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Aluminum Shadowing-Direct Projection Photomicroscopy of Diatom Frustules

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An aluminum shadowing-direct projection method is described which allows high resolution photomicrographic reproduction of diatom preparations in the 200-800 \times magnification range. The technique is simple and provides an inexpensive alternative to low magnification scanning electron micrographs. While resolution and depth of field are constrained by the limits of conventional optical microscopy, the shadowing technique permits size determination in three dimensions. In addition, routine diatom identification and enumeration can be done directly from photographic prints, which provide a permanent record for storage.

Key words: diatom, microscopy, phytoplankton

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On trouvera dans cet article la description d'une méthode par projection directe et ombre d'aluminium permettant une reproduction photomicrographique à haute résolution de préparations de diatomées dans la gamme de grossissements de 200 à 800 \times . Simple et peu dispendieuse, la technique peut remplacer les micrographies au microscope électronique à balayage à faibles grossissements. Alors que la résolution et la profondeur du champ sont restreintes par les limites de la microscopie optique traditionnelle, la technique d'ombre permet de déterminer la grosseur sur trois dimensions. En outre, l'identification et le dénombrement routiniers des diatomées peuvent se faire directement sur les photographies, qui constituent des fiches permanentes pour stockage.

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ANALYSIS of diatom communities is a major tool for assessing present and past environmental conditions in freshwater and marine ecosystems (Kolbe 1932; Patrick 1948, 1963; Hustedt 1939; Stockner 1972; Holland and Clafin 1975). High resolution is often required for taxonomic identification at the specific and occasionally the generic level (Hasle and Heimdal 1970; Hasle 1976; Reimann et al. 1963). Transmission and scanning electron microscopy (SEM) provide high resolution micrographs, but are time consuming, expensive, and generally unsuitable for routine enumeration. Investigators faced with counting and identifying diatom taxa in large numbers of samples must resign

themselves to many hours at the light microscope. Traditional enumeration procedures for cleaned frustules mounted in a high refractive index medium and viewed under bright-field illumination are tedious and provide no record for later rechecking the accuracy of previous identifications and counts. Further, restrictions set by the use of visible light and the numerical aperture of the finest lenses place the highest resolving power attainable with the optical microscope at about 0.17-0.20 μm (Shillaber 1944). This resolution, often needed for critical identification, is infrequently achieved in photomicrographs of diatoms because of inadequate specimen contrast and definition loss associated with transferring the microscope image to film and then from film to print. In certain instances the slight birefringence of silica may also obscure frustular details that are actually within the resolving power of the light microscope (Crawford 1977). In addition, large format, high resolution photomicrographs, which are essential for enumeration from prints,

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are rarely produced because of the ineffective use of planapochromatic objectives by many 35-mm camera attachments (Quackenbush 1975).

As a novel alternative for both diatom photomicroscopy and routine enumeration, we propose here a metal shadowing-direct projection technique that permits critical examination of frustule morphology at the highest resolution possible with the optical microscope. The method is simple, relatively inexpensive, and yields photomicrographs that are suitable for both identification and enumeration.

Materials and Methods

Fresh diatom material was prepared by boiling in sulfuric acid followed by potassium dichromate oxidation and four rinses with distilled water (Patrick and Reimer 1966). An aliquot of the acid-cleaned frustule suspension was smeared onto an ethanol-cleaned glass microscope slide that had been previously coated with a thin layer of 0.5% (w/v) aqueous gelatin. After thorough air drying, slides were placed on the stage of a Mikros vacuum evaporator with the center of the slide 8.0 cm from the tungsten filament basket. The electrode-filament assembly was elevated above the stage so that the angle of shadowing measured at the middle of the slide was 20°. A 2.0-mg charge of acetone-cleaned, aluminum wire was placed in the basket and evaporated under a minimum vacuum of 1.33 mPa.

Shadowed specimens were then mounted in either degassed Araldite or Epon resin and covered with a No. 1- $\frac{1}{2}$ coverslip. Gentle heating in vacuo of the freshly mounted specimens removed any traces of bubbles to provide maximum optical clarity of the hardened mounts.

A Zeiss compound microscope equipped with a direct projection prism or mirror assembly was mounted on an optical bench 25–50 cm from a vertical wall in a darkroom. Selected fields of diatom frustules were focused and projected onto a wall-mounted photoenlarging frame containing standard size photographic paper (20 \times 25 cm). Precisely timed exposures were made by connecting the microscope's halogen lamp to an interval timer. Different magnifications were obtained either by varying the power of the objective lens or the distance of the projection mirror from the printing frame, or both. Exact magnifications were determined by measuring the image of a stage micrometer projected onto the wall.

During exposure it was necessary to shield the photographic paper from stray light reflected from the microscope itself. This was accomplished by placing a rubber cylinder between the field diaphragm and condenser, and also by placing a black cardboard light barrier between the microscope body and the printing frame. Normal exposure times with medium or fast photoenlarging paper ranged between 4 and 30 s.

Since image projection onto the photographic paper is the only optical transfer step involved in the technique, a high quality lens system is important. Flat-field corrected objectives of high numerical aperture are essential with direct projection photography to obtain full field focus and high resolution. We obtained good results using a Zeiss 63-mm Kpl microprojection ocular in combination with either a Zeiss 10 \times 0.32 N.A. planapochromat or a Zeiss 40 \times 1.0 N.A. oil planapochromat objective. Comparable Nikon objectives also provided excellent resolution.

Results

Oblique shadow casting reveals morphological details that are indistinct in unshadowed specimens. Valve surface contours are brought out in great relief and it is possible to distinguish ridges and depressions without refocusing. Raised and depressed central areas of *Cyclotella* valves are evident (Fig. 1) as are elevated costae. External and internal valve views of many pennate forms such as *Frustulia* are easily recognized by valve mantle and raphe shadows (Fig. 2b, c). The puncta of *Stauroneis phoenicentron* are readily seen at a magnification of 760 \times even though they are only 0.6 μ m (Fig. 2a). Even the 0.15- μ m latex calibration spheres visible in Fig. 2c are clear and sharp.

A shadowing angle of 20° gave the best results. At that angle shadows are 2.8 times longer than the specimen thickness. In most cases this is sufficient for accurate measurement. By multiplying the measured length of shadows by $\tan 20^\circ$, the depth of specimens can be determined. Similarly, shadows cast by the spines of some centric diatoms when seen in valve view (Fig. 3) can be used to calculate rapidly their length, a difficult task with unshadowed valves. In addition to size determination, the shadows are helpful in showing the three-dimensional shape of frustules.

The use of flatfield corrected objectives in combination with direct projection photography permits large format prints to be made that do not show empty magnification (Quackenbush 1979). At magnifications of 200 \times and 400 \times , prints 20 \times 25 cm (a standard 8 \times 10 in. (1 in. = 25.4 mm) print sheet) cover fields of ~ 1.25 mm² and 0.31 mm², respectively. By choosing fields of suitable frustule density, the prints can easily display 100–200 specimens at low magnification or 25–50 individuals at higher magnification. With the prints placed in a gridded transparent cover, diatoms can be counted directly from the photographs later. Several prints at each magnification for a single sample will generally provide adequate information on relative species abundance for most purposes. With the use of an appropriate filtration or sedimentation concentration procedure, quantitative results would even be possible.

The 20 \times 25 cm prints are also convenient for storage as a permanent record. A file of photographs of individual species at higher magnifications encountered in samples from specific areas can be assembled for reference.

Discussion

The use of shadow casting for increasing the visibility of objects viewed with the light microscope was first proposed by Williams and Wyckoff (1946). The method has not received wide use by biologists (Fraser and Rogers 1954) because other means of enhancing specimen contrast such as differential dye staining and phase microscopy have proven more effective and are the only choice where visualization of internal detail

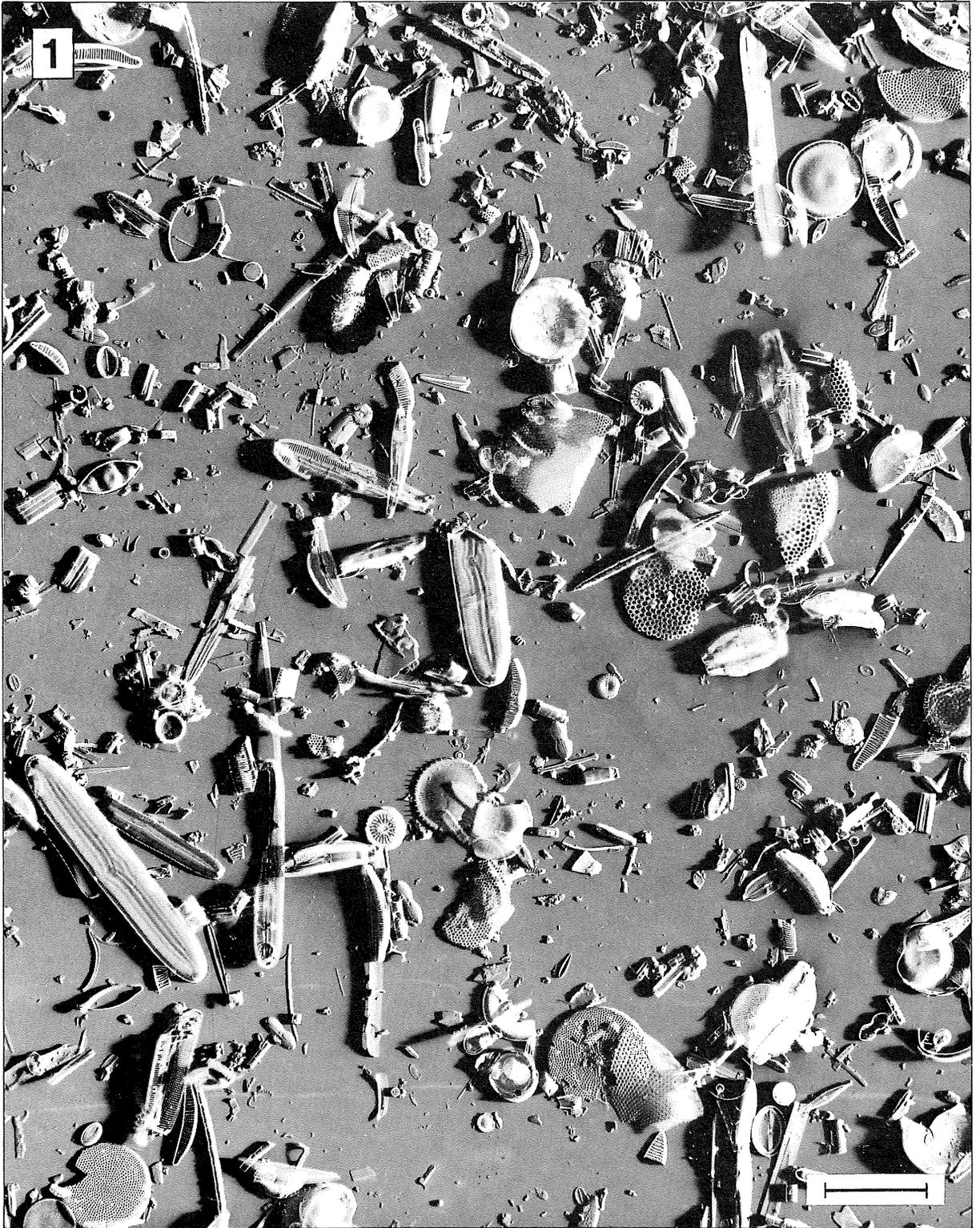


FIG. 1. Mixed marine and freshwater diatoms (supplied by MacMillan Science Inc.). Bar is 100 μm .

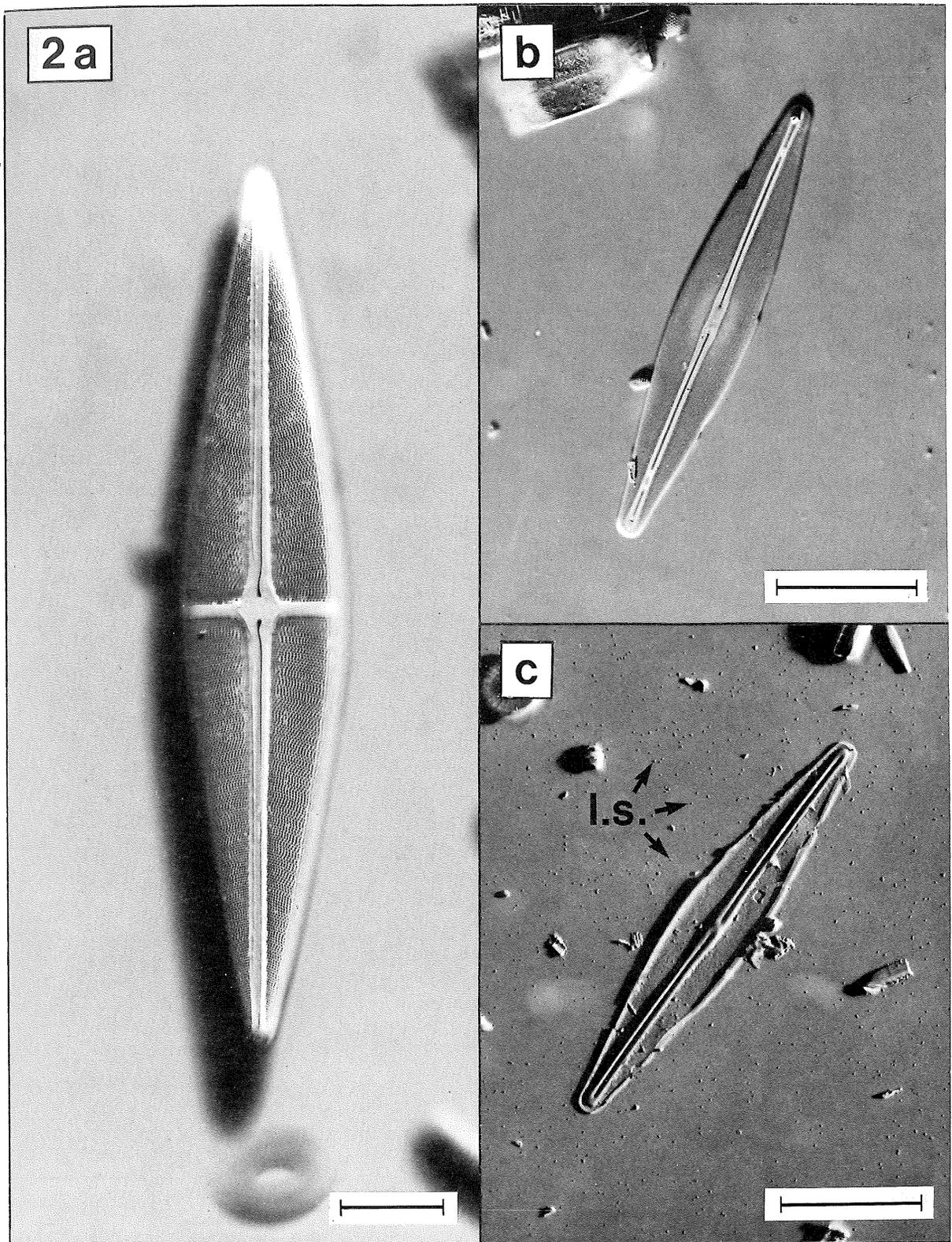


FIG. 2a. *Stauroneis phoenicentron*. Bar is 25 μm . FIG. 2b. *Frustulia interposita*. External valve view. Bar is 50 μm . FIG. 2c. *Frustulia interposita*. Internal valve view. l.s., latex spheres (arrow). Bar is 50 μm .



FIG. 3. Mixed marine and freshwater diatoms (supplied by MacMillan Science Inc.). S.S., spine shadows (arrow). Bar is 50 μm . A higher magnification of a portion of the field shown in Fig. 1.

is required. However, diatom frustules, composed of translucent amorphous silica with intricate surface structure, are perfect subjects for shadowed light microscopic examination. The contrast necessary for adequate observation of frustular detail is traditionally created by mounting frustules in a medium with a high refractive index such as Hyrax (n 1.71) or Pleurax (n 1.77). While these media are an immense improvement over Canada balsam (n 1.54) (Hanna 1930, 1949; Brislee 1932; Cameron 1934), some problems still remain. Structures present on the undersides of some valves can cause confusion in interpreting the morphology of upper valve surfaces. A recent SEM examination of the genus *Cyclotella* has shown, for instance, that the "thickened costae" of *C. atomus* seen with bright field illumination are actually marginal strutted processes on the interior surface of the valves (Lowe 1975). Other examples of misinterpreted light microscopic observation are likely to be found.

The method presented here largely avoids this difficulty. After shadowing, the frustules are mounted in epoxy resin with a refractive index of about n 1.50–1.60 (Lee and Neville 1967), not a great deal higher than that of silica (n 1.45). This optically dissolves some of the finer frustule detail allowing contrast created by the varying thickness of vacuum-deposited aluminum on the valve surfaces to play a major part in image formation. Because much of the image seen is formed by upper surface contrast, interference by structures on the lower side of valves is minimized and the photographs that are produced remarkably resemble SEMs.

What can be seen in a micrograph is a function of both the resolving power of the lens system and the contrast between adjacent regions of the specimen (Williams and Wyckoff 1946). The ability to visualize very small detail with this method is in part attributable to the contrast created by the aluminum shadowing. Another feature of shadowing is that it effectively increases the optical size of very small particles. In this manner, the 0.15- μ m-diameter latex spheres seen in Fig. 2c cast shadows that are about 0.42 μ m long and are readily resolved with the light microscope. This aspect of shadowing has been used fruitfully for revealing the presence of macromolecular-sized particles with transmission electron microscopy (TEM) for many years (Williams and Wyckoff 1945). It also points to at least one other potential use of this method; that of permanent mounts for total, free-living, bacterial counts of water samples.

Finally, the method of direct projection also contributes to the very high resolution obtainable with this technique. The use of metal shadowing with TEM produces prints that are negative, i.e. the shadows are white and the objects are dark. To reverse the contrast, it is necessary to make a positive from the original negative plate and a negative print from this positive (Needham 1958). With the present method, shadowing with aluminum makes the specimens themselves a nega-

tive, and a positive print is made directly by using the microscope as the photoenlarger. As only one optical transfer step is needed to produce the final print, the maximum possible resolution is assured.

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