DENSITY GRADIENT ANALYSIS OF SUSPENDED MATTER

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The research leading to this report was supported in part by the United States Department of the Interior, under the Annual Cooperative Program of Public Law 95-467, Project No. A-085-CAL, and by the University of California Water Resources Center, Project UCAL-WRC-W-613. Contents of this publication do not necessarily reflect the views and policies of the Office of Water Policy, U. S. Department of the Interior, nor does mention of trade names or commercial products constitute their endorsement or recommendation for use by the U.S. Government.

TECHNICAL COMPLETION REPORT

September 1983

ABSTRACT

Development of a simple and inexpensive density gradient centrifugation method, utilizing silica sols, for separating and isolating various fractions of suspended matter and bottom sediments was begun. There is presently no effective means for fractionating suspended material in natural waters into its natural components. The technique will be especially useful and researchers in estuarine productivity and toxicology. The influence of preservatives and density shift reagents on the separating and resolving powers of the centrifugation method has been analyzed. In addition the interaction between the method and the density shift reagents and preservatives on analysis of chlorophyll a, proteins, and carbohydrates was examined. Preliminary results indicate that the development of suitable methodology will require considerable additional effort. The technique has demonstrated that it is an important means of separating certain algal associations.

INTRODUCTION

Lammers (1964) categorized the components of natural waters as 1) non-colloidal, particulate organic matter; 2) non-colloidal, particulate inorganic matter; 3) colloids; 4) solutes; and 5) the water itself. Density is a parameter which allows one to distinguish between organic and inorganic material in the presence of organic densities between 1.0 and 1.65 g/cm^3 and inorganic densities of about 2.6 g/cm^3 (Lammers, 1964). If a mixture of organic and inorganic particles is suspended in a liquid which has a density greater than that of the organic fraction and less than that of the inorganic fraction, separation of the two will occur. The speed of this separation can be increased by centrifugation (Lammers, 1964), making density gradient centrifugation a powerful technique for the separation of particles of diverse size and structure.

Silica sols were first introduced as a density gradient material by Mateyko and Kopac (1963) to provide isopycnotic cushioning for cells undergoing centrifugation. Pertoft and Laurent (1969) demonstrated the general feasibility of silica sols in density centrifugation for the separation of a variety of membrane bound particles.

Bowen et. al. (1972) introduced density gradient centrifugation with silica sols to limnilogical and marine research with their work on the separation of zooplankton from fish eggs and fish larvae utilizing Ludox-AM. Price et. al. (1974) determined the specific weights of some planktonic algae using Ludox-AM in a zonal rotor. Price, et. al. (1977) demonstrated that automatic sorting of some groups of zooplankton is possible with density gradient centrifugation utilizing silica sols.

Price et. al. (1978) used a buffered mixture of the silica sol, Percoll; sorbital; and seawater to isolate marine dinoflagellates by density centrifugation. Jonge (1979) utilized Ludox-TM to separate benthic diatoms from inorganic sediment by density gradient centrifugation. Schwinghamer (1981) separated meiobenthic organisms from sediment materials by density gradient centrifugation utilizing a Percoll-sorbital mixture.

A major objective of our work was to develop a simple and inexpensive (in terms of equipment and time) density gradient centrifugation method for separating and isolating the various fractions of suspended solids in the water column. One aspect was to determine the degree to which we could separate the detrital fraction from the nondetrital fraction of particulate organic matter. We examined the influence of density shift additives and the impact of preservatives on the separating and resolving power of density centrifugation. We also evaluated the influence of additives coupled with density centrifugation on the analyses for particulate protein and carbohydrates.

METHODS

Separation

Discrete density gradients were constructed using Ludox-TM colloidal silica sol produced by E. I. DuPont De Nemours and Co., Inc. Ludox-TM has the highest density of the silica colloids available and offers the greatest range for a density gradient. Our initial work was with Ludox-TM for this reason. We did not sample from any saline waters, eliminating the concern over the possible gelling of the silica sol. To maximize the resolution of our density gradients we utilized

discontinuous gradients as suggested by Jonge (1979). The density steps, expressed in percent concentration of Ludox on a v/v basis with distilled water, were layered into 50 ml polycarbonate centrifuge tubes. A difference of 5% v/v between layers was the lower limit of overlay concentrations that would maintain their integrity. The gradient was established by layering aliquots of the appropriate Ludox concentration in increments of 5% or greater using Pasteur-type pipets. Aliquots of the collected sample were then placed as the final layer using the same method.

Only three to four concentrations of the Ludox-TM could be layered in the centrifuge tubes. To cover the whole range of concentrations required a number of centrifuge tubes with overlapping concentrations, e.g.

Volume	Conc.	Conc.	Conc.	Conc.	Conc.
20 ml	sample	sample	sample	sample	sample
10 ml	10%	30%	50%	70%	90%
10 ml	20%	40%	60%	80%	100%
10 ml	30%	50%	70%	90%	100%

The density gradient for the range shown above would be expressed as sample/10/20/30/40/50/60/70/80/90/100. A specific density might be expressed as 40% Ludox-TM while the interface between 40% and 50% Ludox-TM would be expressed as 40/50.

The tubes were spun in a Clay Adams Dynac II tabletop swinging bucket centrifuge. Centrifugation was carried out at 2400 rpm for 30 minutes to one hour, depending on the objective. Thirty second to one minute periods of acceleration and deceleration between 0 and 500 rpm were used to avoid perturbation.

Chlorophyll a Analysis

Chlorophyll a and phaeopigment concentrations were determined using a slight modification of the method described by Strickland and Parsons (1977). The samples were removed layer by layer, in 5 ml increments, using a 12 ml syringe and a No. 16, 1.5 inch (3.81 cm) blunt needle. The samples were then filtered onto 2.4 cm diameter Whatman GF/C glass fiber filters. The filters were frozen until extraction was performed (approximately 3 weeks). The extraction was accomplished using 90% acetone and was analyzed on a GK Turner Fluorometer, Model No. 111.

Organic Carbon

A modification of Strickland and Parsons (1977) wet oxidation with dichromate procedure was used. Whatman GF/C glass fiber filters with a diameter of 2.4 cm, precombusted at 500 C for 4 hours, were used. The samples were removed with syringes as discussed above for the chlorophylls. The analysis was done using a Baush and Lomb Spectronic 21 and cuvettes with 2.5 cm path lengths. Carbon concentrations were established using a standard curve.

Density Shift Reagents and Preservatives

Four of the density shift reagents studied were polyvinyl alcohol, produced by Sigma Chemical Company; trimetaphosphate, produced by Sigma Chemical Company; Calgon dishwashing detergent, a product of Beecham, Inc.; and polyethylene glycol, distributed by Fischer Scientific. Stock solutions of 3 g/100 ml H_2O were prepared for the first three substances. The appropriate reagent (0.105 ml) was added to each 7 ml aliquot of the silica solutions prior to layering the density gradient.

In other experiments, Tween 20, produced by Sigma Chemical Company, was added to the sample water prior to its placement on the density gradient. To evaluate the effects of preservatives, we added 3 ml of formaldehyde, 0.5 ml of lugols, 2 ml of butanol, and 2 ml of 95% ethanol to the sample aliquots prior to concentrating them (2x) and layering them on the density gradient.

Carbohydrate and Protein Analyses

A modification of the Strickland and Parsons (1968) phenosulfuric method was used to determine carbohydrate concentrations. Proteins were analyzed using the Bonsadoun and Weinstein (1976) modification of the Lowry method.

Samples

Water samples were collected from Mrak Pond on the University of California, Davis Campus; Sherman Lake, at the western-most edge of the Sacramento-San Joaquin Delta; and from lab aquariums containing Daphnia and several snail species. All were concentrated by filtering through a 20 µm Nytex mesh.

RESULTS

We utilized cultures of the alga Ankistrodesmus for our initial evaluations and found that in the exponential growth stage the alga forms a very tight band at the 15/20% interface. A senescent culture of the alga formed a band at 25% Ludox-TM with a few organisms floating at the surface. The addition of a drop of Tween 20 added to the surface caused the floating cells to rapidly sink into the band of cells below.

The Sherman Lake samples (Sacramento-San Joaquin Delta) were centrifuged across gradients with a 10% increment. Most of the material was between the 0 and 70% layers so chlorophyll data was collected at these and the intermediate concentrations and interfaces (Fig. 1). A subsample from each interface was also taken and the phytoplankton enumerated (Fig. 2 and 3, Table 1). When the suspended material in the sample was extremely concentrated, it resulted in a ropy spindle extending the length of the gradient from 0 to 50% Ludox-TM and flaring into bands at the interfaces. A sample of organic bottom sediments from Sherman Lake was found to spread across the gradient from the 35% level through the 100% level and into a pellet at the base of the centrifuge tube.

Samples of Mrak Pond water, collected in July, August, and September, were centrifuged across discontinuous gradients of 5% increments from 0 to 100% Ludox-TM. The suspended solids formed a rope running from the sample/5% interface into a band at the 15% level with a pellet at the base of the tube. Better resolution for the Mrak Pond water was found with a gradient of sample/30/70/100% Ludox-TM. A gradient of sample/15/30/45/60/70/90/100% Ludox-TM was established and phytoplankton collected from the interfaces and enumerated (Fig. 4, 5, and 5, 6; Tables 2, 3, 4).

A sample of aquarium bottom sediments was collected from the aquarium containing a snail and Daphnia culture and centrifuged over a gradient of sample/15/30/45/60/80/100% Ludox-TM. Chlorophyll a and organic carbon data was collected and included in Figures 7 and 8.

Samples of Mrak Pond water were used to examine the impact that Tween 20, polyethylene glycol (PEG), polyvinyl alcohol (PVA), trimetaphosphate (TMP), and Calgon would have on the distribution of chlorophyll *a* (Fig. 9 and 10). Samples of Mrak Pond water were also used to determine the influence that PEG, PVA, TMP, Calgon, EtOH, butanol, formaldehyde, and lugols solution had on the distribution of particulate protein and carbohydrates (Fig. 11-14).

Table 1. Sherman Lake: Distribution of diatom genera by percent across a density gradient Ludox-TM

		Location on Density Gradient									
Diatom	Sample	0	10	10/20	20/30	30/40	40/50	50/60	60/70	70/80	80/90	90
Melosira granulata	95		96.8	93.1	96.5	96.9	99.2	100	100	100	100	76.5
Melosira varians	<1				0.9							
Cyclotella	4			6.9	0.9	0.8						1.2
Thallossiasira decipiens	1		3.2			2.3	0.8					22.4
Centric unid	<1				1.8							

Table 2. Mrak Pond: Distribution of diatoms by percent across a density gradient of Ludox-TM

		Location on Density Gradient					
Diatoms	Sample	0/15	15/30	30/45	45/60	60/70	70/90
Melosira granulata	51	0	40	19	65	95	89
Nitzchia fragilaria	41	100	60	71	26	5	3
Penate unidentified	8	0	0	10	8	0	8

Table 3. Mrak Pond: Distribution of green algae by percent across a density gradient of Ludox-TM

Green algae	Sample	Location on Density Gradient 0/15	15/30	30/45	45/60	60/70	70/90
Micractinium (colonies)	41	69	70	10	9	25	44
Dictyosphaerium (colonies)	16	21	18	0	82	25	41
UGA (unidentified green algae)	39		3	84	9	51	3
Scenidesmus	1	4	0	0	0	0	6
Ankistrodesmus	1	6	9	5	0	0	6
Selenastrium	2	0	0	0	0	0	0
Actinastrium	0	0.4	0	0	0	0	0

Table 3. Mrak Pond: Distribution of blue-green algae by percent across a density gradient of Ludox-TM

Blue-green algae	Sample	Location on Density Gradient 0/15	15/30	30/45	45/60	60/70	70/90
Lyngbya Oscillitoria	29	30	0	0	0	0	0
Microcystis (colonies)	71	52	100	0	100	0	100
Beaded filaments	0	19	0	0	0	0	0

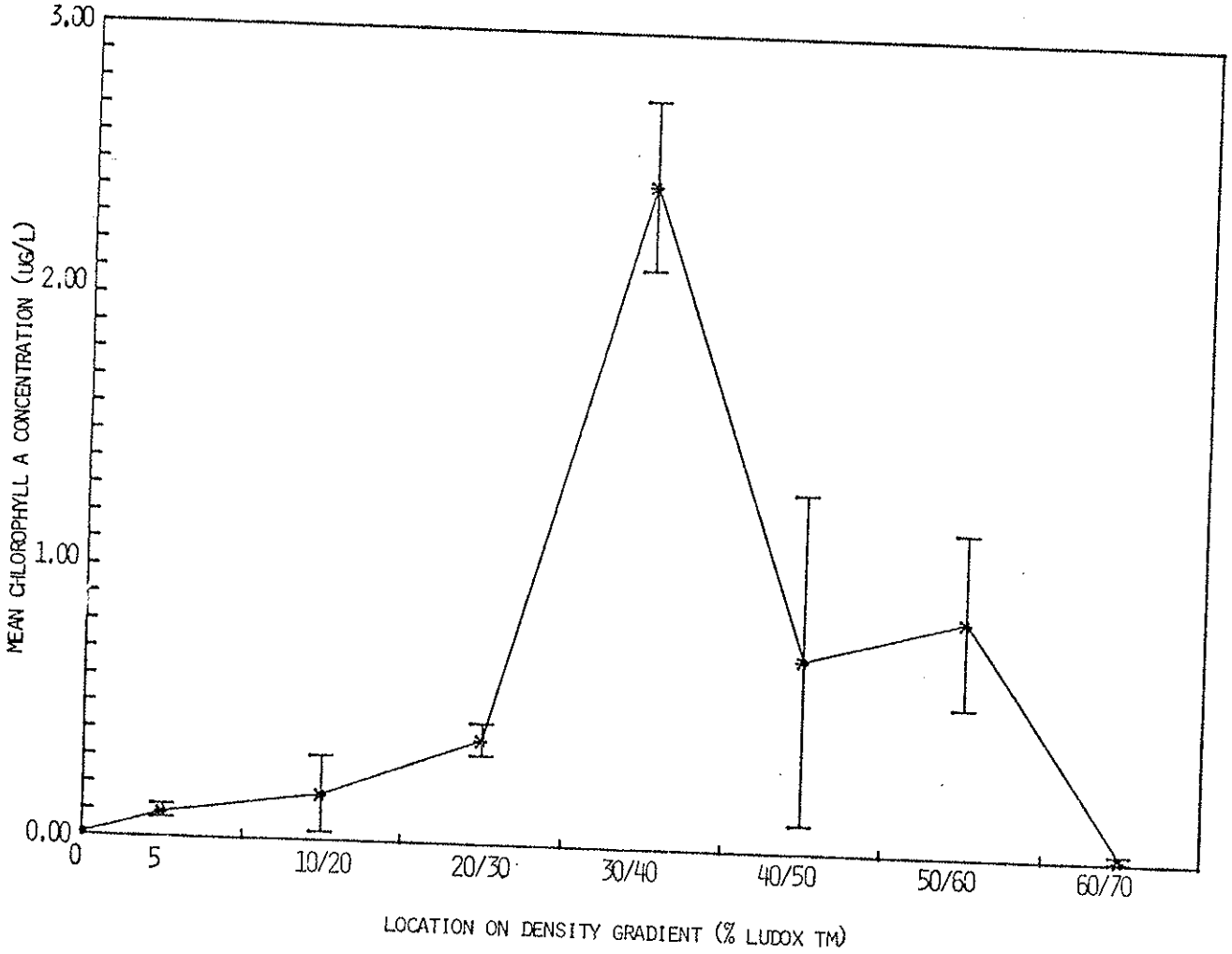

Figure 1. Sherman Lake site: Distribution of chlorophyll a across a density gradient of Ludox TM.

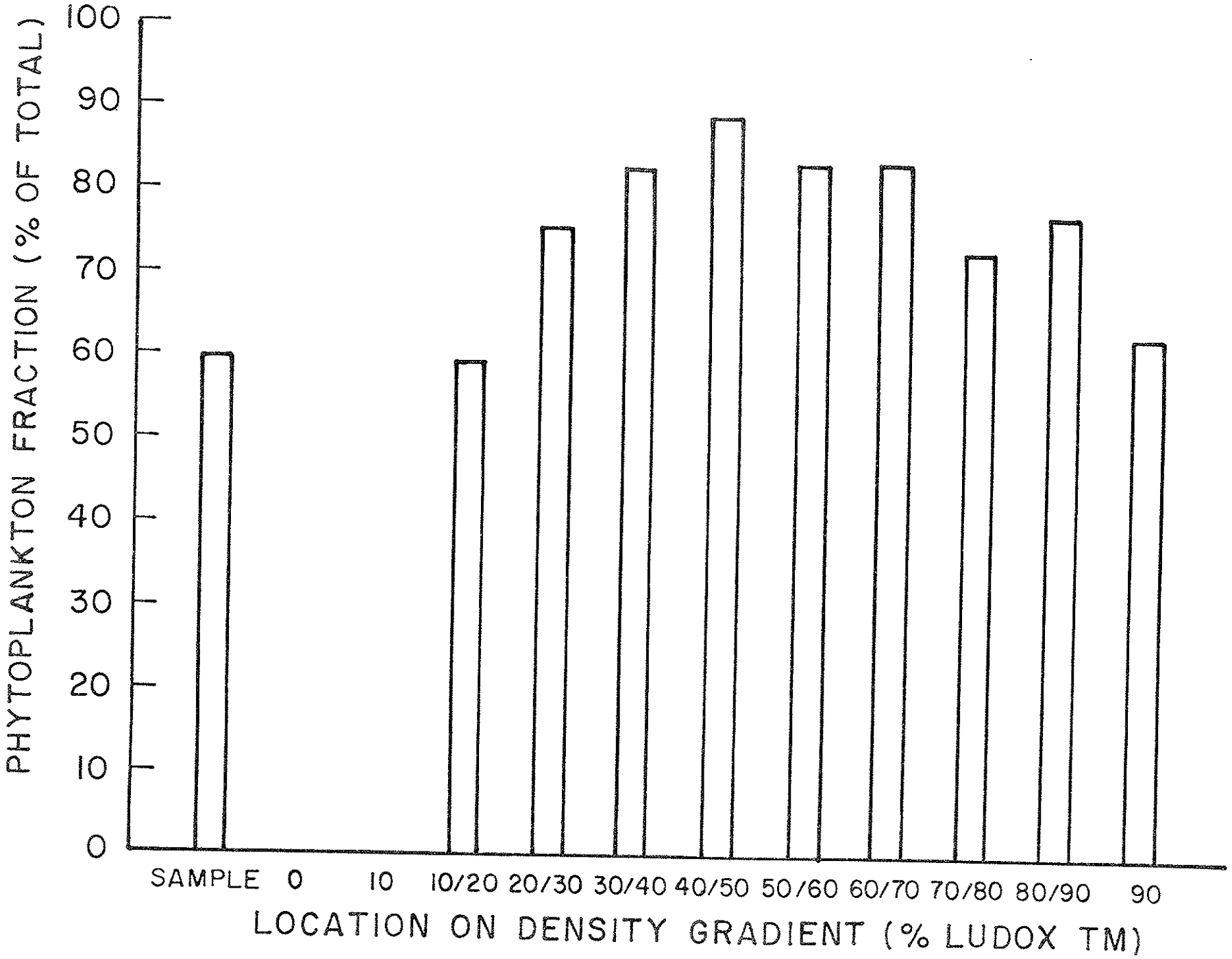

Figure 2. Sherman Lake site: Phytoplankton fraction of particulate matter counted in each layer of a density gradient of Ludox TM

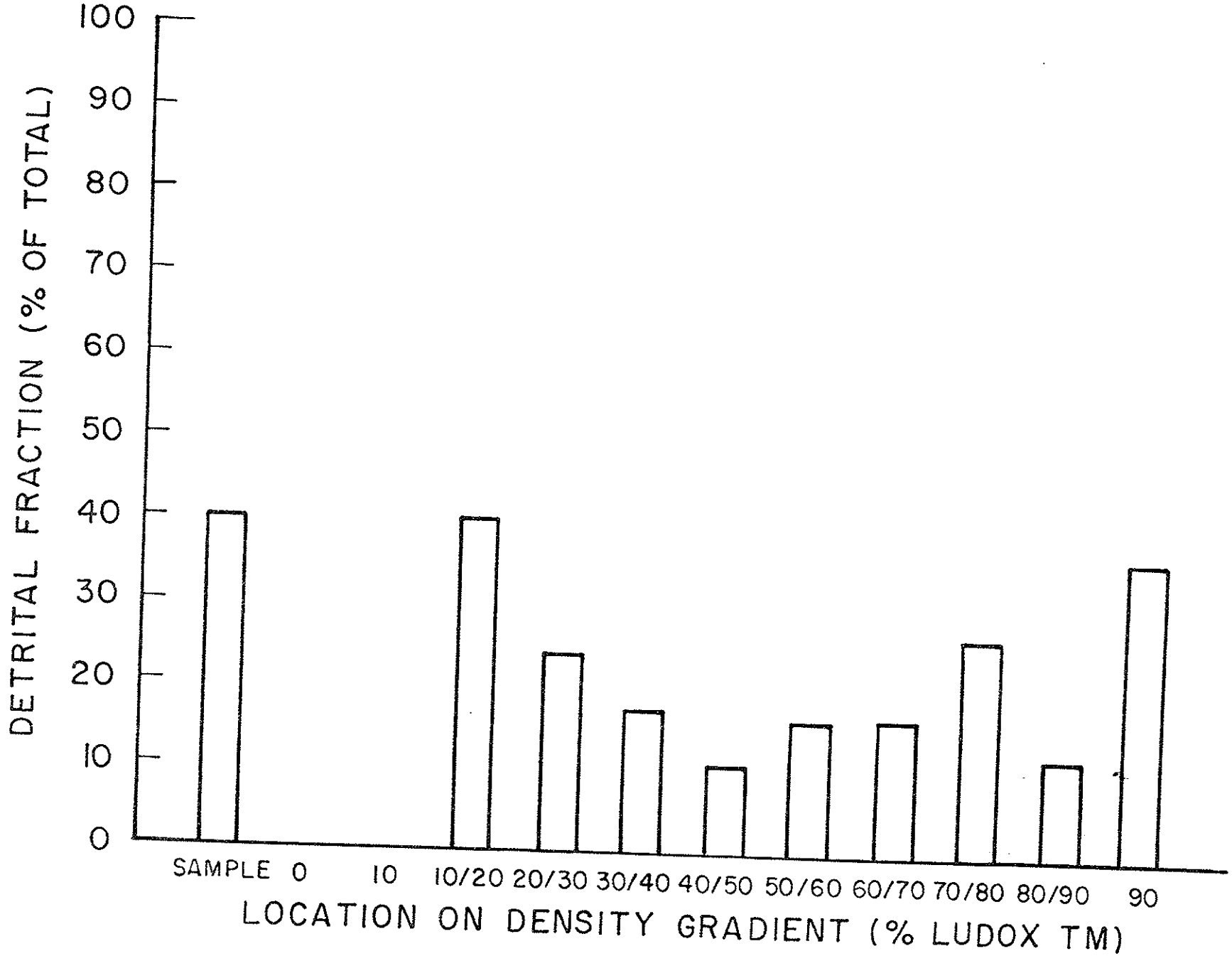

Figure 3. Sherman Lake site: Detrital fraction of total particulate organic natter counted in each layer across a density gradient of Ludox TM.

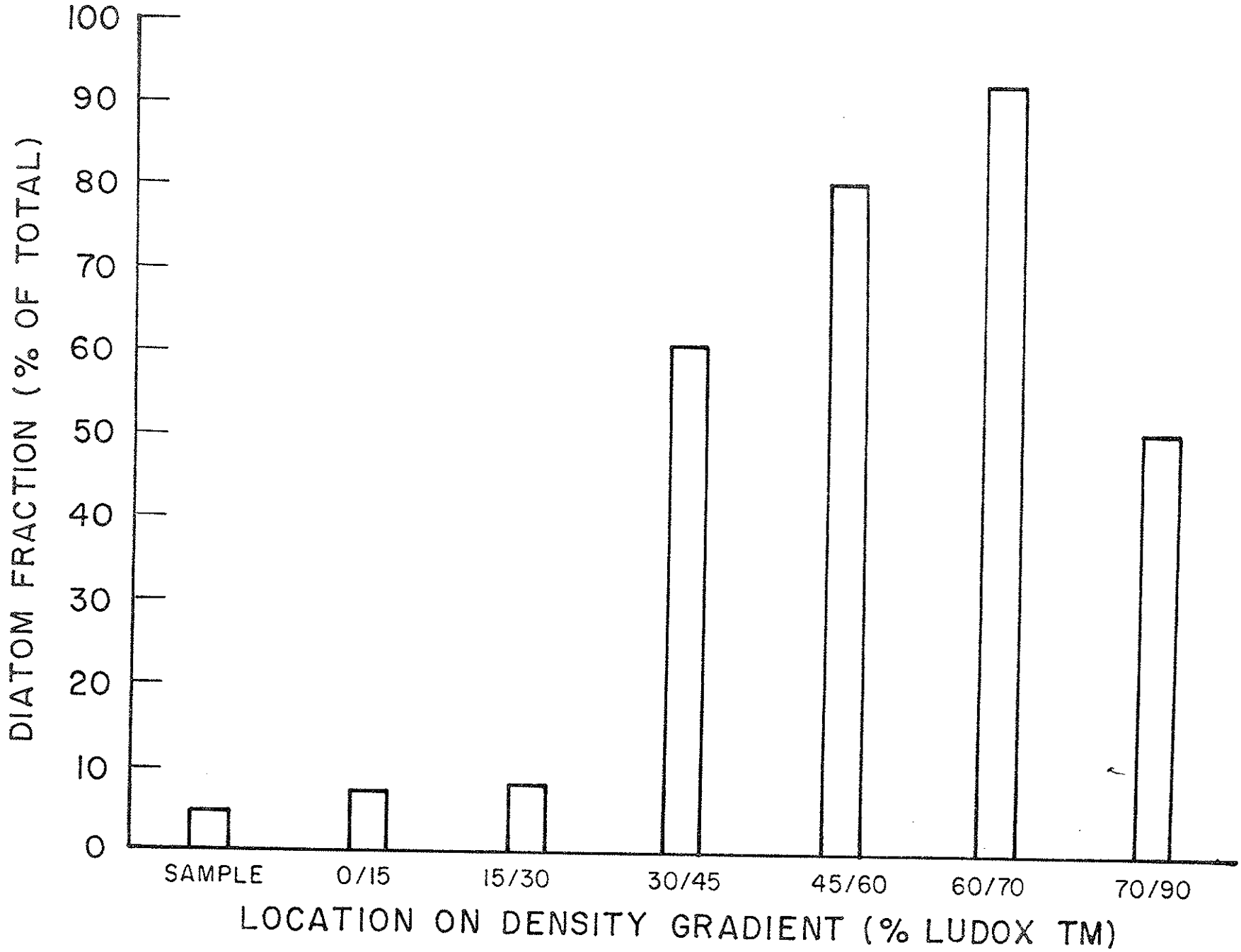

Figure 4. Mrak Pond: Diatom fraction of phytoplankton counted in each layer of a density gradient of Ludox TM.

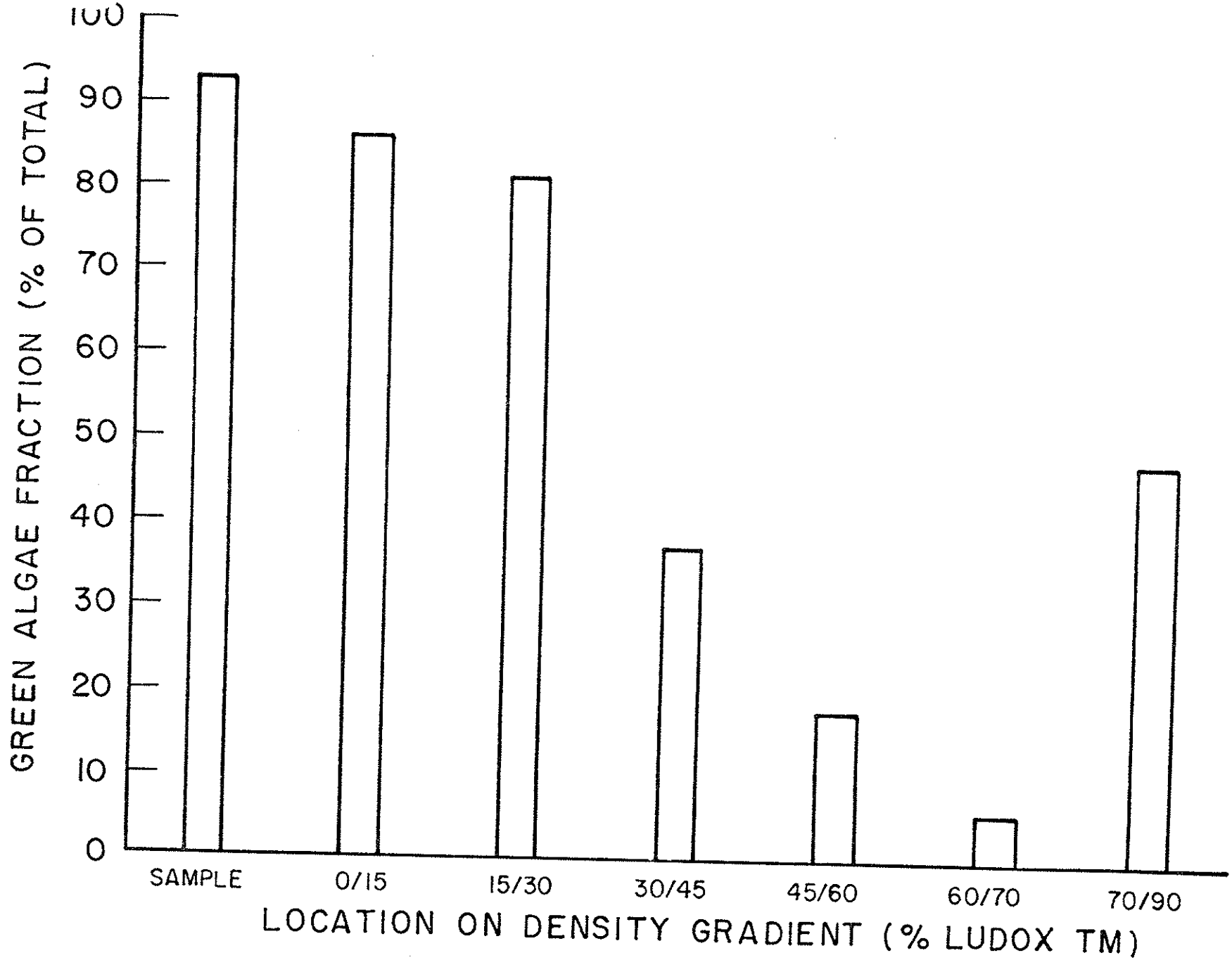

Figure 5. Mrak Pond: Green algae fraction of phytoplankton counted in each layer of a density gradient of Ludox TM.

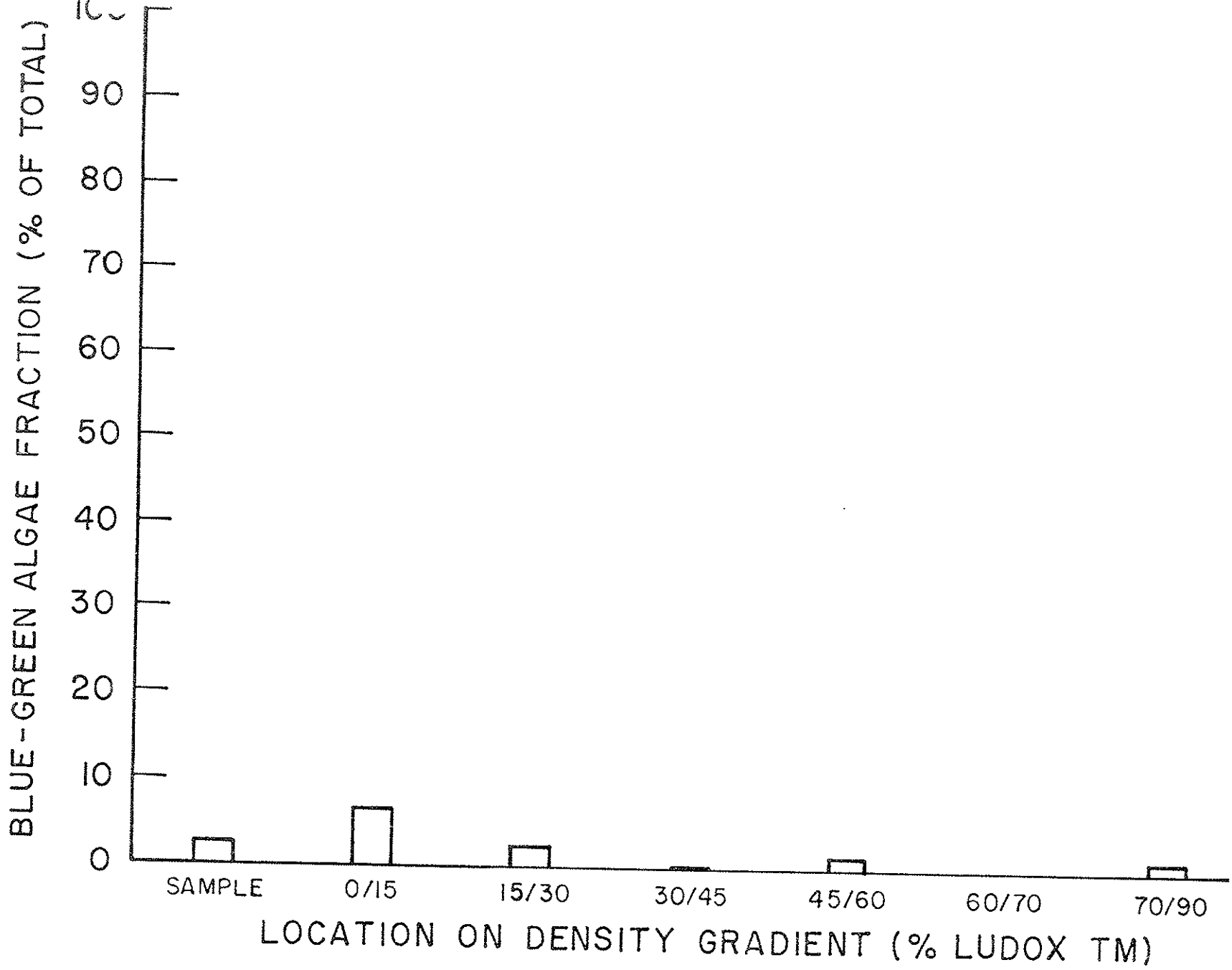

Figure 6. Mrak Pond: Blue-green algae fraction of phytoplankton enumerated in each layer of a density gradient of Ludox TM.

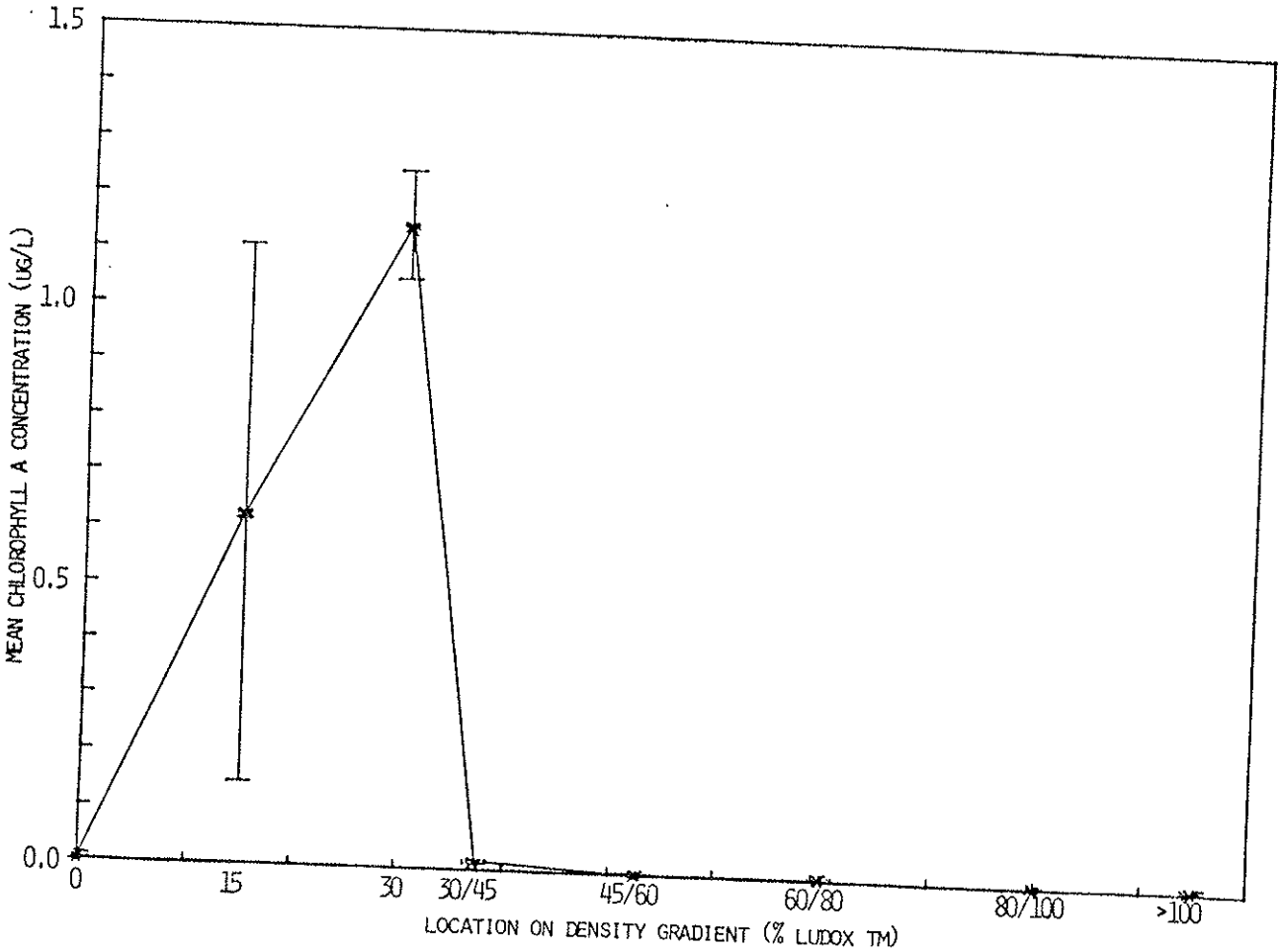

Figure 7. Distribution of chlorophyll a from the sediments of a Daphnia/ snail aquarium culture as measured across a density gradient of Ludox TM.

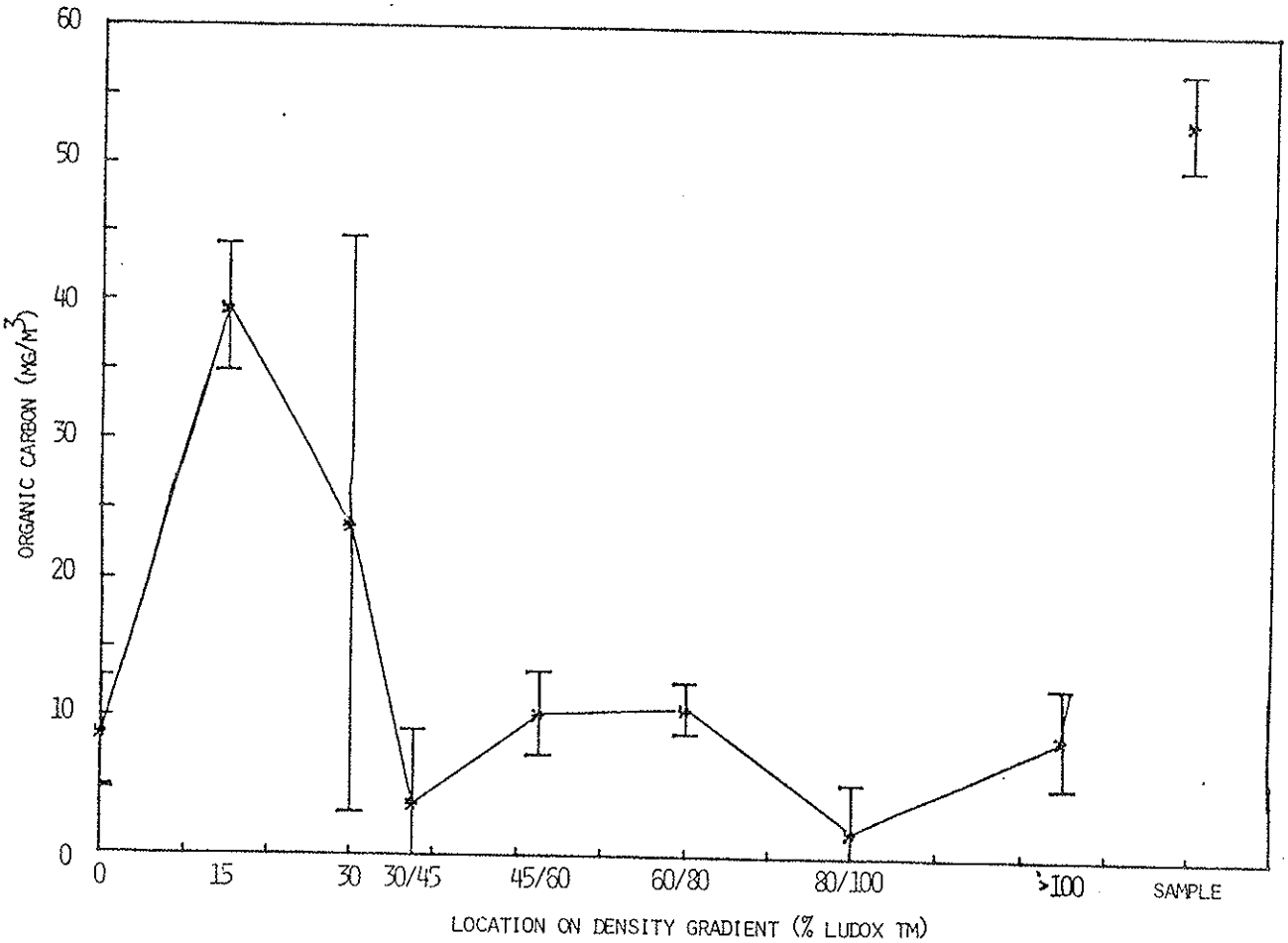

Figure 8. Distribution of organic carbon in the sediments of a Daphnia and snail aquarium culture measured across a density gradient of Ludox TM.

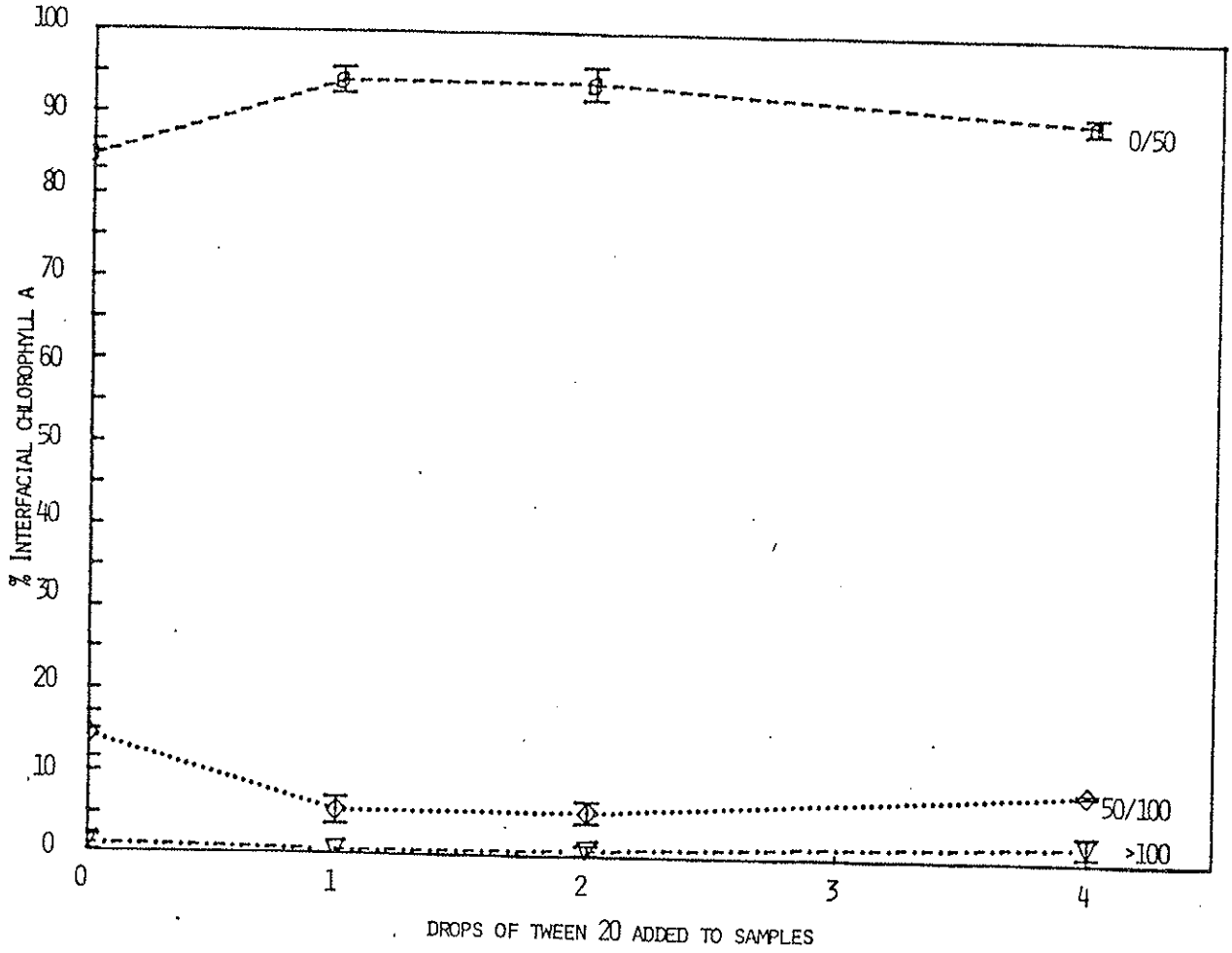

Figure 9 Mrak Pond: Effect of Tween 20 on the % interfacial chlorophyll a found across a sample/50/100 % Ludox TM density gradient.

Figure 10. Mrak Pond: Effect of density shift reagents on the distribution of interfacial chlorophyll a across a discrete density gradient of Ludox TM

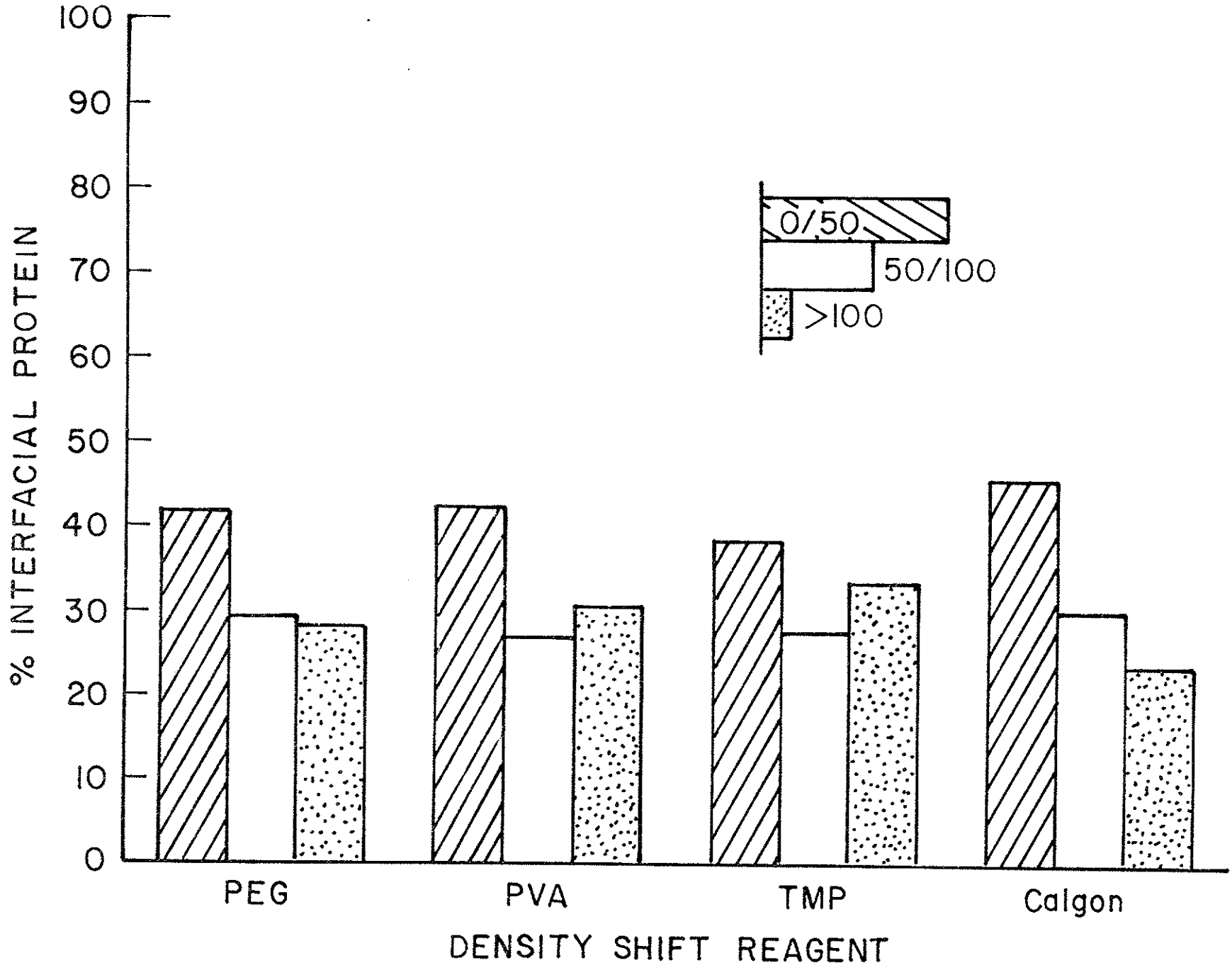

Figure 11. Mrak Pond: Effect of density shift reagents on the distribution of interfacial particulate proteins across a discrete density gradient of Ludox TM.

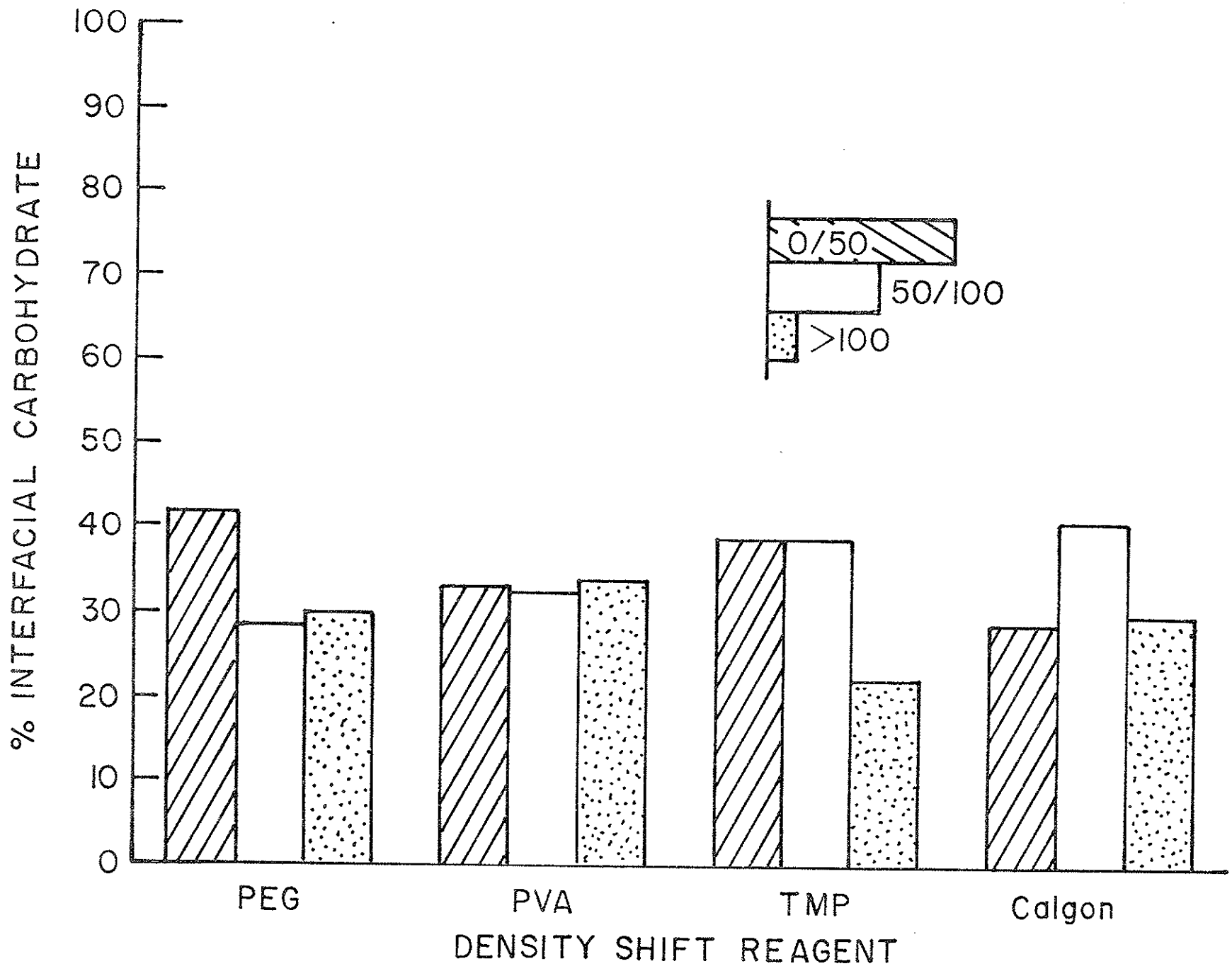

Figure 12. Mrak Pond: Effect of density shift reagents on the distribution of interfacial particulate carbohydrates across a discrete density gradient of Ludox TM.

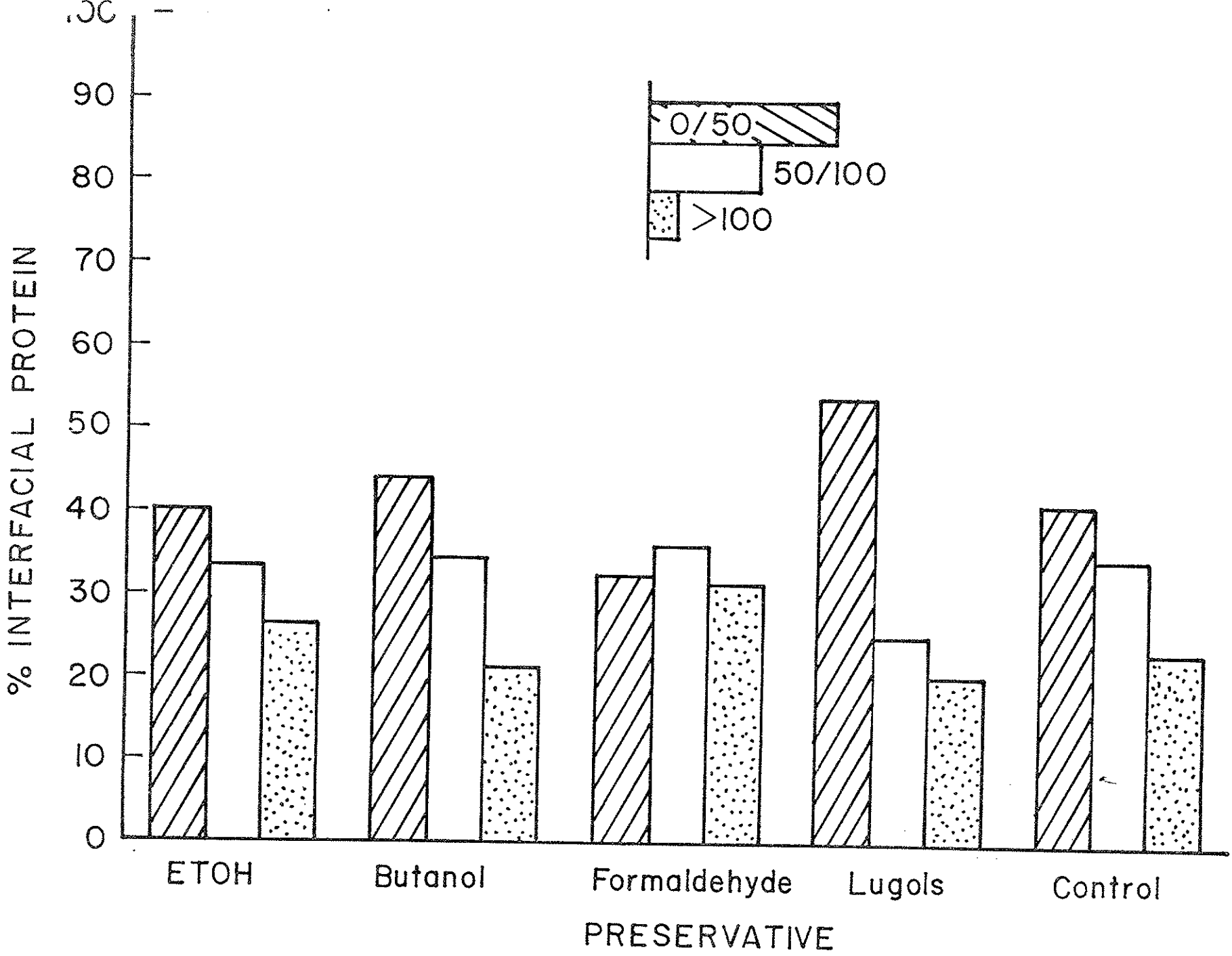

Figure 13. Mrak Pond: Effect of preservatives on the distribution of interfacial particulate proteins across a discrete density gradient of Ludox TM.

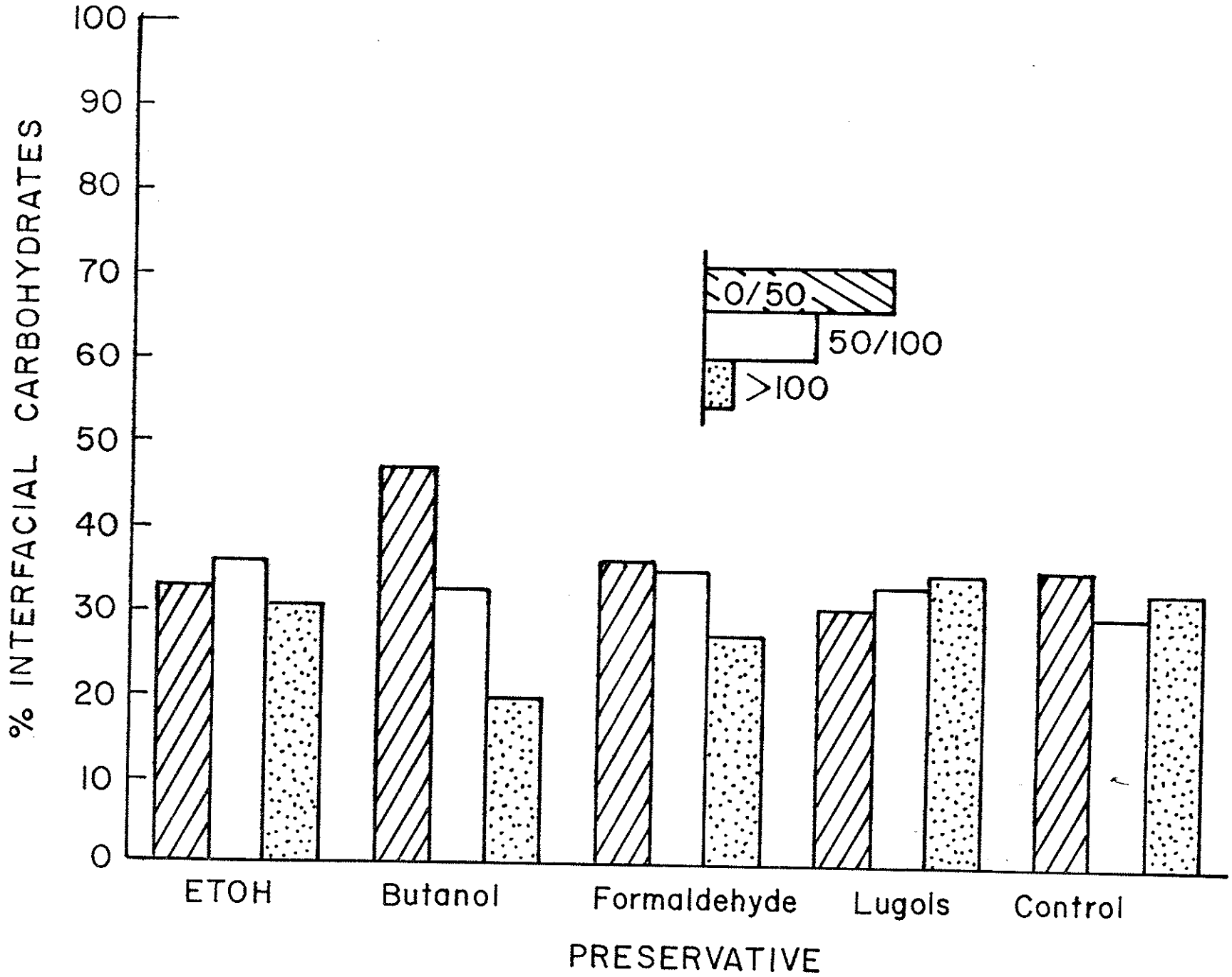

Figure 14. Mrak Pond: Effects of preservatives on the distribution of interfacial particulate carbohydrates across a discrete density gradient.

DISCUSSION

The data regarding the diatoms obtained from the delta (Table 1) indicates inconsistent resolution and separation by the density centrifugation method. Some of the diatoms could be concentrated, e.g., the centric unidentifiable forms and Melosira granulata, while others couldn't, e.g., Thallossiaossira dicipiens and Melosira granulata. None could be effectively isolated from each of the others. The Mrak Pond data (Tables 2, 3, and 4) is even less conclusive as to the resolving capabilities of density centrifugation. This might be a result of overly concentrated samples, a factor which will be discussed later.

Figures 4, 5, and 6 depict some separation of diatoms, green algae, and blue-green algae from each other. However, the resolution is not as good as we expected, considering the buoyancy of the blue-greens and the greater density of the diatoms. Lammers (1964) noted that large organic particles, e.g., filamentous algae, can be carried below their banding level by the mass of inorganic particles, while smaller organic particles, e.g., bacteria and diatoms, have insufficient area to be forced down. Avnimelech et al. (1982) found that high concentrations of algae and clay lead to flocculation and the formation of larger particles with higher sedimentation velocities. Lammers (1971) found that if particles are too concentrated, the density bands will be mixed and distorted in the gradient with large aggregates of mixed particles. If the gradient volume is too large, then the particle band will be too dilute for observation and recovery. A direction of future work might be to determine appropriate limits to the degree a sample should be concentrated prior to density centrifugation.

The lack of resolution between the various blue-green algae (Table 4) might be a function of the density gradient. What may be needed are suitable solutions with specific gravities less than unity which decreases the density of an aqueous solution. A solution could then be layered above the sample layer in the centrifugation processes, extending the density gradient as fractions with a specific gravity <1.0 and thus increasing the resolving power of the method.

A major component of the current research deals with density centrifugation and phytoplankton in an effort to determine the density of the silica sol at which specific genera and species will achieve isopycnic banding (Price et al., 1974; Price et al., 1978; Oliver et al., 1981). Our initial work with the laboratory culture of Ankistrodesmus indicates that there is a range of densities depending on the physiological status of the organism.

Aside from some blue-green algae, freshwater phytoplankton are denser than water and tend to sink, with the sinking rates varying within and between species (Oliver et al., 1981). These rates are a function of the physiological state of the cells which results in changes in the cell density (Smayda and Boleyn, 1965, 1966a, 1966b; Eppley et al., 1967; Titman and Kilhan, 1976) and the morphological characteristics affecting the surface area to volume ratio and the particle orientation (Smayda, 1970). Oliver et al. (1981) found that a wide range of sinking rates may occur within a single field population. Large steps or ranges of density may be necessary to isolate and separate distinct classes or species of organisms. This agrees with the results of Oliver et al. (1981). Additional research will be required if we are to identify the minimum and maximum densities necessary to concentrate various algal species.

Our data (Fig. 2 and 3) indicates that separation of the detrital fraction from the nondetrital fraction of the particulate organic matter is not easily accomplished. The detritus is present across the whole density gradient to varying degrees and is not concentrated or diluted to a great extent in any of them. The chlorophyll a and organic carbon analysis of the daphnia/snail aquarium culture sediments (Figs. 7 and 8) did not indicate any strong trends which would simplify the separation of detritus. This supports the discussion of Lammers (1971) which questions whether or not detritus can be successfully separated from the nondetrital organic fraction by density methods. A future direction of research might be to couple the density centrifugation with appropriate biochemical analysis to determine detrital presence or absence.

Frequently we noted an overlap between the densities of species. When centrifuging a mixed sample through a density gradient, the species do not necessarily become isolated in distinct bands. St. Onge and Price (1975) found that the overlap could be reduced and often removed by the modification of the silica sols. They examined the effects of pH and the addition of trimetaphosphate, dextran sulfate, and polyvinyl alcohol.

A shift of banding densities of fish larvae could be brought about by the addition of low concentrations of anionic polymers to the silica sol gradients (St. Onge and Price, 1975). Morganthaler et al. (1975) found similar shifts for chloroplasts and thylakoid membranes as a result of the addition of polyethyleneglycol to gradients of Ludox-AM. Morgathaler and Price (1976) studied these anomolous density shifts utilizing inert "latex" beads. They found that the beads banded at anomolously low densities when centrifuged in the negatively charged

colloidal silica sols. The addition of density shift reagents increased the banding densities. Qualitatively the fish larvae and chloroplasts studies responded to density shift reagents in a similar manner. The thylakoid membranes of chloroplasts responded by decreasing their banding densities (Morganthaler and Price, 1976).

We were interested in determining the degree to which lignins could be separated from other organic matter in the water column. We analyzed the distribution of chlorophyll a, particulate proteins, and particulate carbohydrates in the density interfaces of the density gradients. In addition, we sought to determine the influence of various density shift reagents and sample preservatives on these distributions. Our preliminary data (Figs. 10-14) indicates little difference between the various treatments. We could not determine any trends in the interfacial distribution of the chlorophyll a as a result of varying the concentration of one of the density shift reagents, Tween 20 (Fig. 9).

The ideal gradient material should have high density, low viscosity, physiologically inert with a minimal osmotic effect, and be soluble in water. Bowen et al. (1972) compared a number of gradient materials in sorting planktonic organisms: Sodium bromides; sucrose; dextran; and Ludox-AM, a silica sol. Sodium bromide is ionic and osmotically active; sucrose is nonionic and osmotically active; and dextran is strongly hydro-phyllic, rendering many of the species in the sample indistinguishable. The silica sol is the only material which met the requirements.

There are a number of colloidal silicas on the market. Much of the density centrifugation work has been done with Ludox-TM and Ludox-Am, both produced by Dupont; and Percoll, a product of Pharmacia Fine

Chemicals. Ludox-AM, with a density of 1.21 g/cm^3, and Ludox-TM, with a density of 1.40 g/cm^3, will gel in saltwater due to their sensitivity to the addition of cations while Percoll, which is a silica colloid coated with polyvinylpyrrodidone and has a density of 1.13 g/cm^3, will not (Oliver et al., 1981). Both Ludox-AM and Ludox-TM are toxic to some biological systems, perhaps as a result of the addition of biocides to increase shelf life (Price and Dowling, 1977) and the presence of zinc and cooper (Jonge, 1979).

Both forms of Ludox are difficult to remove from the sample while Percoll can be easily rinsed out (Schwinghamer, 1981). Oliver et al. (1981) used Fluorinert, with a density of 1.93 g/cm^3 to increase the range of the Percoll density gradients.

Our work did not involve marine waters or the maintenance of live specimens. Future efforts should be directed towards making the methodology compatible with both. Percoll appears to be the silica sol best suited for this. However, additivies need to be utilized which will increase the range of the density gradient. Sorbital has been used (Price et al., 1978; Schwinghamer, 1981), as has fluorinert (Oliver et al., 1981).

The methods of establishing the discrete density gradients and recovery of specific bands should be modified. An apparatus, as presented by Jonge (1979), for layering the step gradients would speed up the procedure and reduce the disturbance of the gradient during loading. An apparatus, as shown in Lammers (1971), would allow samples to be taken from specific points in the gradient with a minimum of contamination or disturbance.

In summary, future efforts should be made in the following areas and directions:

1) The density ranges of specific phytoplankton genera and species at different physiological states should be determined

2) Appropriate limits to concentrating samples prior to centrifugation should be determined.

3) A method for extending the density gradient with less dense solutions to increase separation and resolution with buoyant blue-green algae should be sought.

4) More research needs to be conducted on the separation of the diatoms and blue-green algae from the rest of the phytoplankton.

5) More effort needs to be spent on devising a method for separating the detrital fraction from the rest of the particulate organic matter.

6) The density gradient materials used should be adjusted to allow for testing of saline samples and the maintenance of live specimens. Percoll seems to match the requirements but more work needs to be done on extending its density range.

7) The methodology and equipment for establishing discrete layers and then sampling them without perturbation and contamination need to be refined.

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