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NOTES

A revised method for determining the absolute abundance of diatoms

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Abstract Diatoms and other microfossils are used extensively in reconstructions of past climates and environments, in both terrestrial and marine settings. Both relative and absolute abundances of diatom taxa in sediment are important tools in these reconstructions. However, acquiring these data is a labor-intensive process. Settling-based diatom slide preparation techniques often bias samples through aliquot subsampling and sediment washing. Other techniques involve the use of added markers, which might obscure diatoms on the slide. This paper presents a revision to the widely adopted settling-based diatom slide preparation method presented by Scherer (J Paleolimnol 12:171–179, 1994) and provides a direct comparison to another widely used method. Evenly distributed diatom slides can be created by a settling process, which yields multiple statistically similar diatom slides without needing to clean sediment of salts or do aqueous subsampling, which may impart a bias in the sample when there is a wide range of particle shapes and sizes in the assemblage. Two samples originally utilized by Scherer (J Paleolimnol 12:171–179, 1994) were prepared via the updated method through a series of replicates. These results were compared to the same samples, processed with the method of Schrader and Gersonde (Utrecht Micropaleontol Bull 17:129–176, 1978), utilizing Petri dishes and the original results of Scherer (J Paleolimnol 12:171–179, 1994). The new modification presented here produces smaller standard deviations than the original Scherer method, and order of magnitude better statistics than the Schrader and Gersonde (Utrecht Micropaleontol Bull 17:129–176, 1978) method.

Keywords Methods · Diatoms · Absolute abundance · Light microscopy

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Introduction

The absolute abundance of diatom valves is frequently utilized as a general proxy for past diatom productivity, and relative abundances of species and genera are utilized to reconstruct environmental conditions, such as sea-ice extent, salinity, mixed layer depth, pH, dissolved organic carbon, and nutrient availability (Birks et al. 1990; Kingston and Birks 1990; Fritz et al. 1991; Bennion et al. 1996; Leventer 1998; Armand et al. 2005; Cortese and Gersonde 2007; Crosta 2009; Barbara et al. 2010; Konfirst et al. 2011; Romero et al. 2011). Several distinct methods exist for creating light

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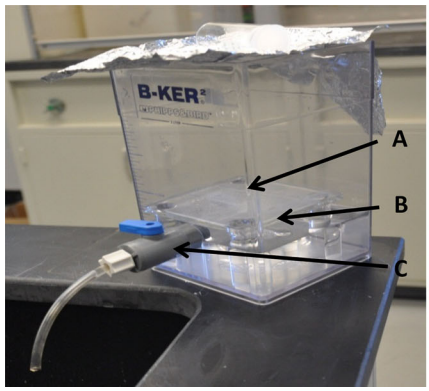
microscope (LM) slides and scanning electron microscope (SEM) preparations for measuring absolute and relative abundances of diatoms, pollen, and other silt-sized particles. Traditional diatom slide preparation techniques for absolute abundance counts involve sampling diatoms and sediments in suspension as an aliquot of a "well-mixed" sample; a specially designed shallow settling dish (Battarbee 1973) or Petri dish (Schrader and Gersonde 1978). This can be problematic, in that diatom and other microfossil populations often exhibit a very large range of shapes and sizes (1 μm^2–100 s μm^2 long and an estimated 21–14.2 $\times$ 10^6 μm^3 volume range), resulting in a wide range in settling speeds (Snoeijs et al. 2002). Thus, some level of bias might be introduced when aliquot subsampling is performed on morphologically diverse assemblages. Diatom suspensions are often settled into specially designed shallow dishes of water via a technique often referred to as the "Battarbee method" (Battarbee 1973). In the Batterbee (1973, 1986) and related Schrader and Gersonde (1978) methods, the suspended diatom material settles in shallow chambers that are completely dried. Ion and clay-rich samples will leave residues, including precipitation of dissolved salts, often necessitating repeated washing steps prior to settling (Battarbee 1986). Each washing step adds time and complexity, and increases the risk of sample loss and cross contamination. Furthermore, some shallow settling dish methods involve direct pipetting of a diatom suspension onto slides and coverslips before pouring water onto the slurry to distribute it throughout a Petri dish (Schrader and Gersonde 1978). Using water to remobilize the diatom slurry which has been directly pipetted onto coverslips results in uneven distribution of particles across the slide (Schrader and Gersonde 1978). Some researchers prefer "added marker" methods, generally involving adding a known quantity of microspheres to a known mass of sample material (Battarbee et al. 2001). Microspheres can interfere with quantitative diatom counts by obscuring some diatoms on the slide.

Scherer (1994) introduced a settled slide method that reduces the number of steps and avoids the problems of subsampling, cleaning, and salt precipitation. This method is frequently used by diatomists working with marine sediments, which frequently have high clay and salt contents. Here we present an update to the method of Scherer (1994) that is easy to implement using commercially available settling chambers. Using the same sediment samples, we compare our method to the original Scherer (1994) method and the Schrader and Gersonde (1978), which we refer to as the dish method.

Methods

Sediment processing

A known mass of freeze-dried sediment is placed into a 20 mL glass vial. For diatomites and other highly diatomaceous sediments, as little as 0.008 g is sufficient, consequently use of a high quality and calibrated analytical balance is critical. Utilizing very small sample sizes has the added benefit of allowing microstratigraphic sampling of, for example, individual laminae in varved sediment. Samples with higher clastic or carbonate components may require as much as 0.050 g of dried sedimentary material. Carbonate, when present, is removed via reaction with 10 % HCl for 3 h. A stronger acid should not be used, as a violent reaction will damage the diatoms. Once the acid reaction is complete, 1–2 mL of 10 % H_2O_2 (slightly stronger, if necessary) is added to each sample to break down organic material and aid in disaggregation of clays. Samples are allowed to react at least 8 h. If the reaction is bubbling, a few drops of dilute ethanol can be added to slow the rate of reaction, to prevent boiling over, and protect diatoms from physical breakage. Dispersing agents, such as sodium hexametaphosphate, may be used in clay-rich samples if necessary, but should be avoided if not needed. For highly lithified sediments, extra chemical or mechanical steps to disaggregate and disperse the sample may be necessary, but care must be taken to avoid dissolution or fragmentation of the frustules. Immediately before adding the sample to the settling chamber, a drop of a surfactant dispersing agent (Kodak Photoflow^{TM} works especially well) is added to the vial, to minimize diatoms adhering to the vial or settling chamber, and prevent diatoms from becoming trapped at the top of the water column due to surface tension. Each sample vial is very briefly agitated in an ultrasonic bath immediately prior to dispersal into the settling chambers. While this step is important in disaggregating clays from diatoms, the ultrasonic bath should be used for only 2–3 s to avoid diatom fragmentation.



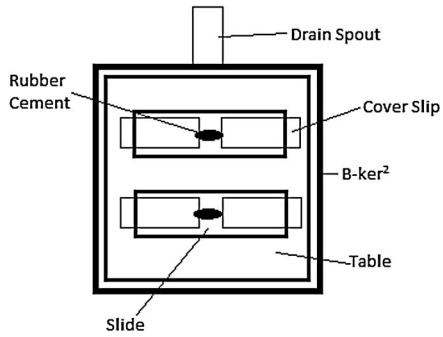


Fig. 1 Settling chamber set-up. Coverslips (*A*) are set on slides which rest on a table (*B*) that sits above the level of the drain (*C*). The beaker is filled to the 2 L mark with water, and the sediment sample settles evenly over the surface of the table. *Each coverslip* represents a proportionate subsample. Four coverslips are shown here, but up to 6 can be accommodated per sample with this set up. A *line* drawing is included for clarification. Each B-Ker^2 is 203 mm tall, with an inner square cross-section of 120 × 120 mm

Settling chamber set-up

In the original method of Scherer (1994), Petri dish bottoms, modified with a 1-cm drilled hole, are placed open-end down into round 1 L glass beakers. A rubber-tipped 20 mL glass pipet snuggly fits in the hole, and a flexible drain hose is attached to the pipet. The pipet and hose are water-filled and a stopcock at the end of the hose is closed. Prepared coverslips are placed on the Petri dish, and the beaker is filled with 1 L of microfiltered and deionized water. The diatom suspension is poured into the beaker and mixed thoroughly. The diatoms are then allowed to settle for several hours. After settling, the clamp is slowly opened, allowing the rubber hose to open and slowly drain the sample over the course of several hours via siphon. The coverslips must be allowed to dry fully before removal from the Petri dish and mounting to glass slides.

The modified method, reported here, utilizes commercially available 2 L square settling chambers with drain spigots, designed for Jar Testing in the water treatment industry (called B-ker$^{2\ TM}$, manufactured by Phipps and Bird, Richmond, VA, USA). The square shape minimizes eddy formation, promoting an even distribution of settled particles, and the stopcock allows easy draining (Fig. 1). If B-ker$^{2\ TM}$ chambers are unavailable, comparable chambers can be fabricated. A square glass or Plexiglas table must be fabricated to provide a platform above the drain port. The table provides sufficient space to allow the creation of multiple slides or SEM stubs per sample (Fig. 1). As with Scherer (1994), coverslips are held in place on clean glass slides with a small drop of rubber cement, and placed on each table. The coverslips will be coated with the sample during settling (the glass slides may be discarded or carefully cleaned after each use). Chemically treated and agitated samples are then poured into the beakers. As with Scherer (1994), a wash bottle should be used to rinse the sample vial to insure that no sample residue is left behind. Sediment should distribute in the water column naturally, but a clean stirrer or spatula can be used to gently mix the sample into the beaker if required. The sample should be allowed to settle without disturbance in covered chambers for at least 6 h; longer for small and gracile diatoms, coccoliths, or other particulates likely to settle slowly. Samples prepared for this study were allowed to settle for 8 h before draining.

Slide preparation

After the samples have settled, the nozzles are opened slightly to allow the beaker to drain. In order to avoid remobilizing the settled sediment, the beaker should be drained slowly, not more than a few drops per second, $\sim$10 mL min^{-1} maximum. When the water has drained below the level of the table, the coverslips are allowed to air dry. Coverslips are permanently affixed to slides using Norland Optical Adhesive, or a preferred resin-based high refractive index diatom mounting medium, per standard methods. Diatoms can

be counted on transects or fields of view, as desired. Because the sediment is dispersed evenly across the platform of the settling table, each coverslip represents a proportional subsample. Diatoms per field of view or transect can be mathematically translated to the absolute abundance (ADA) in diatoms per gram dry weight of sediment via the following equation:

$$ADA = \frac{\#Diatoms\,counted}{\#Fields\,of\,view * Field\,of\,view\,area\,(\mathrm{mm}^2)} * \frac{Beaker\,area\,(\mathrm{mm}^2)}{Dry\,sediment\,mass\,(\mathrm{g})} \quad (1)$$

Counting

To test the reproducibility of the new method and compare it with the dish method, two sets of slides were created using both methods. Both samples used here are retained samples from the original 1994 study: (1) FWD (freshwater diatomite) from a surface exposure (outcrop) in Columbia, South America, and (2) MDM (marine diatomaceous mud) from a fjord on the Antarctic Peninsula, characterized by abundant *Chaetoceros* resting spores. After 20 years in Scherer's collection, the samples had fully desiccated. In the case of MDM, there is evidence of significant shrinking with desiccation, which likely has caused an increase in diatom fragmentation, but FWD was unaltered, due to the high abundance of diatoms and relative absence of clays. Each sample was prepared in four separate beakers. Each beaker was utilized to create four slides per preparation. Each slide was counted twice, once near the edge and once through the center of the slide. All data were collected at least three fields of view inward from the edge of the coverslip. For this study, diatoms were not identified, rather, the numbers of whole valves, including *Chaetoceros* resting spores, as defined by Schrader and Gersonde (1978) and Scherer (1994), were counted. Four original slides from the 1994 study and from the new study were recounted by both authors to internally compare counting methodology.

Table 1 Statistics

Method	MDM	FWD
Scherer (1994)	3.62E+08 [2.07E+07]	4.58E+09 [6.46E+08]
This paper	2.31E+08 [1.28E+07]	1.55E+09 [1.69E+08]
S & G (1978)	3.62E+08 [1.49E+08]	5.28E+09 [2.18E+09]

This table gives the averages and (standard deviations) for all samples and methods compared here

MDM, Marine diatomaceous mud; FWD, Fresh water diatomite; S & G (1978), slides generated via the method of Schrader and Gersonde (1978); Sch94, diatom counts from Scherer's (1994) original version of this method; v gdw^{-1} = valves per gram dry weight of sediment

Comparison to the Schrader and Gersonde (1978) dish method

Both the FWD and MDM samples were prepared in four separate petri dishes according to Schrader and Gersonde (1978). This method uses an aliquot subsample from a 50 mL diatom suspension prepared from a known mass of dry sediment. Each slide was counted twice, as above. ADA in diatoms per gram dry weight of sediment was calculated for comparison to the new method via the following equation:

$$ADA = \frac{\#diatoms\,counted * petri\,dish\,area\,(\mathrm{mm}^2)}{\#Fields\,of\,view\,counted * Field\,of\,view\,area\,(\mathrm{mm}^2) * subsample\,volume\,(\mathrm{mL})} * \frac{50(\mathrm{mL})}{Dry\,sediment\,mass\,(\mathrm{g})} \quad (2)$$

Because the petri dish method utilizes an aliquot subsample of a diatom suspension, a ratio between the 50 mL diatom suspension, and a subsample volume representing the aliquot of the 50-mL sample distributed into the petri dish must be added to the equation.

Statistics

The Tukey–Kramer test for multiple comparisons with unequal sample sizes was used to compare the means

Table 2 Comparison of means

Sample/test	$q_{T\ versus\ S}$	$q_{T\ versus\ P}$	$q_{S\ versus\ P}$	q_{crit}
FWD	0.0002217	0.0002269	0.9999	3.422
MDM	0.0001272	0.001272	0.2485	3.422

Because the samples sizes were unequal, the Tukey–Kramer test was chosen to compare the mean absolute abundances from each of the three methods examined in this paper. The null hypothesis that each compared pair of means is similar is rejected when the calculated q value is larger than a critical value, taken from Zar (2010). All three methods were found to produce statistically similar means ($P < 0.05$). Abbreviations follow Table 1 and used are as follows: T, this method; S, Scherer's (1994) method; P, Schrader and Gersonde (1978) method; crit, critical value

of each of the three methods tested, following Zar (2010). Statistical results are shown in Table 2.

Results

Table 1 presents averages and standard deviations for each method assessed here. Counts (within each sample, between beakers, within beakers, and within single slides), generate similar absolute abundance data (Fig. 2), and both authors' counts are statistically identical. Both the high abundance, pure FWD and the lower abundance MDM sample yielded reproducible results with low standard deviation when produced with the new method. This reproducibility is crucial for interpreting changes to the absolute abundances of microfossils between sediment samples as well as for tracking changes in microfossil absolute and relative abundances within a single sediment core. The Tukey–Kramer test reveals that the means of all three analyses are statistically similar ($P < 0.05$).

For the FWD, the slides counted using the new method yielded an average of 1.55×10^9 valves gram dry weight sediment^{-1} (v gdw^{-1}) with a standard deviation of 1.69×10^8 v gdw^{-1}. The dish method yielded an average of 5.28×10^9 v gdw^{-1} and a standard deviation of 2.18×10^9 v gdw^{-1}. Scherer's (1994) original counts for FWD resulted in an average absolute abundance of 4.70×10^9 v gdw^{-1} and a standard deviation of 6.46×10^8 v gdw^{-1} (much of that deviation came from one anomalously high abundance, possibly due to settling chamber disturbance).

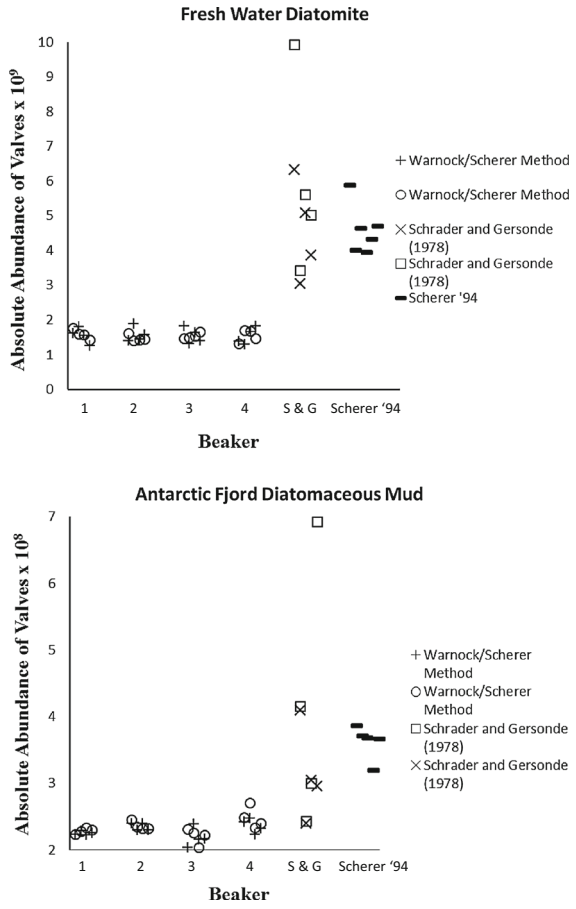


Fig. 2 Comparison of replicate slides. Each sample was processed in four individual beaker trials using the method presented here. Four slides were made within each beaker. Each slide was counted twice. Counts from each slide are arranged vertically (*one circle* and *one plus* per slide). Finally, the samples were processed in four petri dishes using the Schrader and Gersonde (1978) method (abbreviated "S & G", and each slide was counted twice (*one square* and *one times* per slide). The Warnock/Scherer method produces one standard deviation more consistent results compared to the Schrader and Gersonde (1978) method, and slightly more consistent results than Scherer's (1994) original method (*thick dashes*)

For the MDM sample, the new method produced an average of 2.31×10^8 v gdw^{-1} and a standard deviation of 1.28×10^7 v gdw^{-1}, whereas the dish method produces a statistically comparable average of 3.62×10^8 v gdw^{-1} and a standard deviation of 1.49×10^8 v gdw^{-1}. Scherer's original counts for MDM produced an average of 3.62×10^8 v gdw^{-1} with a standard deviation of 2.07×10^7 v gdw^{-1}.

Discussion

The large surface area available in each beaker allows for the simultaneous preparation of duplicate slides from a single sediment preparation. This is useful in creating slide sets to share with colleagues and for the easy generation of slide sets for teaching. Spigots with adjustable nozzles allow for precise control of drain rates, eliminating currents that can remobilize sediment and microfossils that have settled onto coverslips. In comparison to the Petri dish methods, the 2 L square beaker provides a deeper water column, allowing for increased dilution of dissolved ions. Additionally, this water column is calm when the subsample is added, which should reduce the formation of eddies. This can be contrasted with the Battarbee (1973) method where the sample is added to a dry dish, which might cause some eddy formation, and the dish method where the diatom subsample is mobilized by adding water to the Petri dish. The dish method resulted in overall higher diatom counts than the method updated here. Direct pipetting of a diatom suspension over the coverslip, followed by addition of water to mobilize the diatoms, results in a less even distribution of particles across the sample chamber. This causes a higher diatom concentration on the coverslips. In this method, diatoms distribute throughout a stable water column evenly as they settle, creating a more even distribution. Furthermore, we observed more random distribution of diatoms on slides produced in square settling compared to slides produced in Petri dish chambers.

The absolute abundances recorded here are slightly lower than those of Scherer (1994), who counted the same samples. For the FWD sample (4.70×10^9 - v gdw^{-1} Scherer 1994; 1.55×10^9 v gdw^{-1} this study). Circulation patterns that develop in round beakers tend to concentrate larger particles in the center, which may account for the higher numbers generated from round settling chambers in samples containing chain-forming diatoms, which behave as large particles, but individual valves in the chain are counted. FWD confirms this bias. Long chains of *Aulacosiera* are more abundant on the slides prepared using Schrader and Gersonde (1978) and Scherer (1994) methods, with heavy *Aulacosiera* chains settling quickly in the chamber center. Therefore, we consider results from the square beakers to be more accurate than earlier counts.

For the MDM sample, the difference is small (3.62×10^8 Scherer 1994; 2.31×10^8 this study), and can be attributed to enhanced fracturing of diatoms due to dehydration of the sample over 25 years, and to disaggregation methods required now that were not needed then. For both samples, the new method and original Scherer method are found to be more reproducible than the dish method, having lower calculated standard deviations.

In order to further test the robustness of the new method, the outflow from the settling chambers was collected into individual beakers, and allowed to evaporate over a few days to concentrate any diatom loss from the settling chambers. When the settling chamber outflow had evaporated to $\sim$2 mL, it was pipetted onto a single coverslip per settling chamber. The water was then fully evaporated, and the slide was analyzed for diatom content. All of these slides were found to be barren, indicating that the sample is almost entirely captured on the settling platform.

Large settling chambers, small samples, and the absence of aliquot subsampling provide specific advantages in the processing of diatom slides for quantitative analysis. The method described also allows the parallel preparation of multiple slides, as well as SEM stubs. Saline or hypersaline samples may be processed without extra dilution steps, adding an additional advantage for some sectors of the diatomist community. It is important to note that Battarbee (1973), Schrader and Gersonde (1978) and Scherer (1994) methods have been widely adopted, and that each of these methods remains valid and viable options for diatom analysis. The methods tested in this paper, Schrader and Gersonde (1978) and Scherer (1994), and the modification described here all produce reproducible and statistically comparable results (Table 2). All methods tested yield accurate enough results for independent and cross-interpretation, thus it is not necessary to abandon classic methodology. However, we note that the revised method described here appears to improve accuracy, and has the added benefit of being easy to establish and employ in any diatom lab.

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