MICROFILTRATION IN OCEANOGRAPHIC RESEARCH

II. RETENTION OF COLLOIDAL MICELLES BY ADSORPTIVE FILTERS AND BY FILTER-FEEDING INVERTEBRATES; PROPORTIONS OF DISPERSED ORGANIC TO DISPERSED INORGANIC MATTER AND TO ORGANIC SOLUTES[1]

By

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ABSTRACT

To adsorb and retain quantitatively the minute colloidal micelles held in dilute suspensions, fine inert inorganic powders (such as MgO and refined diatomaceous earth) or finely porous cellulose membranes may be employed. Minute micelles, such as molecular hemoglobin, are retained also by setous or ciliary-mucous filter-feeders. Microfiltration is applicable in preparing bacteria-free aqueous filtrates and in determining the preponderance of organic *dispersoids* over organic *solutes* in natural waters. Molecular filter membranes have been used to demonstrate the high variability of total leptopel concentrations and to indicate the inconstancy of ratios between organic and inorganic dispersoids.

Current studies of marine biochemical cycles involve the use of adsorptive filters for the recovery, concentration and quantitative studies of leptopel, the finely particulate organic and inorganic matter suspended in water (Fox, Isaacs and Corcoran, 1952; Goldberg, Baker and Fox, 1952). Although careful experimentation indicated that marine hydrocolloidal micelles must be quantitatively retained either by adsorptive powder filters or by cellulose acetate and nitrate molecular filter (MF) membranes, it was necessary to resolve other critical questions, such as (a) the fineness of micelles retainable by such filters, (b) ratios between quantities of organic and inorganic leptopelic components, and (c) proportions between finely *particulate* and truly *dissolved* organic matter in the sea. These problems have involved (1) testing each type of filter for its retention of extremely small micelles of approximately known size, such as hemoglobins, (2)

[1] Contribution from the Scripps Institution of Oceanography, University of California, New Series No. 662.

[2] The authors wish to acknowledge the generous donation of experimental molecular filter membranes from Dr. Alexander Goetz of the California Institute of Technology.

determining the total dry weight and ash weight of leptopel retained by ashless MF membranes, and (3) measuring the biochemical oxygen demand of endemic marine micro-organisms which utilize total organic matter in raw samples of sea water, comparing such measurements with determinations of the micro-organisms' use of dissolved molecules which remain in filtered samples. The removal of bacteria from sea water systems with each type of filter has also been confirmed (cf. Goetz and Tsuneishi, 1951; Clark and Kabler, 1952).

RETENTION OF COLLOIDAL MICELLES BY ADSORPTIVE FILTERS

Substances Tested and Their Properties

(a) Oxyhemoglobin from a tide-pool blenny (not specifically identified) and from the marine goby *Gillichthys mirabilis;* micellar size of vertebrate hemoglobins = ca 50 Å (McBain, 1950: 128); molecular weight, ca 70,000.

(b) Oxyhemoglobin from the marine polychaete worm *Thoracophelia mucronata;* micellar size two- to ten-fold that of the fish hemoglobin as estimated by ultramicroscopic (dark-field cardioid condenser) examination of both species. Suspensions of about 2 ppm were used in experiments.

Filters and Reagents

(a) Adsorptive powder filters: equal parts of finely powdered MgO (reagent grade) and pure SiO_2 as "Super-cel," a refined diatomaceous earth (Fox, Isaacs and Corcoran, 1952).

(b) Molecular filter (MF) membranes of finely porous cellulose acetate-nitrate (Goldberg, Baker and Fox, 1952).

(c) Freshly prepared (or carefully preserved and always pretested) solutions of benzidine in equivalent parts of glacial acetic acid and hydrogen peroxide (Hawk, Oser and Summerson, 1947).

(d) Special adsorptive refiltering medium consisting of 5 parts Super-cel and 1 part $Ca(OH)_2$ to retain hemoglobin quantitatively from suspensions of 1 part or less in 10^8. This medium, less active than MgO in gradually catalyzing the decomposition of H_2O_2 to yield a "blank" blue color reaction with benzidine, is used for reprocessing filtrates from the other microfiltrational devices. Traces of hemoglobin, retained in suspensions too dilute to yield a positive benzidine reaction on a direct test of the filtrate, are readily detected by passing the fluid through the thin powder pad and by finally applying the test directly to the pad's damp upper surface where any traces of residual hemoglobin would have been concentrated. Powders containing $Ca(OH)_2$ are more effective with sea water than with distilled or

fresh water, since, in the former medium, the sparingly soluble base immediately precipitates gelatinous flocks of insoluble and strongly adsorbent $Mg(OH)_2$.

Precautions

(a) Adsorptive powder-filter suspensions should be allowed to settle for a few minutes in the porous cup, should be firmly packed into position by initial suction before using, and should remain covered with water at all times in order to preclude the formation of cracks or other channels.

(b) Use of excessive amounts of $Ca(OH)_2$ should be avoided in the filtration of sea water systems, since any considerable proportions of the resulting $Mg(OH)_2$ precipitate tend to clog the filter.

(c) An excess of the neutral adsorbent must be present, since this filtrational process is one of *adsorption* rather than mere physical screening. Hence a given quantity or surface area of the adsorbent will possess a limiting saturation value which, if exceeded by the concentration of the adsorbate (e. g. hemoglobin), will allow micelles of the latter to pass.

(d) Numerous types of surface, including the finely powdered MgO-Super-cel mixture, catalyze the decomposition of H_2O_2, thus yielding free O_2 which reacts with benzidine. However, the blue color thus produced appears rather slowly in contrast with the instantaneous manifestation of green or blue-green colors produced by adsorbed hemoglobin. Moreover, this "blank" blue reaction is still more retarded when $Ca(OH)_2$ has been substituted for MgO as a sea water filter. If these factors are kept in mind, there is no difficulty in recognizing the presence of adsorbed hemoglobin.

Results. Four-liter suspensions of fish hemoglobin that had been separated from the laked corpuscular envelopes, and of worm hemoglobin, were resolved by filtration through adsorptive powder columns [0.5 g MgO; 0.5 g Super-cel, and optionally 0.1 g $Ca(OH)_2$] of about 4 mm thickness. Hemoglobin concentrations of 2 ppm or 8 mg total were used in both sea water and distilled water. Following the operation, the damp filter-cake was removed and the benzidine test was applied. Its top surface responded positively at once while the bottom surface was negative; cross sections of the fractured cake showed that the hemoglobin was adsorbed in the upper 1 to 2 mm; its quantitative adsorption was clearly indicated by a distinct color boundary part way through the cake. Complete removal of the hemoglobin was further confirmed by passing the filtrate from the first operation through a second adsorptive powder-cake of 5 parts

Super-cel and 1 part $Ca(OH)_2$; application of the benzidine test to its damp top surface gave negative results. Molecular filter membranes of different porosities were likewise employed for processing similar volumes of dilute, laked and cleared hemoglobin suspensions in sea water and in distilled water. The resulting filtrates were then tested for residual hemoglobin by using the Super-cel-$Ca(OH)_2$ powder as above.

The following filter membranes which were used are designated by their Z-number (time in seconds required for 1 ml of water to pass through 1 cm^2 of surface area at 700 mm pressure) and their VC value (volume ratio of cellulose material to air space).

Filter No.	*Z No.*	*V C*	*Hemoglobin in distilled water*	*Hemoglobin in sea water*
5001–12	0.035	....	Passed	Passed
1152–6	0.26	0.142	Passed	Passed
2010	1.0	0.200	Sometimes passed little*	Sometimes passed traces*
1122–5	2.6	0.213	Sometimes passed little*	Sometimes passed traces*
1122–3	8.0	0.249	——	Retained all
1152–8	20.0	0.269	Retained all	Retained all

* We believe that in some instances adventitious soluble heme degradation products might have passed through the filter, since heme crystals dissolved in water give a strong reaction with benzidine. Furthermore, while the test failed at a dilution of 1 : 25,000, it responded after the heme from this solution had been retained on a Super-cel + $Ca(OH)_2$ filter

From the foregoing experiments it is concluded that the MgO + Super-cel adsorptive powder, as well as cellulose filters of fine porosities, will quantitatively adsorb extremely fine micelles (such as vertebrate hemoglobin) from very attenuated suspensions, and that such microfiltrational operations are therefore suitable for the quantitative resolution of marine colloids. Indeed, most marine colloidal micelles are probably of much larger mean diameter than hemoglobin, and, in view of the presence of the divalent cations and charged micelles, they must occur in various degrees of adsorption, agglomeration or coacervation.

RETENTION OF DISPERSED HEMOGLOBIN BY FILTER-FEEDING INVERTEBRATES

Because our chief interest was concerned with the fineness of colloidal micelles which may be removed by filter-feeders, we conducted a few experiments on the retention of molecularly dispersed fish hemoglobin by two different and widely distributed marine

invertebrates: a mysid crustacean (not specifically identified) which was collected in the vicinity of kelp beds and slicks off La Jolla; the sea mussel, *Mytilus californianus*, which grows in large numbers and is attached to intertidally exposed rocks and pier pilings.

Both control and experimental mysids were placed in previously microfiltered sea water. A small volume of laked and paper-filtered hemoglobin was then added to the microfiltered water that contained the experimentals, which, in contrast with the controls, conspicuously manifested the full dark-colored contents of their alimentary tracts. In numerous instances they had next day voided long dark rod-like fecal strands, which, upon microscopic inspection, especially after mashing under a cover glass, showed green or blue responses to the benzidine test, thus indicating the presence of heme or hemoglobin.

Similarly, both the pseudofeces and stomach rinsings of mussels exposed to dilute molecularly dispersed hemoglobin yielded green colors with benzidine while those of the control animals were negative in this regard. Likewise, the white experimentally talc-laden feces of mussels which had previously received hemoglobin exhibited purple colors with benzidine as well as positive tests for iron in the presence of thiocyanate.

These experiments are regarded as demonstrative of the ability of two representative filter-feeders (one setous, the other ciliary and mucous) to adsorb from suspension colloidal particles of such extremely fine dimensions as those of vertebrate oxyhemoglobin, i. e., 40 to 50 Å. Microfiltrational operations with adsorptive powders or molecular filters may be used to recover the dispersed phases of natural hydrosols, including colloidally dispersed materials commonly gathered by mucous or other filter-feeding aquatic animals.

PROPORTION BETWEEN ORGANIC AND INORGANIC LEPTOPELIC CONSTITUENTS

Goldberg, Baker and Fox (1952) have demonstrated in duplicate samples and with respect to depth the wide variability of finely particulate inorganic matter in the sea, while Fox, Isaacs and Corcoran (1952) have supplied similar information pertaining to organic leptopel. The inhomogeneity of these materials, their changing concentrations with time, and the variability in ratio between organic and inorganic leptopel, were confirmed with the molecular filter in two additional experiments reported here.

Two 4-liter samples, collected in relatively nonwettable polyethylene bottles, were taken from about 20 feet beneath the surface at the end of the Scripps Institution's pier, 1,000 feet offshore. Sample I was

collected during a period of high surf, when the offshore waters contained much visible suspended matter; sample II was taken on the following day when the sea had subsided to a normal surf and when the water was less turbid. In the laboratory, the coarser particles of sand and mica settled out of suspension, and floating algal fragments were removed from the water surface.

Each 4-liter sample was passed through an ashless cellulose membrane filter ($Z = 2.6$). The residue was leached *in situ* with distilled water, and the membrane, with its retained particulate material, was then dried at 80° C for six hours in a vacuum oven. After weighing, each membrane was ignited, and the ash weight was recorded as follows:

	Total dry solids (mg/L)	*Ash (mg/L)*	*Organic matter by difference (mg/L)*	*Organic Matter (%)*
Sample I	10.5	4.0	6.5	62
Sample II	3.8	2.7	1.1	29

These two random samples thus exhibit nearly a three-fold variation in total leptopel content and more than a two-fold difference in the proportion of the organic component.

The value of 29% organic matter obtained from Sample II is close to the figure obtained previously for solid material collected from a marine slick (27%; Fox, Isaacs and Corcoran, 1952) and to the figure derived from several analyses of the flocculent portions of feces from a typical detritus filterer, the California mussel (31%; Fox and Coe, 1943).

PROPORTIONS BETWEEN DISPERSED AND DISSOLVED ORGANIC MATTER IN SEA WATER

Method. Samples of raw sea water were freshly collected in clean and relatively nonwettable plastic bottles. The untreated sea water and filtrates derived from it were assayed for their respective content of (a) original dispersed and dissolved organic matter combined, and (b) organic solutes by determining (with the Winkler method) the biochemical oxygen demand (B. O. D.) of multiple aliquots. The raw sea water already contained a normal flora of micro-organisms and was therefore ready for incubation. However, each of the filtered samples was inoculated with 5 ml raw sea water per liter in order to provide mixed populations of marine bacteria. This inoculation supplemented the organic matter by no more than 0.5% of the original supply. The multiple aliquots, incubated at 27 to 28° C in 60-ml glass-stoppered bottles, were analyzed for residual dissolved oxygen before incubation and at specified intervals (Figs. 1, 2, 3).

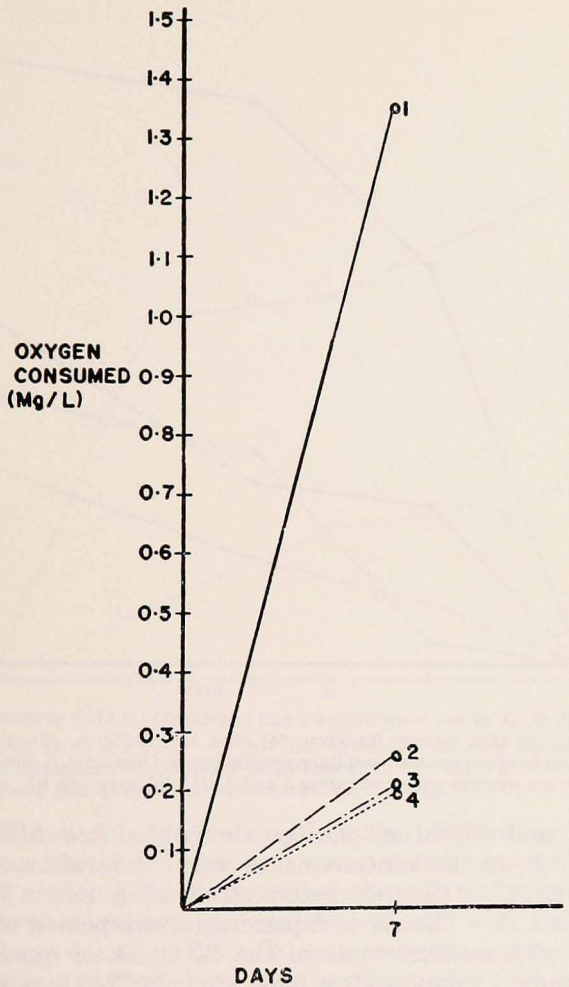

Figure 1. B. O. D. of sea water: (1) raw and untreated; (2) after powder treatment and paper filtration; (3) after powder filtration; (4) after MF filtration. Incubation temperature, 28° C; ratio between retained and filter-passing organic matter (7 days' incubation): ca 85 : 15%.

Results. Experiment I. Paired samples of sea water were incubated in the following stages: (1) in the raw state, (2) after occasional shaking with a little powdered MgO during a two-hour period and subsequent filtering by gravity through a superimposed pair of No. 50 Whatman filter paper cones, (3) following filtration by suction through a packed column of adsorptive powder (equal weights of MgO and

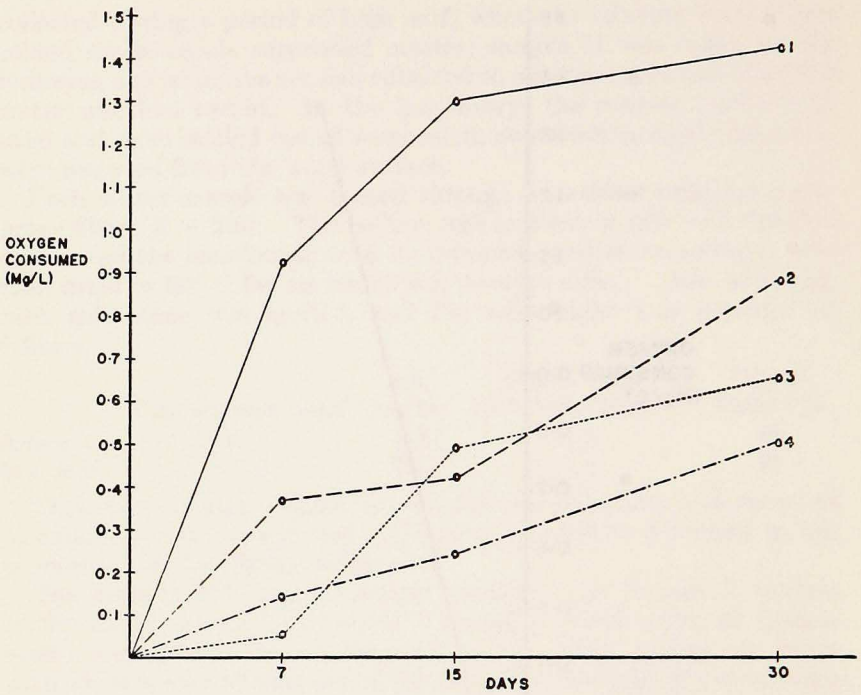

Figure 2. B. O. D. of sea water: (1) raw and untreated; (2) after powder treatment and paper filtration; (3) after powder filtration; (4) after MF filtration. Incubation temperature, 27° C; ratio between retained and filter-passing organic matter (i. e. comparing B. O. D. of curve 1 with the average values of curves 3 and 4 after 30 days): ca 60 : 40 %.

Super-cel), and (4) after passage through a fine MF membrane (Z = 8.5). B. O. D. measurements were made after culturing for seven days at 28° C; the results are plotted in Fig. 1.

Experiment II. The above experiment was repeated with fresh sea water and with measurements of the B. O. D. of quadruplicate or triplicate aliquot samples after incubation for 7, 15 and 30 days at 27° C. Values for the average quantities of oxygen consumed are plotted in Fig. 2.

Experiment III. Since the rubber connections used in the assembly which employed adsorptive powder might have contributed traces of contaminating organic material to the filtrates, comparisons were made between the B. O. D. of sea water (1) before it was treated and (2) after it was filtered through the adsorptive powder in a Morton filter apparatus, which is constructed entirely of glass, i. e., with ground joint and a fine sintered glass disc that is capable of retaining

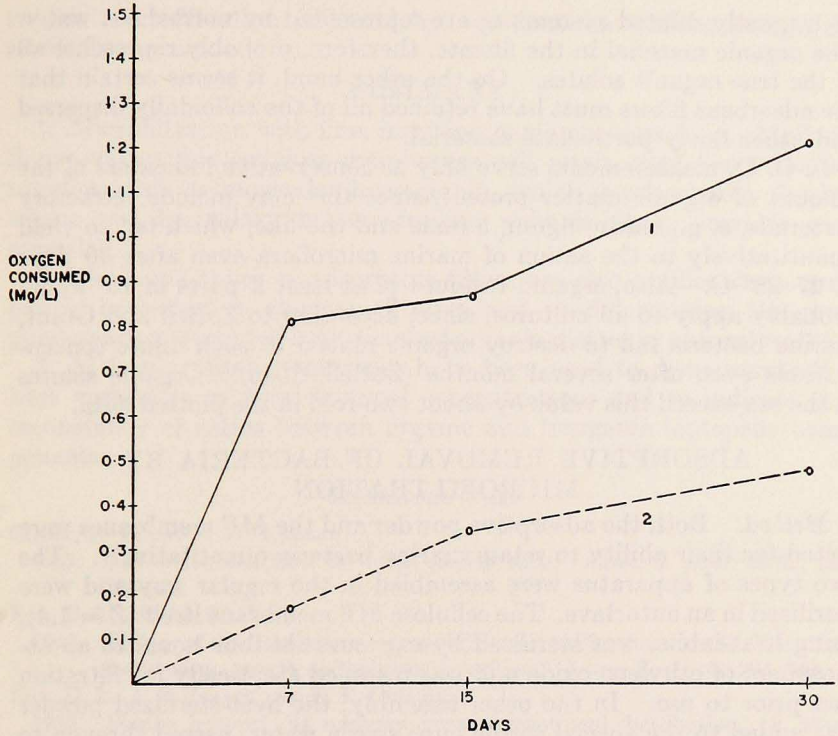

Figure 3. B. O. D. of sea water: (1) raw and untreated; (2) after powder filtration in all-glass Morton apparatus. Incubation temperature, 27° C; ratio between retained and filter-passing organic matter (30 days' incubation): ca 61 : 39%.

bacteria in filtrational sterilization of media. Before use, the whole apparatus was dismantled, its parts were treated separately with hot concentrated nitric or hot concentrated chromic and sulphuric acids, and all parts were ultimately rinsed free of acid with twice-distilled water. Average values of B. O. D. measurements, made on multiple aliquot samples of each medium after incubation for 7, 15 and 30 days at 27° C, are plotted in Fig. 3. Close agreement between the data in experiments II and III suggests that the former involved no contaminants.

From a study of the graphs it is apparent that the major proportion (3/5 or more) of the organic matter in sea water existed in a colloidal or other particulate state rather than in the dissolved condition. While traces of some organic solutes may be adsorbed by the powder or by the cellulose material of the molecular filter, it is highly improbable that such adsorption occurs to a significant extent, especially from

such greatly diluted systems as are represented by normal sea water. The organic material in the filtrate, therefore, probably represents all of the true organic solutes. On the other hand, it seems certain that the adsorbent filters must have retained all of the colloidally dispersed and other finely particulate material.

B. O. D. measurements serve only as conservative indicators of the amount of organic matter present, since this may include refractory materials, e. g., chitin, lignin, humus and the like, which fail to yield quantitatively to the action of marine microflora even after 30 days at 27–28° C. Also, organic residues of at least 2 parts in 10^7 would probably apply to all cultures, since, according to ZoBell and Grant, marine bacteria fail to destroy organic matter at such dilute concentrations even after several months (ZoBell, 1946). Organic solutes in the sea exceed this value by about two-fold in the plotted data.

ADSORPTIVE REMOVAL OF BACTERIA BY MICROFILTRATION

Method. Both the adsorptive powder and the MF membranes were tested for their ability to retain marine bacteria quantitatively. The two types of apparatus were assembled in the regular way and were sterilized in an autoclave. The cellulose MF membrane itself ($Z = 1.4$), being heat-labile, was sterilized by exposure for four hours to an atmosphere of ethylene oxide and was installed aseptically for filtration just prior to use. In the other assembly, the heat-sterilized powder was added to the special sterile cup; sterile water, passed through to seat the powder pad, was received in a sterile test tube which was ultimately removed before the main operation.

Raw sea water, containing approximately 1,000 bacteria per ml, was processed through each filter. Filtrate samples 1 ml in volume were transferred to a series of tubes, each of which contained 5 ml of sterile nutrient sea-water broth (ZoBell, 1946: 41); incubation was then carried out at 27° C.

Results. After 72 hours of incubation, all tubes remained sterile. They were therefore inoculated with mixed marine bacteria to determine whether the filtrate had perchance contained any antibacterial contaminants. After reincubation at 27° C for 24 hours, the contents of all tubes were distinctly turbid, indicating bacterial growth.

It was concluded, therefore, that each type of filter is capable of removing all bacteria from sea water without destroying its ability to support bacterial multiplication. Bacterially sterile and leptopel-free sea water, ionically balanced and of low residual organic (solute) content, may be readily prepared for media or other purposes by

aseptic microfiltration followed by equilibration with a stream of sterile filtered air.

SUMMARY

1. Microfiltration with fine, inert inorganic powders (e. g., MgO + Super-cel) or fine cellulose membranes will retain very fine colloidal micelles, such as molecular hemoglobin, which is adsorbable also by setous and by ciliary mucous-feeding animals (e. g., mysids and mussels).

2. The same types of adsorptive filters are also applicable in preparing bacteria-free aqueous filtrates and in determining the preponderance of *dispersed* over *dissolved* organic matter in natural waters.

3. Molecular filter membranes have been used to demonstrate the high variability of total leptopel concentrations and to indicate the inconstancy of ratios between organic and inorganic leptopelic components.

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