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Estimating phytolith influx in lake sediments

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ABSTRACT

So far, no phytolith extraction protocols have been tested for accuracy and repeatability. Here we aim to display a phytolith extraction method combining the strengths of two widely used protocols, supplemented with silica microspheres as exogenous markers for quantifying phytolith concentrations. Phytolith concentrations were estimated for samples from two sedimentary sequences in which numerical age–depth models make it possible to calculate phytolith influxes (phytolith numbers per cm^2 *per* yr). Analysis of replicates confirmed the statistical robustness, the repeatability and the very few biases of our extraction technique for small phytoliths, since the relationship between grass silica short cells and microspheres was kept stable. Furthermore, we demonstrated that silica microspheres are robust exogenous markers for estimating phytolith concentrations. The minimum number of items (i.e., phytoliths plus silica microspheres) that must be counted to estimate phytolith concentrations and thus influxes depends on the ratio of phytoliths to microspheres (R) and is minimized when $R = 1$. Nevertheless, we recommend using ratios $R \leq 1$ in order to avoid having the counting process become excessively time-consuming, because microspheres are easier to identify and count than phytoliths.

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Introduction

Bio-proxies recovered from sediments are useful for deciphering historical ecosystem dynamics and exploring the interactions between biotic and abiotic features, and their linkage with environmental changes (e.g., disturbances, stress). The development of dating methods, together with the more recent progresses in age–depth modeling (e.g., Blaauw, 2010), has made it possible to accurately reconstruct environmental events and dynamics based on accurate assessment of accumulation rates (or influxes), which enables measuring the number of proxy load per sediment surface area (or volume) per time unit (e.g., year). Proxy influxes have proven useful for demonstrating temporal changes in proxy percentages (Hyvärinen, 1976; Salgado-Labouriau and Rull, 1986; Seppä et al., 2002; Stefanova and Ammann, 2003; Piperno, 2006). Considering influx allows calculating the behavior of each proxy independently from others. In contrast, values are not independent when assemblages are characterized by proxy percentages or frequencies (Brown, 1988; Hill, 1996; Seppä and Hicks, 2006). Bio-proxy influxes can be used to assess changes in vegetation composition and dynamics quantitatively (Brown, 1988; Birks, 1996) as well as to assess changes in biomass, and to compare them to changes observed in other environments (Blarquez et al., 2012). Nevertheless, the accuracy of estimated influxes depends strongly on the sedimentological quality (e.g., no bioturbation along the core), the precision of the radiocarbon dating, and the estimation of the sedimentation rate.

The addition of known quantities of an exogenous marker has long been used for estimating concentrations of bio-proxies by computing their item numbers in the original sample based on the ratio of the proxy to the added exogenous marker (Benninghoff, 1962; Battarbee and Kneen, 1982; Salgado-Labouriau and Rull, 1986; Scherer, 1994). The most common exogenous markers used in pollen analyses are *Lycopodium* spores (Stockmarr, 1971) or *Eucalyptus* pollen grains (Salgado-Labouriau and Rull, 1986), while silica, polystyrene, and glass microspheres are widely used in diatom studies (Battarbee and Kneen, 1982; Scherer, 1994). There are no fast and easy methods, however, for determining phytoliths influx in sediments. Indeed, the aliquot method (e.g., Piperno, 1993; Gil et al., 2006) in which all phytoliths from a known volume of sediment must be counted, is too time-consuming (Piperno, 2006). Studies have used *Lycopodium* spores (e.g., Powers and Gilbertson, 1987; Powers-Jones and Padmore, 1993; Piperno, 1995), but none has tested the reproducibility of the method and, there is no rule on how many markers have to be added for precise concentration estimates (Piperno, 2006).

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We aim (i) to evaluate the reproducibility of a phytolith extraction method adapted from two protocols widely used (Kelly, 1990; Lentfer and Boyd, 1998) on small phytoliths (short cells), and (ii) to test the efficiency of silica microspheres for quantifying phytolith concentrations in sediments. The proposed extraction method combines the strengths of these two protocols (Kelly, 1990; Lentfer and Boyd, 1998), with the addition of useful tips acquired with practice. The physicochemical properties of silica microspheres (inorganic silicate) closely resemble those of phytoliths, but are not identical due to different amounts of water and the presence of organic matter inclusion for phytoliths, as well as its density (2.2 $g \cdot cm^{-3}$ for microspheres and 1.5–2.3 $g \cdot cm^{-3}$ for phytoliths: Jones and Beavers, 1963). Therefore, they are assumed to behave similarly when subjected to common opal phytolith extraction methods. As such, they should be suitable tools for assessing phytolith influxes. Finally, we present guidelines for determining the phytolith numbers needed to achieve acceptable precision when estimating concentrations. The method is evaluated by analyzing two sediment records, one from a Mediterranean forest (grass-poor) and the other from a tropical savanna. We added known quantities of microspheres to a constant sediment volume and used a range of different microsphere concentrations. We checked that silica microspheres are good external markers by examining if the estimated phytolith concentration was independent of the microsphere concentration used with little variation between replicates, and if the extraction procedure did not increase significantly the estimation errors as compared to those due to sampling size only.

Material

Exogenous marker: silica microspheres

Silica microspheres for liquid chromatography from Merck (LiChrospher® Si 1000) with a mean diameter of 10 μm were used as exogenous markers (Fig. 1). The average weight of an individual microsphere (*ca.* 1.1519×10^{-9} g) was estimated based on the density of silica microsphere ($d = 2.20\ g \cdot cm^{-3}$) and the mean diameter of the microspheres (Eq. (1)).

$$\text{volume} = \frac{4}{3} \times \pi \times \text{radius}^3 \quad (1)$$

$$\text{mass} = \text{density} \times \text{volume}. \quad (2)$$

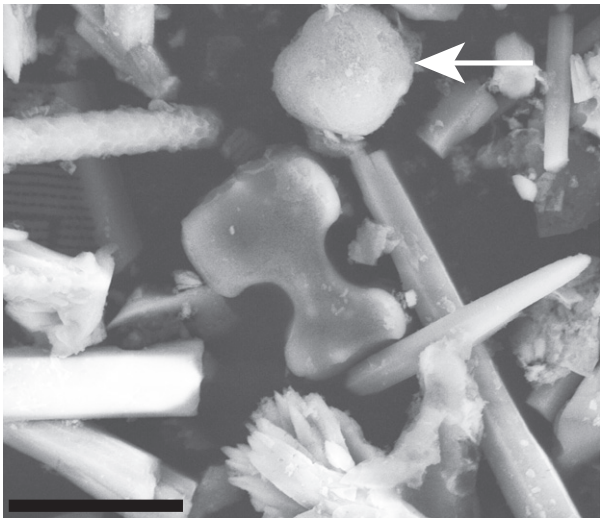

Figure 1. Photograph of a microsphere (white arrow) and a dumbbell phytolith (just beneath) taken with a Scanning Electron Microscope (magnification ×8420; the black bar represents 10 μm).

We prepared a solution containing 10^8 microspheres per ml by diluting 1.1519 g of microspheres in 10 ml of distilled water.

For tropical savanna lake sediments, we added 1.03×10^6 microspheres to each of the five replicate samples taken at a core depth of 193–194 cm (these samples are henceforth referred to collectively as Gbali1) and 2.06×10^6 microspheres to each of the five replicate samples taken at a core depth of 199–200 cm (these samples are henceforth referred to as Gbali2) before performing phytolith extraction in order to test the effect of varying the phytolith:microsphere ratio (*R*).

For lake sediments in Mediterranean forest context, where vegetation *a priori* produces fewer phytoliths compared to the tropical savanna context, three microsphere concentrations were tested (1.14×10^5, 2.06×10^5 and 4.13×10^5 microspheres per cm^3 of sediment) with five replicates per tested concentration. This allowed us to test the reliability of phytolith influx computed with different exogenous marker concentrations. The groups of samples tested with the three different microsphere concentrations are henceforth referred to as Creno1, Creno2 and Creno3, respectively.

The design of the study is summarized in the Supplementary material (Fig. 1S).

Lake sediments

Lake Gbali (4°49′07″N; 18°15′46″E) is located in a flat savanna zone in the Central African Republic where grass (*Imperata cylindrica*, *Cenchrus polystachios* and *Hyparrhenia diplandra*) and typical fire-resistant trees (*Daniellia oliveri*, *Terminalia schimeriana*, *Prosopis africana*, *Hymenocardia acida*, *Piliostigma thonningii*, *Sarcocephalus latifolius*, *Annona senegalensis* and *Crossopteryx febrifuga*) are co-dominant. It is a small (2.3 ha) and shallow (2 m depth) flat lake with a large riparian forest mainly composed of *Hallea stipulosa* and some *Raphia vinifera*. A core sample was acquired from the lake centre.

Two 1-cm-thick samples from the core were taken at positions corresponding to depths of 193–194 cm and 199–200 cm. The age–depth model (not published, see Supplementary material Fig. 2S) dates the two layers at ~2280 and ~2330 calibrated years before present (hereafter cal yr BP, calibrated based on the IntCal09 dataset (Reimer et al., 2009)), respectively. Five sub-samples of 1 cm^3 each were then taken from each sediment slice for phytolith extraction.

Lac de Creno (42°12′18″N, 08°56′45″E) is a small lake (1.5 ha, 6.5 m depth) with no permanent inflow, situated in a glacial basin established in the Mediterranean Corsican mountain, France (Leys et al., 2013). The forest surrounding the lake is composed of *Pinus laricio*, with scattered *Fagus sylvatica*, *Taxus baccata* and *Alnus cordata*, and an understory dominated by *Genista corsica*, *Erica arborea* and *Erica multiflora*.

A single 1-cm-thick layer of the core was sampled at a position corresponding to a depth of 160–161 cm (~3110 cal yr BP, Supplementary material Fig. 2S). Fifteen sub-samples of 1 cm^3 each were then taken from this sediment slice for phytolith extraction.

Method

Phytolith extraction protocol

The extraction protocol was adapted from those described by Kelly (1990), and Lentfer and Boyd (1998), combining their strengths, with added tips improving its implementation. Table 1 depicts the protocol showing its main steps. The heavy liquid flotation procedure was used because it has been shown to produce residues with high levels of clarity and less potential for size/shape selection during extraction than other methods (Lentfer and Boyd, 1998; Madella et al., 1998). Moreover, samples were extracted without fractionating the sediment into particles sizes (silt, clay and sand; e.g., Pearsall

Table 1
Description of phytolith extraction procedure adapted from Kelly (1990) and Lentfer and Boyd (1998). Steps (1) and (12) were used to test the protocol reproducibility but should be replaced with steps (1′) and (12′) for routine extractions.

(1)	Place 1 cm^3 of humid sediment in a centrifuge tube. Do not dry it as this may necessitate grinding that could affect the phytolith count. Add microspheres to the sediment.
(1′)	Place 1 cm^3 of humid sediment in a centrifuge tube.
(2)	Fill up the tube with a 5% weight solution of sodium metaphosphate ($NaPO_3$) at 70°C for deflocculation and shake it for 12 h.
(3)	To rinse the sediment, centrifuge for 2 min at 3000 rpm and carefully discard the supernatant. Fill up the tube with distilled water, shake it and centrifuge for 2 min at 3000 rpm (repeat 2 times).
(4)	Decarbonate with 10 ml of HCl (1 N), vortex and leave the unsealed tube in a sand bath at 70°C for 1 h. Centrifuge for 2 min at 3000 rpm and discard the supernatant. Repeat this process until the reaction stops and rinse the sediment (3).
(5)	Remove the organic matter by adding 20 ml of 33% hydrogen peroxide (H_2O_2). Put the unsealed tubes in a sand bath at 70°C. If the reaction is too vigorous, add few drops of ethanol. Shake hourly and refill with H_2O_2 to avoid desiccation. Repeat the process until the reaction stops and then rinse the sediment (3).
(6)	For lateritic sediments, oxidized iron can be removed by adding 10 ml of tri-sodium citrate (88.4 $g \cdot l^{-1}$) and placing the mixture in a sand bath at 80°C for 1 h before adding 1 g of sodium dithionite ($Na_2S_2O_4$) and shaking gently for few minutes. Repeat this process until the sediment becomes gray, and rinse (3).
(7)	To remove clays, fill the tube with a 5% weight solution of sodium metaphosphate ($NaPO_3$) at 70°C and shake it for 12 h. Sieve the sample through a 250 μm mesh then add distilled water to the residue to a height of 7 cm and centrifuge for 1 min 30 s at 2000 rpm (calculated for a Sigma Aldrich 3–16 centrifuge with an RCF.g of 769 at 2000 rpm). Carefully remove the supernatant and repeat the centrifugation-decanting step until the decanted water is clear.
(8)	To remove water from the sediment without drying it, add 5 ml of 100% ethanol after step (7), shake gently and centrifuge for 2 min at 3000 rpm. Discard the supernatant and repeat the operation. Let the ethanol evaporate for 20 min at 30°C.
(9)	a) Prepare a zinc bromide solution of d = 2.3 by mixing 500 g of zinc bromide powder with 160 ml of HCl (33%). b) This solution can be re-used by filtering it to remove solids, but its density should be checked before such re-use. If it is below 2.3, let the solution evaporate in a beaker at 90°C for at least 3 h and re-check its density after cooling to ambient temperature.
(10)	Add 10 ml of a solution of zinc bromide with a specific gravity of 2.3–2.35. Vortex to ensure good mixing and then centrifuge for 2 min at 3000 rpm. Using a disposable transfer pipet, extract the fine white layer floating on the dense liquor and transfer to a 5 μm PTFE (hydrophobic fluopore) filter mounted on a vacuum glass filtration holder. Repeat this operation until the entire light floating fraction has been transferred.
(11)	Change the flask of the vacuum filtration holder to recycle the zinc bromide solution from step (9) b). Thoroughly rinse the floating residue on the filter with HCl (1 N, 100 ml) and distilled water (minimum 500 ml).
(12)	Transfer the phytoliths to a labeled vial. After decanting for 12 h much of the water can be carefully pipetted off and the sample can be dried in a drying oven. The sample will then be ready for mounting or storage.
(12′)	Stir the microsphere solution for 1 h to homogenize the suspension. Add the required volume of microsphere solution; add a magnetic stir and mix. The amount of microspheres can be adjusted after counting the first slides to achieve a suitable ratio of phytoliths to microspheres (see text). After 12 h of decantation, the bulk of the water can be carefully pipetted off and the sample can be dried in a drying oven ready for mounting or storage.
(13)	Slides are prepared by mixing a drop of ethanol with a pinhead of the sample on a cover slip. After evaporation of the ethanol, add a drop of immersion oil, mix well over the entirety of the cover slip and seal it on the slide. The use of immersion oil as mounting media facilitates 3D observation and counting, and provides a better contrast under the microscope than glycerin.

(2000)) in order to accelerate the processing speed. This ensures that the complete assemblage is available by avoiding potential loss of material when processing several fractions.

For the first step of the extraction protocol, we recommend not drying the sediment to avoid grinding that could induce mechanical damage to the more delicate and ornamented morphotypes (Madella et al., 1998). In step (2), the sediment is deflocculated using sodium metaphosphate ($NaPO_3$) (Lentfer and Boyd, 1998) to disaggregate mineral and organic matters. Then, the decarbonatation of sediment (4) requires HCl heated to 70°C (Kelly, 1990; Lentfer and Boyd, 1998); this step is important to disperse the mineral fraction and prevent secondary reactions (Madella et al., 1998). Decarbonatation has to be performed prior to reaction with hydrogen peroxide because organic matter oxidation by H_2O_2 is more efficient in a slightly acid and non-calcareous environment (Pearsall, 2000). To remove the organic matter (5), we used 33% H_2O_2 (Kelly, 1990; Lentfer and Boyd, 1998) at 70°C to accelerate the reaction. This step must be done carefully since lake sediments are generally rich in organic matter and it is important to obtain clear slides for easier identification and counting. For lateritic sediments, it is recommended to remove oxidized iron (6) using tri-sodium citrate and sodium dithionite (Kelly, 1990). Then, another deflocculation is required using $NaPO_3$ at 70°C (Lentfer and Boyd, 1998) shaken for 12 h in order to remove clay effectively (7). This step is essential since too high concentrations of clay might affect the quality of the data recovered (Madella et al., 1998). For this step, we removed clay by gravity sedimentation using 'low-speed' centrifugation to speed up the processing. Distilled water was added to the residue to a height of 7 cm and centrifuged for 1 min 30 s at 2000 rpm (Stokes' law for particles <2 μm, calculated for a Sigma Aldrich 3–16 centrifuge with an RCF.g of 769 at 2000 rpm). The step was repeated until the float was clear. Before performing densimetric separation of phytoliths, the residue was dried (8) using ethanol to avert dilution of the dense liquor by the water contained in the residue. The density of the heavy liquid is crucial for the densimetric separation step to prevent bias regarding phytolith selection, densities of which range from 1.5 to 2.3. We used as heavy liquid a $ZnBr_2$/HCl solution adjusted to a relative density of 2.3–2.35 (Kelly, 1990). The residue and the zinc bromide were mixed and then centrifuged for 2 min at 3000 rpm. Disposable transfer pipets were used to aspire the fine white layer floating on the dense liquor and transfer it to a 5 μm PTFE filter (Kelly, 1990) mounted on a vacuum glass filtration holder (10). To reduce the costs of the extraction procedure, the dense liquor can be recycled (9.b and 11). The floating residue on the filter has to be rinse with HCl (1 N) and distilled water. The phytoliths are transferred to a vial, decanted for 12 h and then dried in a drying oven (12). Finally, immersion oil was used as mounting media to prepare slides for facilitating 3D observation and counting (13) because it gives a better contrast under the microscope than glycerin.

In this work, the microspheres were added at stage (1) to test the accuracy of the extraction protocol. It was assumed that if this method proved to be accurate and yielded reproducible results, the microspheres would then constitute good exogenous markers and it would be viable to use an extraction protocol in which the microspheres were instead added at stage (12′). Indeed, due to the duration of the extraction procedure (which can last more than 10 days, depending of the sediment type), we recommend proceeding to the extraction for a batch of samples, to test on one how many microspheres are needed and then to add the adjusted number of microspheres in the other samples.

Phytolith counting

Phytoliths were counted and classified according to the International Code for Phytolith Nomenclature (Madella et al., 2005). A minimum of 2 h of counting was performed per sample. The number of slices counted depended on the phytolith concentration, but at least one entire slice was counted in each case. To reduce bias due to counting error (statistical and human), only Grass Silica Short Cells (GSSC) (Mulholland and Rapp Jr., 1992) were considered when calculating the phytoliths to microspheres ratio. By minimizing the error from phytolith counting in this way, it was possible to estimate the errors due to the phytolith extraction procedure and variation in the applied microsphere concentration.

Phytolith influx computation

We computed the phytolith accumulation rate or phytolith influx (I_P, #phytolith·cm^{-2}·yr^{-1}) based on the phytolith concentration (C_P, #phytolith·cm^{-3}) for each sample and the sedimentation rate (S in cm·yr^{-1}), computed using the age–depth models (Eq. (3)). The phytolith concentration for each sample was calculated by multiplying the number of counted phytoliths (N_{CP}) by the total quantity of added microspheres (N_{AM}) and then dividing the product by the quantity of counted microspheres per sample, N_{CM} (Eq. (4)):

$$I_P = C_P \times S \tag{3}$$

$$C_P = \frac{N_{CP}}{N_{CM}} \times N_{AM}. \tag{4}$$

The phytoliths:microsphere ratio $\frac{N_{CP}}{N_{CM}}$ will henceforth be referred to as R. The confidence interval (CI) for R at the confidence level α is (Aleman et al., 2012):

$$R_{\alpha\pm} = \frac{1+R}{1 \mp z_{1-\alpha/2}\sqrt{\frac{R}{n}}} - 1 \tag{5}$$

where $z_{1-\alpha/2}$ is the $(1-\alpha/2)$-percentile of the standard normal distribution. Then, the CI for I_P at the confidence level α is:

$$I_{P\alpha\pm} = S{\cdot}N_{AM}\left(\frac{1+R}{1 \mp z_{1-\alpha/2}\sqrt{\frac{R}{n}}} - 1\right). \tag{6}$$

Statistical analyses

To test the phytolith extraction protocol, we computed the coefficient of variation of R for the five replicates of each sample. In addition, we analyzed the means and variances of the measured phytolith concentrations for each of the temperate Creno1-3 sub-sample groups to determine whether the microspheres were good exogenous markers. The equality of variances within each group of five sub-samples was tested using Levene's test and the equality of means was tested using an ANOVA.

In order to test the repeatability of the extraction procedure, we make the null hypothesis that each replicated extraction leads to the same nominal ratio of phytoliths to microsphere $\tilde{R}$ and that the variability in the measured R is only due to sampling uncertainty, that is to the number n of counted items (phytoliths and microspheres). Under this hypothesis, we estimated the nominal ratio $\tilde{R}$ for each sample by summing the GSSC phytolith and microsphere counts for all five replicates and then dividing one by the other. This represents a high-count sum and therefore provides a more precise estimate of the real ratio R. Then, R is expected to be distributed according to Aleman et al. (2012):

$$R \sim \frac{1+\tilde{R}}{1+\mathcal{N}(0,1)\sqrt{\frac{\tilde{R}}{n}}} - 1 \tag{7}$$

where $\mathcal{N}(0,1)$ is the standard normal distribution. Measured values of R should have 95% probability to lie between $\frac{1+\tilde{R}}{1+z_{.025}\sqrt{\frac{\tilde{R}}{n}}-1}$: and $\frac{1+\tilde{R}}{1+z_{.975}\sqrt{\frac{\tilde{R}}{n}}-1}$. To put it another way, this means that $\sqrt{n\frac{\tilde{}}{R}}\left(\frac{1+\tilde{R}}{1+R}-1\right)$ should follow the standard normal distribution. This was tested with the Shapiro–Wilk test.

To determine the minimum number of counted items needed to achieve a precise estimate of R, we computed the minimum numbers of counted items so that the confidence interval $R_{0.95+} - R_{0.95-}$ (Eq. (5)) is 60%, 40% and 30% of the estimated R, for R ratios ranging from 0.1 to 10.

Results and discussion

The low coefficients of variation ($Coef_v$) between replicate concentrations (Table 2, <23%) show that there is no bias in relative density or size during the extraction procedure, which would have caused the relationship between microspheres and GSSC to change. The $Coef_v$ are much lower for the Lake Gbali samples (<13%), almost certainly because far more items were counted for these samples. Nevertheless, the results for all of the replicates fell within the computed 95% CI (Fig. 2) and the distribution of $\sqrt{\frac{n}{R}}\left(\frac{1+\tilde{R}}{1+R}-1\right)$ is not statistically different from a standard normal distribution (p-value for the Shapiro–Wilk test of 0.53). This means that the extraction procedure did not add significant differences to those expected because of phytoliths and microsphere sampling. Without proving that the extraction was complete, it however proved that it was repeatable. Moreover, the ANOVA and Levene's test results revealed no significant differences between the results obtained at the three different microsphere concentrations for the single core slice from the Mediterranean lake. This demonstrates that the computed influx is independent of the initial microsphere concentration used over the range tested. Silica microspheres thus appear useful as exogenous markers for determining absolute phytolith concentrations.

The protocol was tested on small phytoliths (GSSC) in order to reduce bias due to counting error. We therefore did not show that the relationship between microspheres and other larger phytoliths (e.g.,

Table 2
Summary of phytolith and microspheres counts for the five samples studied. S is the sedimentation rate in cm·yr^{-1}, Q_m is the quantity of microspheres injected in the replicates, R_m is the mean of the R ratios for all five replicates per sample, N_{CP+CM} is the mean of the total items counted per sample, C_P is the mean concentration of Grass Silica Short Cells (GSSC) phytoliths per sample in GSSC·cm^{-3}, I_P is the mean GSSC phytolith influx per sample in GSSC·cm^{-2}·yr^{-1} and $Coef_v$ is the coefficient of variation between the replicates' GSSC concentrations.

Sample	S	Q_m	R_m	N_{CP+CM}	C_P	I_P	$Coef_v$
Creno1	0.036	1.14×10^5	0.591	177	6.71×10^{4a} ($\pm 1.1 \times 10^4$)	2.41×10^{3a} ($\pm 4.0 \times 10^2$)	0.17
Creno2	0.036	2.06×10^5	0.265	217	5.47×10^{4a} ($\pm 1.3 \times 10^4$)	1.97×10^{3a} ($\pm 4.6 \times 10^2$)	0.23
Creno3	0.036	4.13×10^5	0.169	247	6.97×10^{4a} ($\pm 1.6 \times 10^4$)	2.52×10^{3a} ($\pm 5.7 \times 10^2$)	0.23
Gbali1	0.104	1.03×10^6	0.527	782	5.44×10^5 ($\pm 3.5 \times 10^4$)	5.67×10^4 ($\pm 3.6 \times 10^3$)	0.08
Gbali2	0.107	2.06×10^6	0.336	712	6.97×10^5 ($\pm 7.5 \times 10^4$)	7.47×10^4 ($\pm 8.1 \times 10^3$)	0.12

[a] Indicates no significant differences between the concentrations for the 3 samples from the Lac de Creno (based on an ANOVA to test the equality of means and a Levene's test for the equality of variance).

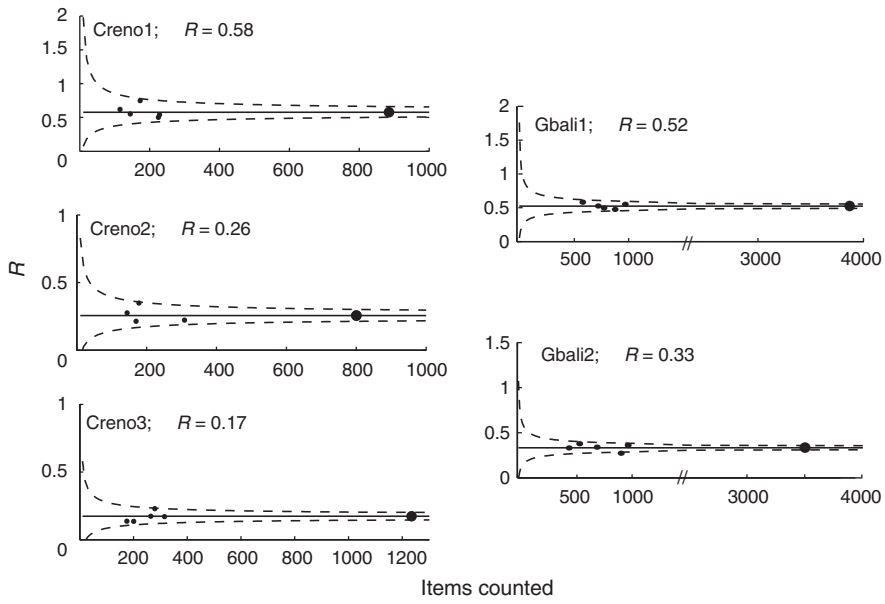

Figure 2. Dotted lines show the 95% confidence interval (CI) computed for each sample. Small dots indicate the *R* ratios for individual replicates; large dots indicate $\tilde{R}$.

bulliforms, elongated or blocky) was kept intact. Nevertheless, heavy liquid flotation is known to have less bias in recovering large particles as spherical and block, and adequate disaggregation and oxidation of sediments usually leads to few size/shape selection (Lentfer and Boyd, 1998). The other phytolith types are also expected to be recovered well by this extraction protocol. Thus, the extraction protocol used in this study appears precise and replicable with little error, at least for small phytoliths since the relationship between microspheres and GSSC was kept intact between replicates.

Figure 2 shows that as the count sum increases, the 95% CI decreases significantly. This finding is confirmed by the results of the statistical analysis (Fig. 3). Moreover, the minimum number of items that must be counted to precisely estimate the phytolith concentration depends on *R* (Eq. (5), Fig. 3). Indeed, for ratios $0.5 \leq R \leq 2$, only 200 items must be counted to obtain an estimation of *R* (and thus of the phytolith concentration) with an error of less than ±30%. When $R < 0.5$ or $R > 2$, the minimum count required increases rapidly. To obtain an estimated *R* with an error of less than ±20%, more than 400 items must be counted. Independently of the desired precision, the count sum is minimized when $R = 1$, which supports the results of Wolfe (1997) on diatom concentrations. Figure 3a can be used to determine the count sum required to yield a score within a CI of ±30%, ±20%, or ±15% for *R* values ranging from 0.1 to 10. An estimated influx with a precision of ±30% is enough for detecting changes in phytolith influxes (e.g., values of phytolith influxes computed in Abrantes (2003) and Gil et al. (2006) long-term records). The results for each group of five replicates are also plotted on this figure. All of the estimated *R* values for replicates from the Lake Gbali samples (with the exception of one replicate of the Gbali2 sample) had errors of less than ±20%, while the estimated *R* values for the Lac de Creno samples had errors of more than ±30%. Figure 3b shows the number of phytoliths that must be counted as a function of *R*. When $R < 1$, the number of phytoliths is low (<200 for a precision of ±30% and ±20%, and <400 for a precision of ±15%); but when $R > 1$, the number of phytoliths needed increases exponentially. It is much more easy and rapid to identify and count microspheres compared to phytoliths. Thus, when $R < 1$ even if the number of items required is high (>600) the number of phytoliths that need to be counted stays reasonable and not too time-consuming.

The computed concentrations for the tropical and Mediterranean samples differed significantly from one another, with those for the tropical samples being greater by a factor of 10 (Table 2). Nevertheless, concentrations in themselves do not mean much since they represent the amount of phytoliths in a stratigraphic level that can

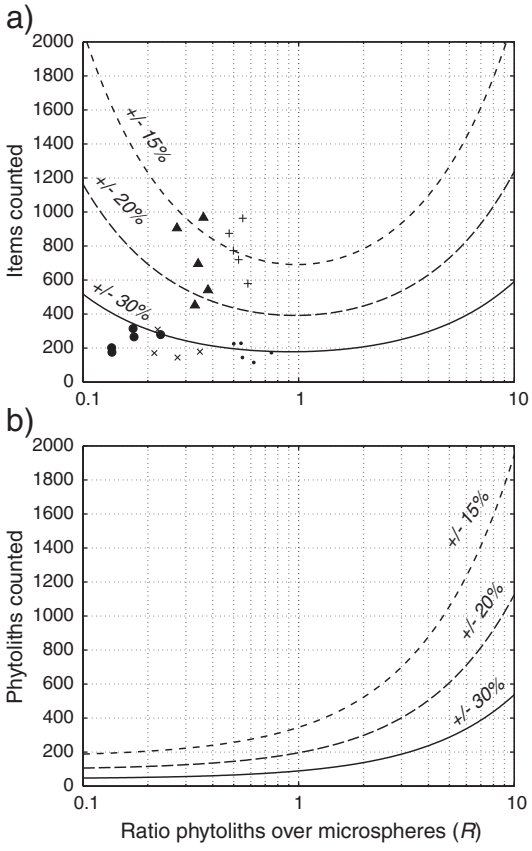

Figure 3. The first panel (a) represented the number of items that need to be counted as a function of the *R* ratio (represented using a logarithmic scale) to obtain an estimate of *R* with less than ±30%, ±20% and ±15% errors. Results for individual replicates are shown using the following symbols: • Creno1; × Creno2; ● Creno3; + Gbali1; ▲ Gbali2. The second panel (b) represented the number of phytoliths that need to be counted as a function of *R* depending on the associated error.

greatly vary between sedimentary systems. Phytolith accumulation rates or influxes enable a comparison between lakes, as they register the quantity of phytoliths that settle onto a unit of surface sediment per year. Thus, a change in the lake hydrology which would result in changes in the quantity of sediments accumulated in the lake would be taken into account by the sedimentation rate and, thus in the phytolith influx calculation. In the present case study, the phytolith influxes between the tropical and the Mediterranean lakes were different, with higher influxes in the tropical sediments, as expected. In addition, there were significant differences between the phytolith influxes at the two studied core depths for Lake Gbali, demonstrating that the rate of GSSC phytolith accumulation changed over the corresponding time period (i.e., between 2280 cal yr BP and 2330 cal yr BP; Table 2). However, the part of the sequence in which Gbali1 and Gbali2 were sampled corresponds to the most extrapolated part of the age–depth model, so the difference between the two sedimentation rates could be higher inducing an uncertainty associated to these two influx values. But, taking into account this uncertainty, the two influxes are still significantly different. There are several possible reasons for such changes in phytolith accumulation. Changes in the abundance of different tree and grass species in the environment around the lake would affect each phytolith type influx but may not affect the type of phytolith forms due to the taxonomic level of identification. If the total phytolith influx is constant or decrease, a decrease in trees abundance in favor of grasses would cause an increase in GSSC influx. Another hypothesis for the increase in GSSC influx would be a change in grass composition for species more phytolith-productive. Alternatively, decreases in the lake water level would increase the grass cover area on the surrounding land and thus increase GSSC production and deposit without a real change in the vegetation cover (i.e., tree:grass ratio). These results illustrate how calculating phytolith influxes can improve the comparisons of the productivities of different ecosystems and make it possible to identify changes in productivity within the same ecosystem.

Conclusion

Our results demonstrate that silica microspheres are useful tools for quantifying absolute phytolith concentrations in sediments. By adding microspheres before phytolith extraction, we demonstrated the reproducibility of the method reported herein. However, for subsequent studies on phytolith concentration based on this method, we recommend that the microspheres should be added at stage (12′) of the extraction procedure (Table 1). Indeed, the phytolith:microsphere ratio, R, determines the number of items that must be counted per sample and is crucial for estimating concentrations. The interest of adding microspheres at stage (12′) is that it is possible to test on one sample the number of microspheres that needs to be added to the remaining samples. The error of adding the microspheres at the end of the extraction is weak compared to the error introduced if the ratio R is much higher than 1. If there are too many phytoliths relative to microspheres, the minimum phytolith count required to obtain a concentration estimate with an error of less than $\pm 20\%$ may be quite high (>600 phytoliths for $R > 5$). We recommend that ratios $0.5 < R < 1$ in order to avoid having the counting process become excessively time-consuming.

The question of how many phytoliths must be counted to obtain accurate estimates of relative abundances has been addressed (Stromberg, 2009) in terms of the precision of phytolith indices (e.g., $\frac{D}{P}$ and I_{ph}), but accuracy in calculating absolute concentrations of specific phytolith types was not yet investigated. Consequently, this new procedure is a major improvement for phytolith studies. But, computing influxes of very frequent phytolith types requires a different implementation than computing influxes of rare ones. For these rare phytoliths types, the quantity of microspheres that must be added would be very high and to avoid wasting time it is advisable to count only microspheres and the rare types for these samples, leaving aside the most common ones. Finally, we recommend the use of silica microspheres as exogenous markers for paleoenvironmental reconstructions based on lake sediments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yqres.2013.05.008.

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