Use of Membrane Filters for Microscopic Preparations of Sponge Spicules[1]

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Abstract. A new method for preparation of sponge spicules for microscopy utilizes membrane filters for separation of spicules from cleaning fluids. The membranes with adherent spicules are cleared during mounting on microscope slides for direct analysis by light microscopy. The basic filtration procedure is also used to produce preparations for scanning electron microscopy. The main advantages of this procedure over a variety of traditional methods include preclusion of spicule loss during cleaning and significant reduction of procedure-introduced variation. The technique described offers promise for quantitative analysis of spicule complements.

Incentive to develop a standardized procedure for spicule preparation stems from several problems noted in publications of both veteran and neophyte sponge taxonomists. Most prevalent is a general absence of detailed reporting of methods employed in spicule examinations. The result is a disturbing lack of certainty in taxonomic identifications, deriving partly from real differences in the specimens themselves, but also is an artifact of the methods of spicule preparation and analysis. The most commonly reported problems are the rare occurrence or absence of a spicule type and differences in size of spicules from specimens that otherwise appear to belong to the same species.

We surveyed 123 primary taxonomic papers on Porifera published during the last 40 years[3]. Although the sample is biased by both ease of access and language of publication (untranslated Eastern languages were excluded), it does include all of the important contemporary treatments: 75% (n = 92) of these papers include no mention of the method of skeletal preparation; 19% (n = 24) include a short mention of, or reference to, a technique; and only 6% (n = 7) provide a description of methods in sufficient detail to enable duplication. Potential investigators looking to these publications of methods to use in identification, and, more importantly, seeking an authoritative format for preparation of taxonomic publications, are provided with little or no guidance.

A detailed set of standard procedures for (1) making spicule preparations, (2) examining them microscopically, and (3) carrying out and reporting quantitative statistical analyses on the data generated has not been formally proposed or tested. Our purpose is to put forward a basic procedure for producing permanent spicule preparations. This procedure is new, requires minimal spe-

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cialized apparatus, is inexpensive, and rapid. Most importantly, it eliminates many of the procedure-introduced sources of variation common to all currently used methods.

PROCEDURE

Our procedure for making permanent spicule preparations differs from others in the use of a membrane filter. The filter surface serves for spicule capture and retention from the digested suspension stage through preparation of the final microscopic slide mount. The method minimizes the loss of small spicules during rinsing. We have developed two slightly different devices: (1) a standard filter unit (Fig. 1A) for usual qualitative analysis and measurement of spicule dimensions, and (2) a rinsing-filter unit (Fig. 1B) for analysis of uniform spicule distributions, and, ultimately, for detailed quantitative analyses (enumeration of spicule types, ratios between types, etc.). Both units have proven to be dependable and durable over five years of use in processing hundreds of specimens in the Redpath Museum collections and at remote field stations.

To illustrate the performance of these units, we have selected a Caribbean haplosclerid demosponge, *Niphates erecta* Duchassaing & Michelotti, 1864. This sponge has one class of megascleres (ca. 210 μm in length), predominantly oxeas, but including strongyle and style variants, and one class of sigmoid microscleres (ca. 15 μm in length). The microscleres have been reported commonly as rare or absent in many specimens from local populations throughout the range of the species (Hechtel, 1965, 1969; Soest, 1980).

Preparation of Spicule Suspension

In processing an unknown specimen, broad taxonomic placement and expected spicule pattern are determined by examining a tissue fragment digested on a microscope slide with sodium hypochlorite. Chemical composition is often obvious from the spicule shapes encountered, but verification can be made easily with crossed polarizers ($CaCO_3$ spicules are anisotropic, bright) or dilute acid ($CaCO_3$ spicules dissolve, often releasing CO_2). On the basis of chemical content, the spicule cleaning solution is selected: sodium hypochlorite for calcareous spicules and concentrated nitric acid for siliceous spicules. A fragment of the specimen, usually a rectangular block perpendicular to the surface and 1–10 mg dry weight (3–4 mm on a side), is removed for digestion. Choice of size and location of the fragment varies with specimens, spicule types, and experience of the investigator. Fragment size is governed by the need for an adequate sample of spicules for survey; if necessary, specimens of 1.0 μg dry weight, or portions of a single gemmule, can be processed easily.

The fragment is placed in a small Erlenmeyer flask (25 ml) and 2–5 ml of the appropriate cleaning solution is added. To prevent eruptive reaction, excessive calcareous material should be removed from samples to be cleaned by nitric acid digestion. The sample fragment and cleaning fluid are brought to a gentle boil over an alcohol lamp or Bunsen burner in a fume hood, with total boiling time of 30 sec to 2 min. Heating to dryness should be avoided. Organic components may be dissolved at room temperature, but complete cleaning in

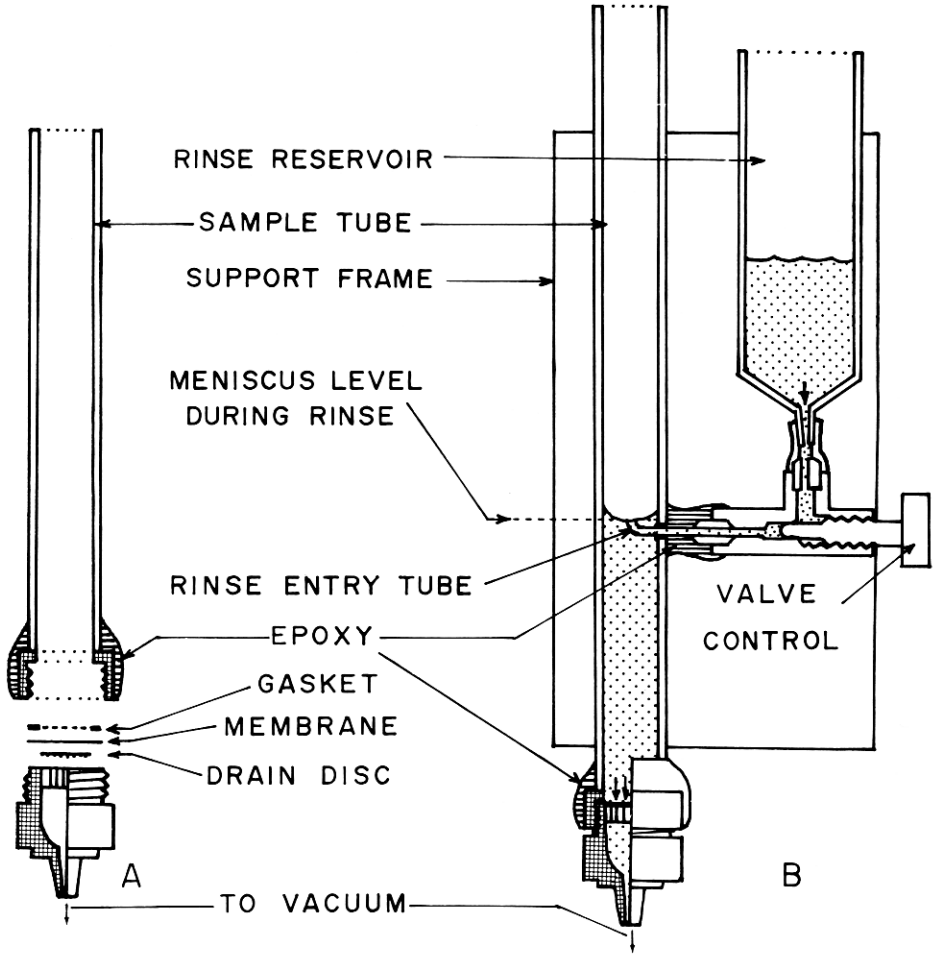


FIG. 1. Diagram of standard filter unit (A, exploded) and rinsing filter unit (B, during operation).

this case may take over 24 h. Use of a double boiler or heating block to regulate temperature just below boiling is efficient for processing many samples at once. Caution should be exercised to avoid significant loss of spicules that could introduce a non-systematic bias between the original sample and the final spicule preparation.

Spicules may be lost in the aerosols generated during boiling, artifactual size-fractionation of spicules being a potential source of sample bias. We have examined spicules in fumes driven from boiling flasks, and found that both spicule types are liberated in the aerosol by direct ejection during active boiling. From an examination of the fluid condensed from flask openings on cold (0°C) glass surfaces, the megascleres constituted 0.3% of those estimated to be present

in the fluid contents of the flask. Surprisingly, microscleres were liberated at only 25% of total megasclere counts. Considering abundance of the two spicule types in the suspension, fractionation is estimated to be 13:1 (megascleres: microscleres). Within the 2-min boiling period, therefore, the loss of spicules in aerosols is an insignificant source of between-sample variation.

The cooled flask contents are diluted with distilled water to a volume suitable for obtaining an acceptable density of 10–100 megascleres/mm^2 on the filter surface. Required dilutions may range from 1:5 to 1:500, depending upon original sample size and spicule abundance in the sample fragment.

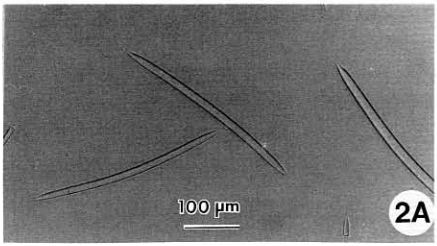
Standard Filter Unit

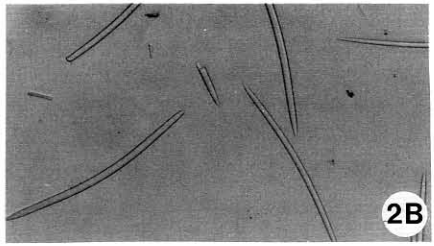
The filter apparatus (Fig. 1A) is loaded with a 13-mm diameter, 0.2-μm pore-size membrane filter, drain disc, and gasket. The unit is clamped to a support in a vertical position and the vacuum line and fluid trap are connected. An aliquot of the diluted spicule suspension, usually 2–5 ml, is transferred to the sample tube, either by large-bore pipette, or directly by rapid stir-pour manipulation of the dilution vessel. Vacuum is applied and the sample is filtered. When the aliquot has been filtered completely, the sample tube is rinsed while filtration continues, with 5–10 ml of distilled water dispensed from a wash bottle. When the rinse water has passed the filter surface, the vacuum is removed at the holder, the filter is transferred by forceps to a labelled glass microscope slide and placed in an oven maintained at 50°C for drying. The filter unit, gasket, drain disc, fluid trap, vacuum lines, etc. are rinsed thoroughly to remove excess cleaning solution and adherent spicules which might contaminate a subsequent sample. After at least 15 min of drying, the filter and slide are removed from the oven for final mounting. The filter is cleared while warm with a drop or two of xylene and mountant is added to one edge. Bubbles between membrane and slide can be removed by manipulation with a needle and forceps, followed by addition of a standard 22-mm^2 (No. 1) cover glass.

The completed spicule-membrane slide usually will require addition of mountant once or twice during early drying because of residual solvent evaporation over a 48-h period on a slide warmer at 40–50°C. Air bubbles pulled into the filter matrix during this period can render the preparation opaque. We prefer Canada balsam to rapidly-contracting media (e.g., DPX) or media that crystalize readily (e.g., Permount). The completed preparations are most suitable for phase-contrast or dark-field optics, but image quality in standard bright-field illumination with the substage diaphragm partially closed is only marginally less favorable than that provided by direct slide-spread preparations (Figs. 2, 3).

Filter Selection: Size, Composition, and Pore-size

The 13-mm diameter membrane size is most suitable for the following reasons: (1) the entire filter can be mounted on a standard microscope slide under cover glass without trimming or subdivision; (2) the entire effective filter sur-


100 µm
2A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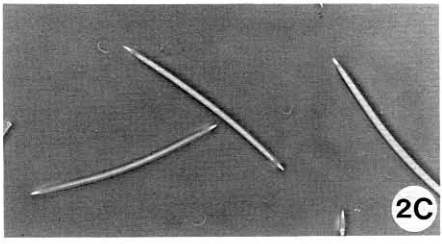
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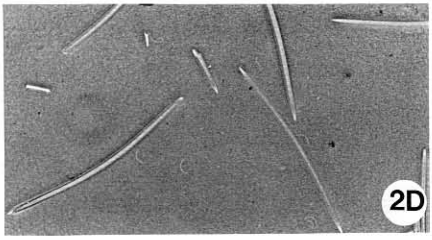
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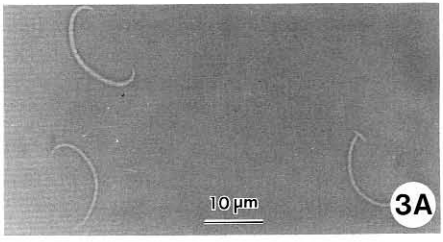
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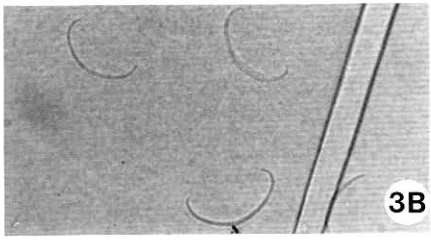
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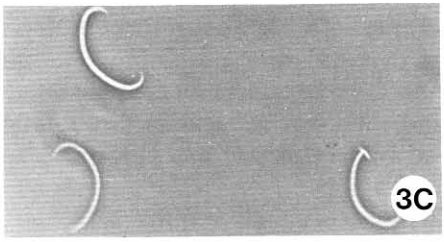
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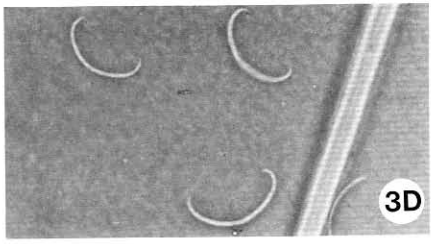
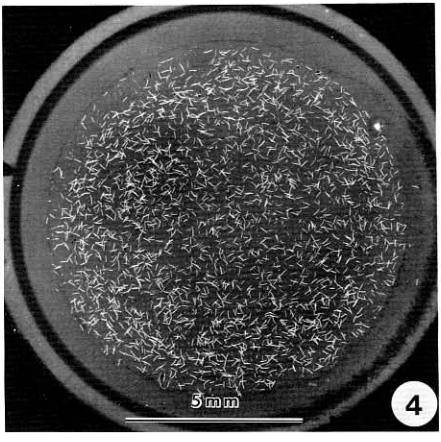

3D


5 mm
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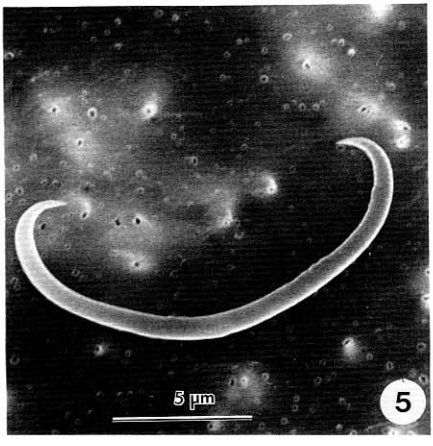
5 µm
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face (ca. 10-mm diameter) can be mounted on most commonly used scanning electron microscope (SEM) pegs; and (3) the spicules can be surveyed from smaller sample fragments on a small filter surface area.

A standard filter unit that accommodates 13-mm filters is not available from commercial sources, but such a unit can be fabricated easily with short lengths of 10-mm internal-diameter glass tubing, 13-mm polypropylene Swinney filter holders (Millipore SX000130 or equivalent), and waterproof epoxy. The syringe connection and conical upper portion of the holder is removed above the gasket seat by fine coping saw and abrasive. The threaded gasket seat is then attached with epoxy to a 10-cm length of glass tubing. After several layers of epoxy have been applied, the holder unit is trimmed internally to conform to the internal sample tube diameter, thus providing a smooth column through which spicules can fall to the filter surface.

Although we have found the 13-mm diameter format entirely satisfactory for our needs, some workers may prefer 25- or 47-mm diameter filters for ease of physical handling and for their capacity to store larger spicule samples. The larger filters require subdivision or trimming for mounting on standard microscope slides, a process which imposes the attendant hazard of physically disturbing adherent spicules.

The white, mixed cellulose-ester membrane filters (Millipore MF type or equivalent) are most suitable for light microscopy (LM) because of their relatively high transparency when cleared in solvents and mountants. Selection of the 0.22-μm pore size reflects a compromise between speed of filtration and efficiency of retention. We are unaware of minute spicules smaller than this pore size, but we do not ignore the possibility that very small spicular elements, visible only by SEM, indeed may occur in some sponges. The small commas of *Neofibularia nolitangere* and microchelas of *Rhaphidophlus schoenus* are near the limits of resolution using LM, and smaller elements are thought to occur in some demosponges.

For SEM preparations, we employ the Nuclepore 0.2-μm pore-size polycarbonate membrane (or equivalent) because of the smooth surface and discrete pore design; the basic procedure remains the same as that for LM. When oven drying is completed, filters for SEM are transferred to metal pegs coated with adhesive or double-sided tape. The excess filter border is trimmed away and a drop of silver conductive paint is added on the margin to minimize surface charging during viewing. Finally, metal coating is usually determined by convention of the local SEM facility.

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FIGS. 2, 3. Spicule preparations of *Niphates erecta* at low (Fig. 2, 20× obj.) and high (Fig. 3, 100× oil) magnifications; LM. Pour-slide preparations are on the left (A, C) and RFU preparations on the right (B, D) with transmitted (upper) and phase-contrast (lower) optics, respectively. Scale bars in micrographs labelled A apply to entire figure in each case. FIG. 4. A complete RFU preparation of spicules from *Niphates erecta* photographed under dark-field illumination showing uniformity of spicule distribution (approx. 2,800 megascleres total). FIG. 5. Microsclere of *Niphates erecta* prepared by RFU method; SEM.

We maintain 12 filter units for simultaneous use with an inexpensive, multiplace vacuum manifold constructed of polyvinyl chloride pipe and polyethylene syringe valves; this permits rapid sequential processing of a set of samples with greater efficiency. A vacuum pump or faucet siphon provides filtration suction in a laboratory setting, but with the small-volume fluid displacement required for this process, small hand-operated vacuum pumps are adequate in a field station environment.

Rinsing Filter Unit

Spicule preparations made with the standard filter unit described above are adequate for determination of spicule types and basic measurement of spicule dimensions. However, they have limited potential for more detailed quantitative work because of uneven distribution of spicules, especially of edge clumping that results from rinsing. To obtain a more even spicule distribution across the filter surface (Fig. 4), we use a slightly modified version of the standard filter unit that includes an integrated rinse dispenser (Fig. 1B). A 15-cm long, 10-mm internal diameter glass tube is perforated by a blown-in 1-mm diameter hole 5 cm above the filter holder. A small glass tube (ca. 0.8-mm diameter Pasteur pipette tip), with upward-directed aperture, is epoxy-seated into the hole and connected, in turn, to a controlling needle valve (an inexpensive aquarium air-controller is adequate) with epoxy. The valve and lateral entry tube permit continuous addition of distilled water from a reservoir (polyethylene syringe barrel) directly into the sample tube while filtration is in progress. The entire unit with sample tube, valve, and reservoir, is constructed on a small Plexiglas plate to allow easy vertical attachment to a laboratory stand.

Following loading of the filter and positioning, the rinse reservoir is filled (15 ml distilled water), and vacuum lines are connected to filter holder and fluid trap system. The sample aliquot is added to a level covering the rinse tube and usually to nearly fill the sample tube (ca. 10 ml), vacuum is applied, and filtration begins. As the sample fluid level falls to the top of the rinse tube aperture, the valve is opened and distilled water is gently layered at the fluid surface, displacing sample fluid at the same rate as it is drawn through the filter and removed from below. As the sample is pulled through the filter and spicules are deposited on its surface, rinsing occurs without disturbing spicule distribution. When the reservoir is exhausted, the rinse fluid is finally pulled through the filter with addition of an albumen adhesive solution to help maintain spicule distribution (discussed below). The filter is then dried and processed for LM or SEM as outlined for standard filter unit preparations.

For an adhesive, add 1 ml of a 0.2% aqueous solution of filtered Mayer's albumen affixative to the rinse reservoir while it still contains about 1 ml of rinse water. The final albumen solution is delivered to the sample tube by finger closure of the tube aperture, allowing the vacuum to pull the final contents of the reservoir through the delivery tube. Added in this way, the albumen creates no detectable difference in appearance of spicule detail in

either LM or SEM (Fig. 5), but greatly enhances adhesion of spicules to the filter surface after drying.

Performance of the Rinsing Filter Unit

The pattern of spicule distribution on the membrane preparations produced by the rinsing filter unit (RFU) was tested statistically. Separate spicule suspensions were prepared from two sample fragments of a specimen of *Niphates erecta*, taken from sites separated by no more than 5 cm. Spicule suspensions cleaned with nitric acid were divided repeatedly by plankton splitter (hexagonal, Plexiglas, two-chambered, knife-edge splitter tested to have less than 1% error in fluid division) to $1/128$ fractions. Fractions of the two initial samples were processed separately to assess different measurement methods.

From the first sample set, 10 replicate RFU preparations of the $1/128$ dilution fractions were analyzed for megasclere length, megasclere and microsclere densities, and differences in densities of both spicule types between central and edge regions of filters. Megasclere length was determined by direct ocular micrometer measurement (40× objective lens, 2.5-μm scale resolution) of every megasclere encountered by the micrometer scale in a continuous scan until a total of 51 spicules was measured from each filter preparation. Spicule densities were determined by counting all whole spicules and parts (at field edges or broken fragments) exceeding ½ spicule in contiguous microscope fields progressing in horizontal and vertical series across the entire filter area. Microscleres were enumerated under a 40× objective (0.49 mm field diameter) and megascleres under a 10× objective (1.24 mm field diameter). Differences in central and edge fields were assessed by partitioning the field counts of each filter to the two classes and comparing the set of 10 RFU's on a filter-by-filter basis.

The second sample was processed as six replicate RFU preparations of the $1/128$ dilution fractions. Megasclere length was determined by measurement of every complete spicule included in photographic fields taken at 1.5-mm intervals in a horizontal series across the filter surface (10× objective; mag. 29.2× at negative). Two methods were used for measurement: (1) projection of negative and ruler measurement (1,250×, 0.8-μm scale resolution), and direct ocular micrometer measurement of negative image under a dissecting microscope (2.3-μm scale resolution). Total number of spicules measured per replicate filter ranged from 102 to 160. For comparison of the RFU's with non-filter methods, traditional pour-preparations of spicules rinsed by centrifugation/resuspension (see Hartman, 1975) were made from ⅛ splitter fractions of each of the two samples. Measurements and enumerations were carried out as outlined for the respective RFU's.

Measurement of megasclere lengths showed no discernible differences (Mann-Whitney *U*-test for central tendency, Kolmogorov-Smirnov test for differences in frequency distribution of length, Kruskal-Wallis ANOVA test) between means of any preparation whether made by filter or slide-pour methods, or whether measured by ocular micrometer or photoprojection. The megascleres were

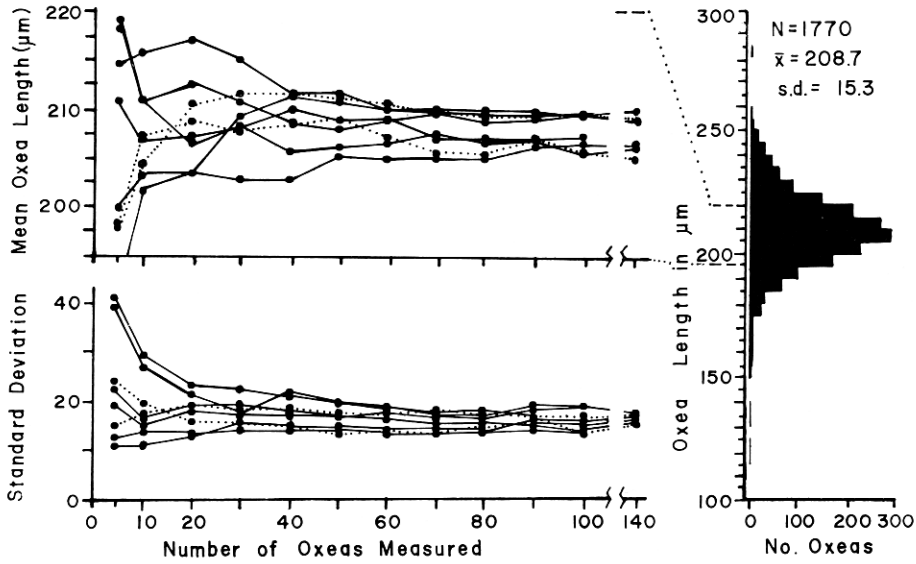

FIG. 6. Mean lengths of oxeas (megascleres) from *Niphates erecta*. Cumulative mean lengths and standard deviations with sample size of six RFU preparations (solid lines) and two traditional pour-slide preparations (dotted lines) from the same spicule suspension are shown on the left. The unimodal length-frequency distribution of all combined RFU (n = 16) and pour-slide preparations (n = 3) is given on the right.

normally distributed and consisted of a single size-class (Fig. 6). Uniformity of both megasclere and microsclere distribution across filter surfaces (center vs. edge clumping, or rarifaction) was tested using the Kruskal-Wallis ANOVA. No significant differences were detected for either spicule type. These results strongly support the use of the RFU system as a suitable format and procedure for detailed quantitative spicule analysis.

Although uniform spicule distribution has been obtained across individual filters, the logical extension of the method for measurement of spicule abundance, ratios of abundances of types, etc. requires that replicate preparations from a single suspension be statistically indistinguishable. In testing for between-replicate differences in spicule densities (abundances) and ratios of microscleres to megascleres, we were prepared to find differences between the two sample sets since regional differences in spicule densities within a specimen are expected. We found significant differences (in all tests) between filters of a replicate set. The differences were unrelated to splitting "geneology" of the replicates, and thus cannot be attributed to a systematic error in volume division by the splitter (as negated by prior testing). Apparently unequal distribution of spicules between replicates occurs in the splitting process, most probably in the single splitter chamber just before rotation and division of the sample. Such splitters, although widely used for particle division (Guelpen et al., 1982), have contradictory requirements for sample quiescence (allowing

even distribution of fluid) and sample turbulence (permitting even division of suspended particles; see Allen, 1981). Complete success cannot be realized until a method providing dependable subdivision of a heterogeneous spicule suspension is developed.

Discussion

Spicule characters derived from microscopic preparations (size, shape, abundance, abundance ratios of types, etc.) represent only a subset of phenotypic characters available for taxonomic studies of Porifera. Like all other characters, those of spicules exhibit considerable intraspecific variation. Part of this may be due to genetic differences between populations or individuals of a single population; part may be due to the influence of environmental variables (temperature, salinity, pH, etc.) on spicule formation in individuals and populations (Stone, 1970); and part may be due to differential expression of a single genotype (intrinsic variation) in an apparently uniform external environment over short ("blink") or long (developmental or aging) periods (Ayling, 1982; Jones, 1984). To demonstrate and quantify real variations in spicule characters, we must avoid artificially modifying their variance during sample processing (making spicule preparations) and analysis. This method of preparation, applicable to a wide spectrum of both siliceous and calcareous sponges, helps to avoid added variance. Our procedure cannot be employed for extremes of spicule form. Cases of exceedingly long, rare, and rapidly settling spicules (e.g., sterrasters) require special handling.

The number of spicules that should be measured to obtain an adequate estimate of a given spicule population will clearly depend upon the degree of variation within that population. A standard number of measurements is not adequate for all specimens or for all spicule categories. In this study of the megascleres of *Niphates erecta* (Fig. 6), 10 measurements clearly are too few and 100 measurements probably excessive for estimation of true mean and variation for taxonomic purposes. Fifty measurements seem to be an acceptable compromise for *Niphates erecta* in estimating mean to within 2% of the apparent true value (large sample mean) and estimating standard deviation within 30% of the apparent true value. Apparently, little advantage is gained in additional measurements unless specific tests between samples are required.

The major problem encountered in extending the RFU method of spicule preparation to areas of quantitative analysis is the subdivision of mixed spicule suspensions without fractionation between fluids and spicules. Currently, there seems no way to avoid subdivision of the suspension by "end-run" strategies of reducing tissue sample size or increasing filter size. Reducing sample size concomitantly increases the proportion of spicules damaged (broken) by handling, again introducing variance artificially, since damage is partly size-dependent. Employing a larger filter to distribute a greater volume of original suspension provides only a partial solution, since the largest filter convenient for use (i.e., 47-mm diameter) has only 16 times as much effective surface area as the small filters (13 mm) used in our procedure. With dilutions of $1/128$ required for most common demosponges, this would not improve the situation

significantly. Performance tests of methods that attain satisfactory subdivision of spicule suspensions, yet retain the attributes of convenience, minimal expense, and simplicity of equipment, are needed.

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