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Black ceramic spheres as marker grains for microfossil analyses, with improved chemical, physical, and optical properties

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ABSTRACT

Marker grain method is commonly used by palynologists to quantify absolute pollen concentration in sediments. DuPont NEM plastic microbeads and *Lycopodium* spore tablets are two of the most commonly used marker grains. The DuPont NEM series has an obvious advantage over *Lycopodium* tablets because *Lycopodium* is not guaranteed 'exotic' in many ecosystems (especially in the past). Moreover, NEM also has high visibility, easily identifiable spherical shape, and availability in different size ranges. However, the production of NEM has been discontinuous since more than a decade, and palynologists of the world are using the precious remainders of their own stock in very small (=suboptimal) quantities. Here we propose a solution. Microspheres of black ceramic can be used as marker grains for a wide range of microfossil analyses (in this paper, however, we mainly discuss the use of the material for pollen analysis because of the expertise of the authors). The ceramic spheres are tolerant to all chemicals commonly used during pollen extraction processes and is extremely resistant to physical stresses. The particles are matt black, spherical, available in a wide range of size clusters, have a density very close to that of fossil pollen grains (1.424 g/cm^3 vs. 1.494 g/cm^3 in average), and don't change color and density over a long storage in acidic liquids. The behavior of ceramic spheres was closer to the pollen grains than the *Lycopodium* spores regardless of the pollen concentration and composition in sediments. In combination, these properties make the black ceramic spheres an even better solution for palynologists than DuPont NEM microbeads or *Lycopodium* spore tablets. The absolute pollen concentrations estimated from ceramic spheres and those estimated from volume method were indistinguishable within errors. On the other hand, when the samples were relatively poor in fossil pollen grains, the concentrations calculated by *Lycopodium* method tended to be significantly overestimated. The ceramic microbeads are available either as a dry powder in different size ranges, or as a mixture of two size ranges blended in a defined ratio and dispersed in a buoyancy-neutral liquid, ready to be added to sediment samples. Mixing two different size ranges in a known ratio serves to detect any laboratory failures that differentially favor recovery of larger or smaller pollen types.

1. Introduction

The absolute quantity of microfossils is a very useful parameter (Bonny, 1972 and references therein). For instance, with quantitative data we can discuss pollen productivity, presence or absence of sorting or ultraviolet decomposition of organic matter and biomass in sparse vegetation (e.g. boreal forest or arid zone). Because the absolute pollen concentration is independent from frequencies of pollen grains of other taxa, it often reflects environmental changes more directly than percentage values. Adding known-amount of marker grains, such as *Lycopodium* spores and DuPont NEM plastic microbeads series was making those scientific approaches possible (Stockmarr, 1971; Ogden, 1986).

The DuPont NEM microbeads series has been, however, sorely missed since its production was discontinued. The microbeads method was first proposed in 1986 (Ogden, 1986) and, since that time, the DuPont product has attracted a number of enthusiastic users (e.g. Wang and Geurts, 1993; Turetsky et al., 2004; Fedotov

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et al., 2012; Sugaya et al., 2016). Unfortunately, since the DuPont NEM series was discontinued, no alternative marker grain product of comparable quality has been introduced. The DuPont NEM microbeads were supplied in various size ranges and had high-visibility under the microscope due to their uniform spherical shape and matt black color. With an average density of 1.369 g/cm^3, they were also close to that of fossil pollen grains (1.494 g/cm^3 in average; Regnéll and Everitt, 1996). The microbeads were also inert and tolerant of the chemical treatments used in conventional palynological procedures (HCl, KOH, $ZnCl_2$, acetolysis, and even conc. HF). The synthetic microbeads from DuPont NEM therefore offered what seemed to be an ideal marker grain for palynological research and so the discontinuation of the product line sent shock waves throughout the palynology community.

Here we report a high quality alternative to the DuPont NEM microbeads. As well as being tolerant of all palynological processing chemicals, including HF, this novel marker grains does not decolorize or swell after lengthy storage in liquid, which was something of an issue with the DuPont NEM materials. Its specific gravity is also closer to that of fossil pollen grains than the DuPont microbeads, which ensures more coherent movement of pollen and marker grains during pollen extraction. This paper aims to assess the performance of this new product in comparison with the conventional *Lycopodium* spore tablets.

2. Materials and methods

We tested the black ceramic spheres (PALYNOSPHERES; Fig. 1a–b) obtained from a UK-based company called Palynotech (http://www.palynotech.com/). The density of the microbeads is 1.424 ± 0.008 g/cm^3 (2σ) (Table 1), which, according to our measurements by Archimedes method, does not depend on their size clusters. The ceramic spheres are available in five size ranges centered at ca. 23 μm (coded "Orange"), 27 μm ("Blue"), 34 μm ("Green"), 38 μm ("Yellow") and 46 μm ("Red") (Table 2). (N.B. The color codings are simply for convenience; the actual color of the powder is matt black in all size ranges). Two marker grains, coded "Red" and "Orange", are easily distinguishable by sizes under the microscope (Fig. 1a, b, d). Using the mixture of small and large marker grains enables to check the size dependency of the microfossil recovery rate after the treatment of sediments.

In this research, PALYNOSPHERES SG06 Special Blend (Batch no. SG0605; Fig. 1a and b) was used for the experiment. The SG06 Special Blend is a mixture of two easily distinguishable size clusters of ceramic microbeads (coded "Orange" and "Red" – see above) suspended in a buoyancy-neutral carrying media. The Orange/Red mixing ratio is ca. 2.9 (exact ratio is variable across batches; the product label provides more precise and lot specific value), which counterbalances the higher visibility of larger grains. The carrier media (sodium bromide; NaBr) has specific gravity of 1.40 ± 0.05 (measured at 20 °C; temperature dependence unknown). This media density has been chosen to be quasi-buoyancy neutral with the marker grains so that re-dispersal is easily achieved. The media contains non-ionic detergent, Tween 20. The marker grain concentrations differ to a small degree from lot to lot, but are generally in the region of 1.8 ± 0.1 × 10^4 grains/ml and 6.2 ± 0.4 × 10^{3}grains/ml for the small and large grains, respectively. Exact concentrations to 3 decimal places are provided by the manufacturer for each production lot and are detailed on the product label. The lot No. SG0605, the one that we used in this experiment, contains 6.17 ± 0.42 × 10^3 large ('Red') grains and 1.78 ± 0.13 × 10^4 small ('Orange') grains per ml, according to the lot-specific product label. The number of grains in 1 ml of the formulated material is equivalent to approximately two *Lycopodium* tablets manufactured by Lund University (Stockmarr, 1971), which makes it easy to convert between *Lycopodium* tablets and PALYNOSPHERES in the laboratory procedures.

The pollen analysis was carried out to compare the behavior of the black ceramic spheres and *Lycopodium* spores. The DuPont NEM

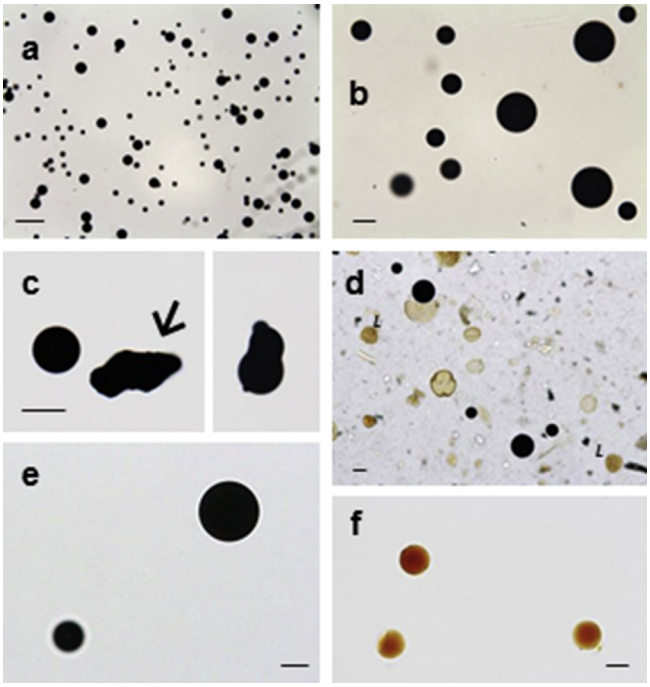

Fig. 1. Photos of (a, b) the black ceramic spheres. (c) Amorphous (i.e. non-spherical) grains. (d) The marker grains and *Lycopodium* spores added to the sediment, treated and extracted by a normal preparation protocol (Nakagawa et al., 1998) along with fossil pollen grains. *L* denotes the *Lycopodium* spore. (e–f) New and conventional microbeads after storage in suspension liquid for long-term. DuPont NEM microbeads (f) are loosing its color after storage in suspending solution for 13 years. The marker grains proposed in this paper (e) were developed very recently and hence such lengthy stability check has not been carried out. However, the oldest available lot, which has been stored in the buoyancy neutral medium described in this paper for almost 5 years, is not showing any recognizable changes in optical properties. Scale bars represent 100 μm and 20 μm in (a) and (b–e), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of density.

	Density (g/cm^3)
Fossil pollen grains[a]	1.494 ± 0.028
Black spherical ceramic powder[b]	1.424 ± 0.008
DuPont NEM series	1.369 ± 0.013
Lycopodium spore[c]	1.377 ± 0.016

[a] After Regnéll and Everitt (1996).
[b] The product (PALYNOSPHERES) discussed in this paper.
[c] *Lycopodium* tablet (Batch no. 3862) made by Lund University.

Table 2
Available size clusters of black ceramic spheres.

Color code	Particle size (μm)
Orange	22.6 ± 2.0
Blue	27.2 ± 3.6
Green	33.7 ± 3.7
Yellow	38.0 ± 3.2
Red	45.5 ± 3.86

The color codes are simply for convenience. The color of microspheres is matt black in all size clusters.

series was excluded from the comparison simply because the product is no longer available. The SG12 core from Lake Suigetsu, Japan (35°35′ 08″N, 135°52′ 56″E, Suzuki et al., 2016) were used for the analysis. Based on the preliminary result of pollen analysis of the core (unpublished data), six 1-m long sections (7.7 m, 12.5 m, 18.5 m, 25.8 m, 33.0 m, 38.9 m), which contain most of typical pollen taxa in Japan (Gotanda et al., 2002), were chosen. The rejected materials from each target depth were homogenized into a batch of "standard sample" (Nakagawa et al., 2013). Then four samples of 1.50 ± 0.05 wet g were taken from six standard samples. We then treated those 24 samples in an identical procedure as follows: two *Lycopodium* tablets and 1 ml of well mixed PALYNOSPHERES SG06 Special Blend were added to all samples (24 samples in total); pollen slides were prepared by the "dense-media separation" method of Nakagawa et al. (1998), *i.e.* 10% HCl treatment (room temperature for 12 h); 10% KOH treatment (90 °C for 10 min); washing with water for 7 times; washing with 10% HCl; dense media separation ($ZnCl_2$ of density 1.90, 1800 rpm for 10 min); acetolysis treatment (sulfuric acid: acetic anhydride = 1:9 v/v, 90 °C for 10 min); washing with water twice; ethanol treatment twice; mounting with 100% glycerin. The method is widely used by the palynologists of the world as it allows preparing remarkably clear pollen slides with relatively small effort, with no significant distortion of pollen composition data (Campbell et al., 2016). At least 400 pollen grains were counted under the optical microscope at 400 × magnification. The pollen grains were classified into two groups: pollen-L and pollen-S. Pollen-L means large grains with air sacs, *i.e.*, *Abies*, *Picea* and *Tsuga*. Pollen-S type contains all other taxa. Spores, *Lycopodium* spores and black ceramic spheres were also counted at the same time.

Absolute pollen concentration and its error were calculated based on the volume method and two marker grain methods using black ceramic sphere and *Lycopodium* tablet. The absolute pollen concentration by volume method was calculated using the following equation:

$$c = p/m \times a/a' \times v/v', \quad (1)$$

where c denotes pollen concentration per unit mass (grains/g); p is the number of pollen grains counted (grains), a is the area of cover glass (cm^2), a' is the observed area (cm^2), v is total volume of residue (cm^3), v' is the volume of mounted residue (cm^3), and m is the sample mass (g).

The pollen concentration by marker grain method was calculated using following equation:

$$c' = p/m \times dc/g, \quad (2)$$

where c' denotes pollen concentration per unit mass (grains/g); p is the number of pollen grains counted (grains), d is the concentration of the marker grain suspension (grains/cm^3), c is the volume of the added marker grain suspension (cm^3), g is the number of marker grains counted with pollen grains (grains), and m is the sample mass (g).

3. Results and discussion

3.1. Usability of PALYNOSPHERES

During the experiment, we observed a total of 17,321 black ceramic grains under microscope. The product that we tested exhibited the following characteristics.

3.1.1. Chemical and physical properties

We did not observe any chemical or physical degradations of the ceramic spheres through treatments by acids and alkalis. After the treatment, the black ceramic spheres were not decolorize and damaged (Fig. 1d). It was not even possible to squash the spheres by fingers between slide and cover glasses, which was not the case with the discontinuous DuPont NEM series.

Amorphous (i.e. non-spherical) marker grains are occasionally found amongst more healthy ceramic spheres (Fig. 1c). The frequency of such amorphous grains which were not clearly distinguishable from organic matter was <4%. Presence of the amorphous grains, however, does not cause distortion of pollen concentration data because they are not included in the marker grain concentration specified on the product label.

3.1.2. Optical properties

The black spherical ceramic powder exhibits a high level of visibility and a distinct character under the microscope (Fig. 1d), being matt black and spherical. The grains do not suffer from edge diffraction artifacts, nor do they decolorize over time (Fig. 1e).

3.2. Comparison of pollen concentration between different methods

The absolute pollen concentrations were calculated from three methods, *i.e.* volume method and two different microbeads methods using ceramic spheres and *Lycopodium* tablet, respectively. The result of comparative experiment is shown in Table 3 and Fig. 2. The error bars in Fig. 2 denotes standard deviation among four analysed samples deriving from the same 'standard' batch. The absolute pollen concentrations estimated from ceramic spheres and those estimated from volume method were indistinguishable within errors, regardless of the pollen composition and sample depth. By contrast, the quantification by *Lycopodium* tablets were very variable depending on the pollen composition. Using the pollen-rich samples, which contain more than 98% of Pollen-S type (7.7 m and 12.5 m), the absolute pollen concentration obtained by volume and *Lycopodium* methods were relatively close. The absolute pollen amounts estimated from *Lycopodium* is only 1.3–2.1 times larger than that from volume method. When the samples were relatively poor in fossil pollen grains, however, the concentrations calculated by *Lycopodium* method tended to be significantly overestimated. The absolute pollen concentration estimated by *Lycopodium* method is by 16–164 times larger (18.5 m and 25,8 m). This indicates that *Lycopodium* spores were preferentially lost in the process of pollen extraction. Relatively low density of *Lycopodium* spores, when compared with fossil pollen grains and PALYNOSPHERES is the most likely cause of the loss (Table 1). PALYNOSPHERES, by contrast, has almost identical behavior with the true fossil pollen grains because their densities are remarkably similar (Table 1).

4. Advantages over alternative/conventional products

4.1. Comparison with DuPont NEM microbeads

We believe that when used as marker grains, the black ceramic spheres are not only a quality alternative to the now discontinued DuPont NEM microbeads, but that they actually offer a number of physical, chemical and optical advantages over the historic product. The black ceramic spheres do not break, dissolve, swell or decolorize, maintaining good visibility over a long period of storage. This was not necessarily the case with the DuPont NEM microbeads, which faded in color on prolonged exposure to liquid (mixture of 1% HCl solution, approximately 30% $ZnCl_2$ and a small amount of wash-up liquid) (Fig. 1f). The specific gravity of the black ceramic spheres is closer to that of fossil pollen grains than the DuPont NEM material (Regnéll and Everitt, 1996) (Table 1), making them a better

Table 3
Pollen concentrations calculated using three different methods.

Method	7.7 m	12.5 m	18.5 m	25.8 m	33.0 m	38.9 m
Volume method	$2.12 \pm 0.19 \times 10^5$	$1.16 \pm 0.15 \times 10^5$	$6.96 \pm 1.40 \times 10^3$	$3.38 \pm 0.46 \times 10^3$	$1.35 \pm 0.23 \times 10^4$	$6.26 \pm 2.27 \times 10^3$
PALYNOSPHERES[a]	$2.77 \pm 0.85 \times 10^5$	$1.42 \pm 0.10 \times 10^5$	$8.65 \pm 1.74 \times 10^3$	$4.25 \pm 0.69 \times 10^3$	$1.50 \pm 0.19 \times 10^4$	$8.66 \pm 3.32 \times 10^3$
Lycopodium[b]	$3.38 \pm 0.47 \times 10^5$	$1.97 \pm 0.20 \times 10^5$	$1.68 \pm 0.34 \times 10^5$	$3.61 \pm 1.12 \times 10^5$	$7.16 \pm 1.55 \times 10^5$	$5.18 \pm 1.36 \times 10^5$

[a] PALYNOSPHERES SG06 Special Blend (Batch no. SG0605).
[b] *Lycopodium* tablet (Batch no. 3862) made by Lund University.

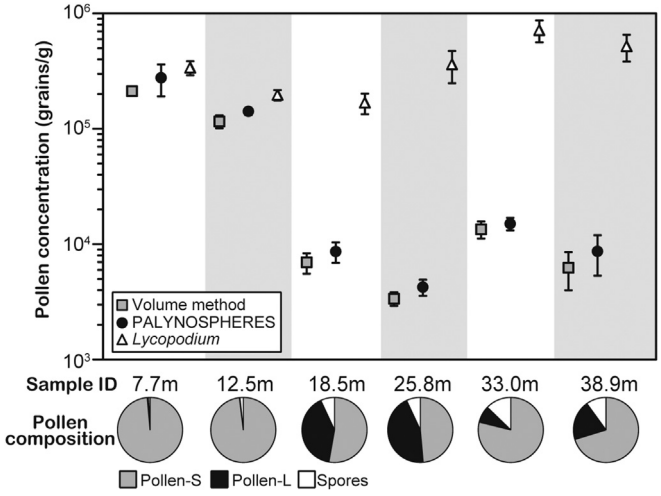

Fig. 2. Comparison of the pollen concentrations quantified by three different methods. The error bars are 1σ. The pie charts show the pollen and spore composition of each sample. The identified palynomorphs were classified into three groups: pollen-S, pollen-L and spores. Pollen-L group contains *Abies*, *Picea* and *Tsuga*, the pollen grains of which are relatively large and have air sacs. Pollen-S contains all the other taxa. Spores include both monolete and trilete spores.

microfossil surrogate for extraction control.

4.2. Comparison with Lycopodium spore tablets

The dense color, spherical shape and uniformity of black ceramic spherical marker grains (Fig. 1a, b, d, e) offers overwhelming advantages to operators compared to the *Lycopodium* tablets produced by Lund University (Stockmarr, 1971). In contrast to *Lycopodium* spores, the artificial marker grains do not exist in nature and thus can be used for all types of naturally derived samples from any location (for example, *Lycopodium* spontaneously grows in Japan). Microscopic examination is made much easier courtesy of their uniform shape and high visual contrast, allowing even inexperienced users to significantly reduce their analysis time (Fig. 1d). Interestingly, the density of the black ceramic spheres is closer to that of raw fossil pollen grains than that of the commercially available acetolysed *Lycopodium* spores (Table 1). The supply of the black ceramic spheres is stable whereas that of the *Lycopodium* spore tablet essentially relies on the volunteer spirit of the Lund University.

5. Conclusion

Black ceramic marker grains substitute discontinuous DuPont NEM marker grains. They inherit all essential properties of the conventional product, and have added advantages such as even closer density to fossil pollen grains, increased chemical and physical durability, and availability in either format as a dry powder or formulated suspension in a buoyancy-neutral liquid with controlled concentration. The ceramic spheres are not lost significantly during the course of the dense-media separation treatment, which was not necessarily the case with *Lycopodium* spores when the sample is pollen-poor.

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