced an R package, called R-Ratepol, for the identification and summary of biotic and abiotic rate of change (ROC) in palaeoecological sequences. Assuming continual and consistent use by palaeoecologists, this tool could improve our

global datasets and help the discipline to adhere further to open science principles. R-Ratepol could be particularly helpful in the study of lake deposits and understanding the role of spore transportation within a sequence. Another statistical sub-sampling tool was developed by Keen et al. [83] to enhance accurate pollen assemblage data. The tool provides the best pollen count for your sample to determine richness and evenness. Modifying such a tool to determine the best SCF count for herbivore density would be beneficial. Overall, this practice will help to expand the study of SCF through multidisciplinary approaches.

### 6.3. Improve ID of SCF through Morphological and DNA-Based Studies

Although ancient DNA studies have, to date, concentrated on bacteria or plants [59], there is potential for its use in fossil fungi through the fusion of techniques. In fact, it is usable on all organisms [61]. SedaDNA is consequently becoming increasingly included within palaeoecological studies. Furthermore, the practice of ancient DNA analysis seems likely to increase in popularity, accelerated by its reduction in cost over the years [60]. Not only does this open many new development possibilities, but reduces time and need for technical replicates [59,61]. Moreover, the use of online archives adheres more to open science principles. Overall, it enhances our ability to understand the dynamics of past ecological changes [84]. This will ease the very difficult task of spore species identification and provide insight into spore evolution. A comprehensive investigation of the SedaDNA literature should be conducted so we can accelerate its practical usage. Specifically, our understanding of how different environments affect the preservation of DNA needs to be improved [60]. The synthesis of this and the Non-Pollen Palynomorph Image Database (NPP-ID) will allow improved interpretation and identification and broaden accessibility for researchers [85]. Overall, SedaDNA is another tool that will enhance palaeoecological studies and should be used consistently along with studied coprophilous fungi.

Multiproxy studies are always more concrete than single proxy studies. When using SCF, data should always be related to pollen and macrofossil proxies to provide more evidence on the timing and size of changes in previous agricultural practices [75]. For example, if fruitbodies are found in macrofossil analysis then this provides an indication that SCF were not transported by air [30] and therefore evidence for samples reflecting local information. Van Geel et al. [47] found fruitbodies of coprophilous fungi, still full of ascospores, inside mammoth faeces and explained this as evidence for coprophagy. Although SedaDNA can be used for species identification, it will likely be used as an additional proxy. This would, similarly, allow the validation of records and add further information to the reconstruction.

There are currently only four established, relatively commonly used, SCF for indicating herbivore presence: *Sporormiella*, *Podospora*, *Cercophora*, and *Sordaria*; however, it should be noted that *Cercophora* is not obligate to dung. An extensive outline of all SCF is provided in van Asperen [13]. Identifying and describing more fungal remains will expand this list of established SCF, fundamentally improving this palaeoecological tool [30]. The development of SedaDNA should speed up this process. Johnson et al. [20] found that by including all SCF taxa, no matter their interpretation reputation, that uncertainty within their reconstructions was reduced.

The value of expanding the range of fungal spores examined was recently highlighted by Wei et al. [86]. In their study, modern fungi spore assemblages and communities were assessed based on topsoil and dung samples from the Qinghai–Tibetan Plateau [86]. The results showed that the *Urocystis* type correlated highly with herbivore abundance, although until this study was carried out, *Urocystis* was only known as a parasitic fungus [86]. This not only highlights why we should be counting SCF other than *Sporormiella*, but also expresses the need for studies to take place in a wider range of environments and geographic locations. Although improving, palaeoecology needs to become more globally applicable as studies that take place outside of North America and Europe are at a disadvantage due to the lack of indicator species identified [13,38]. Yet, progress is being made as studies,



such as Montoya et al. [87] in South America, Loughlin et al. [88] in Ecuador, and Goethals and Verschuren [55] in Africa, increase in number.

#### 6.4. Present SCF Data Independent of Other Proxies

Despite large outputs of data, the progress of SCF studies has been hampered because it is often difficult to compare results, especially over different research organisations. This is due to the variations in research methodologies [13].

Pollen and SCF should be counted separately when researchers are interested in the coprophilous fungi separate to vegetation. This was similarly suggested by van Asperen et al. [13]. Although no minimum spore count can be created, when possible, researchers should try and reach a relatively high *Lycopodium* spore count as this should maximise validation when data reveals an absence of herbivores [13,89]; following counting protocols calculate the confidence of detection to be estimated, such as those set out by Keen et al. [83].

Finally, in future studies, each SCF type should be counted and presented separately, along with a total sum of all the SCF types [13]. When quantifying results, more than one method should be used for validation [13,14]. Based on the literature reviewed, we recommend the use of pollen and SCF concentration. However, it is recognised that the percentage method is overall reliable and accessible.

## 7. Conclusions

The palaeoecological tool of using SCF to indicate past herbivore presence is sensitive to spore dispersal and transportation, lack of standardised methods, and influences from climate, environment, and livestock. Considering the information presented in this literature review, the following conclusion is made: Yes, spores of coprophilous fungi are accurate indicators for herbivore presence and relative changes in abundance, but the tool is not yet developed enough to be used in herbivore biomass estimations. Further research must be conducted into the factors that influence spore abundance and subsequently how to isolate these for SCF to present accurate biomass estimations. Future studies should consider the use of SedaDNA and spore biology as this will likely improve our understanding of fungal communities on the faeces of different herbivore species. However, for this to be successful, our knowledge on the use of SedaDNA and other statistical tools must be improved.

An outlook into the future of the field is presented, one which is more considerate of the biological interactions that influence fungal communities. Although this subdiscipline is interdisciplinary, it is clear that biological aspects require more consideration. Such attention in future SCF studies should allow the expansion of capabilities in the palaeoecological tool.

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