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SPECIAL SCIENTIFIC REPORT–FISHERIES No. 433

UNITED STATES DEPARTMENT OF THE INTERIOR
FISH AND WILDLIFE SERVICE

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United States Fish and Wildlife Service
Special Scientific Report--Fisheries No. 433

Washington, D. C.
June 1962

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by

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ABSTRACT

The use of the molecular filter in the preparation of marine phytoplankton for microscopic examination and enumeration is discussed, and certain modifications in the method of Goldberg, Baker, and Fox (1952) are suggested.

The modified technique involves the filtration of a fixed and preserved sea-water sample. The organisms, retained on the upper surface of the filter, are washed with successively diluted volumes of sea-water, dehydrated with ethanol, stained with alcoholic Fast Green, and finally rinsed with absolute ethanol. The filter disk is cleared with beechwood creosote, xylene, or anisole and mounted directly on a microscope slide with xylene or toluene balsam under a thin cover glass.

Many nannoplankton species may be readily identified and counted on the filter disk. The method is quantitatively comparable to the Utermöhl technique. The distribution of organisms on the filter surface is nonrandom, and the organisms on the entire filter should be counted.

INTRODUCTION

Goldberg, Baker, and Fox (1952) described a method of preparing marine phytoplankton for microscopic examination and enumeration directly on molecular filters. While similar membrane filters (wet-type) have been employed for such purposes by German limnologists (Utermöhl, 1931; Heinrich, 1934; and Schmitz, 1950), Goldberg, Baker, and Fox (1952) apparently were the first to employ similar techniques utilizing the new dry-type molecular filters.

On the basis of extensive experience with the method of Goldberg, Baker, and Fox (1952), we have adopted modifications in the technique which reduce the time required for making the individual preparations and provide preparations which are optically superior. The responses of a variety of organisms to the treatment have been examined, and the

[1]Support for this research was provided by the Marine Life Research Program of the California Cooperative Oceanic Fisheries Investigations and the Scripps Tuna Oceanographic Research program, financed by the U.S. Bureau of Commercial Fisheries under Contract No. 14-19-008-9354 with funds available under the Act of July 1, 1954 (68 Stat. 376), commonly known as the Saltonstall-Kennedy Act.

keeping properties of such preparations have been followed for periods exceeding 5 years. For certain types of study, this method seems to be superior to all others. Its advantages make it a useful supplement for nearly any examination and enumeration technique.

MATERIALS AND METHODS

We have employed 1-inch or 47-mm. diameter molecular filters (pore size 0.45μ) together with the glass and/or stainless steel filter holders. Additional top elements of different diameters have been fabricated for the base of the stock filter assemblies so that the filtering area of the molecular filter disk may be reduced.

The revised method of sample treatment differs from the original in the following details: (1) a basic formalin fixative is substituted for Lugol's iodine, (2) alcoholic rather than aqueous Fast Green stain is added at a later stage in the dehydration process, and (3) beechwood creosote or anisole have been substituted for cedar oil as clearing agents.

Sample Preparation

Prior to sample preparation, the organisms contained in 100 ml. of a freshly collected sea-water sample are killed and fixed by adding 3-5 ml. of 5 percent formalin made basic (pH 11-12) by the addition of sodium carbonate. If the sample does not contain organisms of interest bearing calcium carbonate skeletons (e.g. coccolithophorids, foraminifera), a more satisfactory fixative may be substituted. This fixative (10 g. iodine, 20 g. potassium iodide, 20 g. glacial acetic acid, and 200 g. water) suggested to me by Wilhelm Rodhe has been found to preserve the naked flagellates and dinoflagellates better than the basic formalin in that flagellae are not shed and cell deformation and shrinkage are less severe (Lund, Kipling, and LaCren, 1958). Enough of the mixture is added to the sample to give it a weak tea color; the samples are stored in the dark. In the concentrations normally employed, this solution makes the sea-water sample sufficiently acidic to dissolve skeletal parts composed of calcium carbonate.

Following fixation, the sample is vigorously agitated, and an aliquot is withdrawn with a wide-bore pipette and passed through the filter under suction. Before the organisms have dried, the filter is washed with successively more dilute sea-water (75, 50, 25, and 10 percent) and finally with distilled water made basic (pH ca. 8.0-8.5) by the addition of ammonium hydroxide. The material on the filter is next dehydrated by washing successively with 10, 30, 50, 75, and 95 percent aqueous ethanol. The amount of reagent used in each of the washings depends upon the filter diameter--generally 10-15 ml. is adequate.

The filter disk is completely covered with alcoholic Fast Green (0.1 percent in 95 percent ethanol) and allowed to stand for about 20 minutes. The Fast Green solution is then passed through the filter disk (with suction) and the disk rinsed with 10-15 ml. of absolute ethanol. Staining with Fast Green, although an optional step, makes it easier to locate organisms on the filter disk and to distinguish small detrital particles from small flagellates.

The filter disk is carefully removed from the filter assembly, its edges are trimmed, and it is placed, filtering surface up, on a few drops of clearing agent on a glass microscope slide. A variety of clearing agents have been investigated. Beechwood creosote clears well, and samples using this agent are still in good condition after 5 to 6 years. Anisole (methoxybenzene) is a better clearing agent; its keeping properties have been followed for 18 months with no signs of deterioration. Although toluene and xylene also clear the filter disk well, there is a tendency for the filter disk to cloud with age unless the filter is completely free of water prior to mounting in balsam.

After the filter disk is cleared (usually less than 10 minutes) the excess clearing agent is removed from the bottom of the filter disk by dragging the filter across a clean microscope slide. The filter is transferred with forceps onto a few drops of toluene or xylene balsam on a clean microscope slide. A few additional drops of balsam are added to the upper

surface of the disk, and a clean, thin (No. 0 or No. 1) cover glass is carefully lowered on the preparation. The slide is dried at room temperature or in an oven at about 40° C.

Identification of Organisms

The method is well suited for field operations where a vacuum system is available and has been used extensively on shipboard for both water samples and aliquots of samples collected with fine nylon nets (mesh size 30 x 30 μ).

Various organisms and groups of organisms respond differently to the treatment. Summarized below are the results obtained with a variety of planktonic plant groups.

Bacillariophyceae.--The majority of the pelagic diatom species respond well and suffer few if any morphological changes which complicate identification. Frustule deformation has been observed in only a few thin-walled *Chaetoceros* species. Positive identification in this instance demands other techniques.

It is not always possible to resolve the fine detail necessary for the identification of very small centrate and pennate diatoms. A mounting medium with a different refractive index may reduce these difficulties. If resolution is still impossible, the use of the electron microscope is indicated.

Dinophyceae.--Experience with thecate members of this class has been limited. It is apparent, however, that certain thecate genera respond well to the treatment and others do not. Species belonging to the genera *Exuviaella*, *Phalachroma*, *Prorocentrum*, *Dinophysis*, *Ornithocercus*, *Histioneis*, *Goniaulax*, *Heterodinium*, *Podalampas*, and *Ceratium* are well preserved, usually with visible plate sutures. In species of the genera *Peridinium* and *Oxytoxum* the plate structure is not consistently visible, and occasionally pronounced deformation of the theca occurs. Identification of any thecate dinoflagellate may be difficult once the mounting medium has hardened. While the mounting medium is still viscous, it is possible with the aid of needles to exert pressure on the cover glass and orient specimens in different ways, greatly facilitating identification.

The majority of naked dinoflagellates are difficult to identify after fixation with basic formalin. The iodine fixative mentioned earlier yields much better fixation images. Regardless of the fixative employed, however, this technique seems to distort and damage many naked dinoflagellate species and therefore cannot be recommended for samples in which this group is an important component and for which positive identification must be made. In such cases, examination of living material is necessary.

Chrysophyceae.--The coccolithophorids are well preserved, and no evidence of coccolith deterioration has been evident in 3-year-old preparations. Identification of these organisms is seldom easy, but once a species has become familiar, it can usually be recognized on the filter disk.

Silicoflagellates.--Representatives of this group have been readily identified on the filter disk.

Chlorophyceae and Myxophyceae.--On the basis of limited experience, the technique appears satisfactory for these organisms.

Tintinnidae.--Nonarenacious genera have been readily identified. Too few arenacious genera have been encountered to permit evaluation of the method.

Mu flagellates.--Generally members of this large and heterogeneous group of organisms are not well preserved with basic formalin. The iodine fixative (see section on Sample Preparation) is certainly superior. The members of this group, however, do not seem to respond too well to the multiple filtrations involved. The more delicate species seem to be damaged, presumably by the mechanical stresses. Certain robust species (e.g., *Chilomonas marina*) have been obtained in good condition. Ballantine (1953) says that they can usually be easily identified on the filter disk.

Enumeration of Organisms

Although McNabb (1960), using a similar technique, concluded that organisms are randomly distributed on the filter, microscopic examination of filter surfaces shows clumping of organisms just inside the edge of the filter and in the central region. Clumping was also

noted in Ballantine's (1953, p. 135) preparations which were made according to Goldberg, Baker, and Fox (1952).

Using a typical filter disk, a count was made of the numbers of *Gymnodinium* sp. on each of 537 nonoverlapping, equal area ocular fields. The observed counts (table 1) were tested for conformity to a Poisson distribution, and the hypothesis of a random distribution was strongly discredited ($\chi^2_{536} = 1070$; $p \ll 0.001$). These results are in agreement with those of Ballantine (1953, Table III).

TABLE 1.--Number of *Gymnodinium* sp. per ocular field on entire Millipore filter surface and calculated index of dispersion

Number in field	Frequency	
0	26	
1	11	
2	20	
3	42	
4	47	
5	49	
6	56	
7	62	$\bar{x} = 6.898$
8	55	
9	48	$\chi^2_{536} = 1069.7$
10	32	
11	37	
12	18	
13	12	
14	4	
15	7	
16	3	
17	2	
18	4	
19	2	
Sum	537	

If a complete count is to be undertaken, it is advisable to make two preparations of each sample. One disk should be small (ca. 0.5 cm. in diameter), the other larger (i.e., 1-2 cm. in diameter). The small and generally more abundant organisms should be counted on the small disk, and the larger organisms on the larger disk. The number of organisms in a water sample naturally varies with location and time of year and other factors, so no firm rules can be given as to the amount of water to be filtered for each of the preparations. In tropical offshore waters, 3-5 ml. and 50-100 ml. are appropriate volumes for the small and large disks, respectively. In richer waters it becomes necessary to reduce the volumes filtered by a factor of 2 to 5.

Comparison of the Molecular Filter and Utermöhl Methods

Since the inverted microscope method of Utermöhl (1931) is virtually the standard method of phytoplankton enumeration, several comparisons have been made between it and the molecular filter method. The results of one such analysis are shown in table 2. No differences between counts obtained except in the case of the naked or mu flagellates (see section on Mu flagellates) have been found; the two methods seem to be comparable.

Molecular filtration of phytoplankton has a number of desirable features which compensate for some of the difficulties previously observed. These are (1) the preparation is long-lasting and therefore is useful for teaching purposes, reference, and exchange; (2) the preparations require little storage space; (3) little expensive specialized equipment is required; and (4) the method can be used on shipboard.

TABLE 2.--Comparison of Utermöhl and molecular filter methods

Species	Utermöhl technique			Molecular filter technique
	2 ml. examined			
	Number in 2 ml.	Expectation limits for 6 ml. for 95 % confidence[1]		6 ml. examined
		Min.	Max.	
Asteromphalus heptactis	2	0	22	4
Coscinodiscus spp.....	4	3	31	16
Fragilaria sp. (no. chains).....	258	660	870	667
Nitzschia seriata (no. chains).....	105	246	372	293
Thalassiothrix longissima	28	54	126	83
Dinophysis sp.....	0	0	11	1
Exuviaella baltica	1	0	17	1
Peridinium spp.	4	3	31	4
Distephanus speculum	11	15	58	51

[1] Values from graphs in Holmes and Widrig (1956).

SUMMARY

Two modifications seem desirable in the molecular filter technique of Goldberg, Baker, and Fox (1952) for preparing marine nannoplankton for examination and enumeration. These modifications involve the substitution of alcoholic Fast Green for aqueous Fast Green and of beechwood creosote or anisole for cedar oil in the clearing process.

A variety of phytoplankton may be readily identified and counted on a filter disk, although difficulties in identification have been encountered with the mu flagellates and a few genera and species belonging to other plant groups.

Because the organisms are not distributed randomly on the filter disk, all the organisms should be counted. When the entire disk is counted, the results are quantitatively comparable with those obtained with the Utermöhl (1931) technique.

The technique is rapid and apparently produces a permanent preparation of the organisms; no signs of deterioration have been observed in preparations 5 years old.

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