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THE SURVIVAL OF SEWAGE BACTERIA AT VARIOUS OCEAN DEPTHS

By

H. P. Vind, J. S. Muraoka, and C. W. Mathews

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Sewage outfalls in the ocean are usually relatively close to shore at depths of 200 feet or less. An investigation was undertaken to ascertain if Escherichia coli, the principal species of bacteria in sewage, would survive for shorter or longer periods if the sewage were discharged at depths of 1,000 feet or so, where there is no light, and where the pressure is greater and the temperature is lower. Cultures of the Seattle strain of E. coli in autoclaved seawater were placed in 25-ml bags made of dialyzing tubing. Some of the

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bags were suspended near the surface of the ocean, some at depths of 200 and some at depths of 1,000 feet. Some of the bags were suspended in opaque containers to protect them from sunlight; others were suspended in translucent containers. All of the E. coli cultures exposed near the surface of the ocean in translucent containers died in approximately 4 hours. Those suspended near the surface in opaque containers survived for periods of an estimated 2 weeks. Cultures of E. coli suspended in either translucent or opaque containers at depths of 200 and 1,000 feet (where little or no light penetrates) also survived for periods of an estimated 2 weeks, with only slight differences in the mortality rates at these two depths. If the sewage were discharged at a depth of 1,000 feet, there would be no danger of contaminating surface waters because the cold deep water does not mix with the warmer surface waters. If the sewage were discharged at a depth of 200 feet, there would probably also be no danger of contaminating surface waters unless the thermocline was deeper than that. If the sewage were discharged at shallow depths, there would be contamination of surface waters; but at least one species of the contaminating microorganisms would probably survive for only a few hours in sunlight.

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Sewage outfalls in the ocean are usually relatively close to shore at depths of 200 feet or less. An investigation was undertaken to ascertain if Escherichia coli, the principal species of bacteria in sewage, would survive for shorter or longer periods if the sewage were discharged at depths of 1,000 feet or so, where there is no light, and where the pressure is greater and the temperature is lower. Cultures of the Seattle strain of E. coli in autoclaved seawater were placed in 25-ml bags made of dialyzing tubing. Some of the bags were suspended near the surface of the ocean, some at depths of 200 and some at depths of 1,000 feet. It was found that if the sewage were discharged at a depth of 1,000 feet, there would be no danger of contaminating surface waters because the cold deep water does not mix with the warmer surface waters; if the sewage were discharged at a depth of 200 feet, there would probably also be no danger of contaminating surface waters unless the thermocline was deeper than that; if the sewage were discharged at shallow depths, there would be contamination of surface waters, but at least one species of the contaminating microorganisms would probably survive for only a few hours in sunlight.

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INTRODUCTION

The disposal of sewage in the sea and adjoining estuaries is widespread. The outfalls are usually relatively close to shore and the sewage is discharged at depths of 200 feet or less. The purpose of the study undertaken by the Civil Engineering Laboratory (CEL), Port Hueneme, CA, and described in this technical note is to determine whether sewage bacteria would survive for longer or shorter periods if the sewage were discharged at greater depths, where there is less light, where the pressure is greater and where the temperature is lower. Bacteria of the species Escherichia coli, the most abundant bacterial species in human wastes, were employed in the study as representative sewage bacteria.

METHODS AND MATERIALS

Ocean Exposure

Small containers of sewage bacteria were exposed in the ocean by a team of Navy divers who had had extensive experience in conducting oceanographic experiments. A modified aluminum LCM-8 was employed in the undertaking.

The exposure site was approximately 3 miles offshore, 5 miles southeast of Point Mugu, California. The water depth was 1,300 feet. Ocean exposure stations were established near the surface of the ocean and at depths of approximately 200 feet and 1,000 feet. The exposure stations were simply tethering hooks or loops for suspending samples at various positions on a 1,000-foot line (Figure 1). The line was supported from the surface by three steel drums serving as buoys (Figure 2). A 500-pound weight at the end of the line held it taut in the water (Figure 3). The assembly was prevented from drifting by a separate 600-foot-long line attached to the 500-pound weight on one end and to an ocean-bottom anchor on the other. Powerful winches (Figure 4) were used to raise and lower the assembly when samples were removed or placed in the ocean.

Bacterial Cultures

Pure cultures of Seattle strain E. coli as supplied by Roche Diagnostics, Division of Hoffman-LaRoche, Inc., Nutley, NJ 07110, were employed in all of the experiments. The bacteria were supplied as BACT-CHEK discs, which are composed of dried bacterial cells and have a diameter of approximately 4 millimeters and a thickness of approximately 1/2 mm. Each disc contains 100,000 to 10 million viable microorganisms.

Bacterial Counts

Coliform counts were made by a widely used modification [1] of the Standard Total Coliform Membrane Filter Procedure [2]. The counts were made with field monitoring equipment developed and supplied by the Millipore Corporation, Bedford, MA. The equipment included a syringe with two one-way valves. With the syringe, water samples were drawn through membrane filters having pores sufficiently small (0.45 millimicron) to retain most microorganisms. The membrane filters were mounted in ''Millipore Field Monitors'' which are sterile, disposable, plastic devices, serving both as filter holders and culture chambers. The membranes are supported on absorbent pads for retaining culture medium.

The water samples to be counted were serially diluted (Figure 5) in test tubes, each containing 9 ml of an autoclaved 3:1 mixture of distilled water and seawater. The diluted fractions of the samples were then drawn through the membrane filters contained in the Millipore Field Monitors (Figure 6). In this step, all of the bacteria in the diluted fractions were deposited on the surfaces of the membrane filters. The absorbent pads on which the membrane filters were mounted were each moistened with 0.8 ml of sterile ''MF-Endo Broth,'' a proprietary medium prepared specifically for the isolation and identification of coliform bacteria. The monitors were then closed and incubated at 35°C for 24 hours.

Each coliform bacterium on the surface of the membrane filters multiplies many times in the course of 24 hours and ultimately forms a macroscopically visible aggregate or colony. Most other bacteria species do not multiply on the selective MF-Endo Broth. The number of colonies which develop is assumed to be the same as the number of coliform bacteria in the serially diluted fraction of the water sample that was filtered. Because most samples were counted in duplicate and several dilutions were made of each, several estimates were obtained of the number of microorganisms in a unit volume of each sample. When the results were averaged, greatest reliance was placed on plate counts in the range of 20 through 80.

EXPERIMENTAL RESULTS

Effect of Sea Salts on the Survival of *E. coli*

In preparation for experiments which were to be conducted at sea, several preliminary tests or exercises were conducted in the laboratory.

In the first exercise, one BACT-CHEK disc was placed in a test tube containing a 3:1 mixture of distilled water and seawater. The test tube was incubated for 2 hours at 35°C. At the end of that time the disc was sufficiently soft to disintegrate when the test tube was shaken. The bacteria were then uniformly distributed throughout the tube. The contents of the tube was added to a 1-liter flask filled with filtered autoclaved seawater. Bacterial counts were made at various intervals and the following counts were obtained:

Time (hours)	Count (per ml)
0	2,000
1	2,000
3	2,700
24	300

The exercise was repeated with the following results:

Time (hours)	Count (per ml)
0	2,100
22	800
25	900
91	400
139	300

In a similar test, a BACT-CHEK disc was incubated in a test tube of water at 35°C for 24 hours, instead of for 2 hours, before it was added to a flask containing 1 liter of filtered autoclaved seawater. Immediately after mixing, the bacterial population of the seawater was approximately 200,000 per ml instead of approximately 2,000, as was the case when the discs were incubated for 2 hours.

In a final laboratory exercise, the effect of added nutrient on E. coli populations was tested. Ten-ml quantities of a freshly prepared E. coli culture were added to individual 250-ml flasks of filtered seawater and to flasks of seawater containing added tryptic soy broth (a proprietary mixture of nutrients and salts sold by Difco Laboratories, Detroit, MI for preparing culture media for microorganisms). The following coliform counts per ml were obtained.

Time (hours)	Count in Filtered Seawater	Count in Tryptic Soy Broth and Seawater
0	45,000	35,000
26	4,000	25 million

Effect of Ocean Depth on Survival of E. coli

Comparisons were made of the numbers of E. coli surviving in seawater cultures exposed for various periods of time at various depths in the ocean. The cultures included E. coli suspended in filtered autoclaved seawater and in an autoclaved mixture of seawater and human feces. Some of the cultures were contained in 25-ml bags made of cellulose acetate dialyzer tubing tied at each end (Figure 7). The dialyzer bags permitted water soluble substances to diffuse freely between the bags and the ocean but retained the cultures of E. coli in the bags and prevented the

entry of microorganisms and proteins from the ocean. Because of the possibility that the dialyzer bags might rupture, pliable, translucent, polyethylene bottles were also employed as containers for some of the E. coli cultures. Although the bottles were essentially impermeable, they did permit the cultures to be exposed to ocean pressures and temperatures.

Synthetic sewage water was prepared for the experiment by adding approximately 10 grams (wet weight) of human feces to a 2-liter flask of seawater. The flask was autoclaved for 20 minutes at 15-psi steam pressure. A 2-liter flask of filtered seawater was autoclaved at the same time. Both flasks were inoculated with E. coli in the usual manner. Two of the BACT-CHEK discs were used in the preparation of each of the two cultures.

Some of the seawater culture was distributed into the dialyzer bags and some into 25-ml polyethylene bottles. The synthetic sewage culture was distributed only into the polyethylene bottles.

The dialyzer bags and the 25-ml polyethylene bottles containing the E. coli cultures were placed in wide-mouthed, 500-ml polyethylene bottles in which numerous 1/4-inch round ventilation holes had been drilled. The larger bottles served as cages for the smaller containers. They were securely fastened to the anchored nylon line and suspended at various depths in the ocean.

Bacterial counts were made after various time intervals. Counts were also made on the remains of the original cultures which were maintained in the laboratory at room temperature. The results of the experiment are summarized in Table 1.

Effect of Sunlight on the Survival of E. coli

An investigation was made of the effect of sunlight on the survival of E. coli. Cultures of the microorganisms were exposed in opaque and in translucent containers at the ocean surface and at depths of 200 and 1,000 feet. Comparisons were then made of the numbers of microorganisms surviving in the opaque and translucent containers. The experiment was conducted in the following manner.

A culture containing 100,000 E. coli per ml was distributed into 54 dialyzer bags with a capacity of approximately 25 ml each. Six of the bags were retained in the dark in the laboratory for control tests, three being maintained at room temperature and three at 3°C. The other 48 bags were divided into pairs and distributed into 24 cages made of polyethylene bottles in which numerous 1/4-inch round ventilation holes had been drilled. Twelve of the cages had been made opaque by a covering of electrician's black tape; the other twelve remained translucent (Figure 8).

The following day, two of the opaque cages and two of the translucent cages containing dialyzer bags of E. coli culture were exposed near the ocean surface for periods of 1 and 4 hours. The exposures were made by simply fastening the cages on the end of a length of nylon parachute cord and lowering them over the side of the ship. The bottles floated and remained on the surface. The third of the opaque and the third of the translucent cages were not exposed in the ocean and were employed as zero hour controls.

Table 1. Survival of E. coli at Sea

Sample	Depth (feet)	Temp (°C)	Count per ml at Various Time Intervals					
			0 hr	1 hr	24 hr	48 hr	148 hr	288 hr
Filtered seawater in dialyzer bags exposed in ocean	0	12		–[a]	2	3	–	–
	200	10	1,500	1,400	800	100	8	1
	1,000	8		1,500	400	120	40	15
Filtered seawater in polyethylene bottles exposed in ocean	0	12		–	0	0	–	–
	200	10	1,500	1,800	220	60	0	0
	1,000	8		900	200	270	11	0
Synthetic sewage in polyethylene bottles exposed in ocean	0	12		–	40	17	–	–
	200	10	4,000	3,400	1,500	1,200	12	17
	1,000	8		3,100	1,800	970	10	1
Filtered seawater in flask in laboratory	0	22	1,500	–	–	–	40	–
Synthetic sewage in flask in laboratory	0	22	4,000	–	–	–	8×10^6	–

[a] Dash indicates no data available.

The other 18 cages were attached to the nylon line anchored in the ocean. Three each of the opaque and translucent cages were suspended from the line at a depth of only a few feet; three each, at a depth of 200 feet; and three each, at a depth of 1,000 feet. One opaque and one translucent cage was removed from each depth at the end of 24, 48, and 120 hours.

The bags were removed from the cages and samples for bacterial counts were obtained by piercing the bags (Figure 9). Figures 10 to 13 show the colonies which developed on the field monitor plates for the final or 120-hour samples after the series of dilutions of 10 ml of the contents of the bags. The results of the experiment are summarized in Table 2.

Sunlight Effect on E. coli in Glass and in Polyethylene

A comparison was made of the survival of E. coli exposed to sunlight in containers made of pyrex glass and containers made of polyethylene. The shorter wavelengths of ultraviolet light are less able to penetrate the glass than the plastic containers.

A culture was prepared by inoculating 1-1/2 liters of autoclaved seawater containing 0.5 gram of ''Eugenbroth'' (a proprietary mixture of nutrients and salts sold by the Division of Bio Quest, Cockeysville, Maryland for preparing bacteriological media with E. coli). The culture was incubated for 24 hours at 32°C, at which time growth of E. coli was sufficient to cause the medium to be cloudy. One hundred ml of culture was added to each of three small polyethylene bottles and three pyrex glass flasks. The six containers were placed outdoors in the sunlight for 6 hours. During the first 2 hours, the sun was obscured by fog and low-lying clouds. During the next 2 hours the cloud cover disappeared, and the sunlight was very bright for the last 2 hours of the exposure. Periodically, bacterial counts were made of the contents of the 6 flasks. The following values were obtained for the counts per ml:

Containers	0 hr	2 hr	6 hr
Plastic	24×10^6	26×10^6	<10
Glass	24×10^6	28×10^6	<10

Effect of Sunlight on Seawater

An experiment was conducted to ascertain if sunlight altered the chemical composition of seawater in such a way as to make it unsuitable for the growth of E. coli. Four Erlenmeyer flasks, each containing 100 mg of Eugenbroth preparation and 400 ml of seawater, were autoclaved at 15 psi for 20 minutes. Two of them were then exposed to sunlight for one complete day and the other two were stored in the dark. All four flasks were then inoculated with 1-ml portions of a vigorous E. coli culture, and all four were incubated in the dark for 24 hours. Counts of E. coli were then made, and the following results were obtained:

Table 2. Effect of Sunlight on Survival of E. coli

Container	Depth (feet)	Temp (°C)	Bacterial Count Per ml at Various Time Intervals					
			0 hr[a]	1 hr	4 hr	24 hr	48 hr	120 hr
Translucent cages in ocean	0-10	12	42,000	15,000	175	0	0	[b]
	200	10	–[c]	–	–	7,400	3,300	200
	1,000	8	–	–	–	16,000	8,000	3,500
Opaque cages in ocean	0	12	34,000	37,000	32,000	15,000	6,000	[b]
	200	10	–	–	–	8,000	6,000	500
	1,000	8	–	–	–	20,000	7,000	2,000
Flask in laboratory	0	22	–	–	–	11,000	–	1,400
Flask in ice box	0	3	–	–	–	–	11,000	2,000

[a] All containers were filled from the same original culture of E. coli in filtered seawater which at time zero minus 24 hours had a population of approximately 100,000 microorganisms per ml.

[b] The containers on the surface station were torn loose during high winds.

[c] Dash indicates no data available.

E. coli in medium preexposed to sunlight, 19 x 10^6 per ml.
E. coli in medium protected from sunlight, 14 x 10^6 per ml.

DISCUSSION OF RESULTS

Numerous investigators have studied the effects of seawater, sunlight, pressure, or ocean exposure on the survival of sewage bacteria. So many of their results are conflicting [3] that no attempt is made to compare the results of the study reported here with the results obtained by other investigators.

E. coli cells gradually died off in filtered autoclaved seawater. Apparently they died from lack of nutrients rather than from any harm caused by the sea salts, because they flourished and multiplied rapidly in seawater containing added nutrients such as tryptic soy broth or human feces. It appears that the filtered seawater contained insufficient nutrients for the microorganisms to multiply. The BACT-CHEK discs themselves may have contained some nutrients because the discs produced cultures with a hundredfold greater E. coli population when they were incubated in test tubes of water for 24 hours than when incubated for 2 hours.

In both experiments performed at sea, the counts for samples exposed at 1,000 feet tended to be higher than the counts for samples exposed at 200 feet. The differences were not great, however, and were surely within the range of possible experimental error. In both experiments striking differences were noted in the counts on the samples exposed at 200 or 1,000 feet and those exposed near the surface. Unless protected from light, the bacteria died very rapidly at the surface. There were no great differences in the mortality rates of E. coli in filtered seawater maintained in the laboratory at room temperature, in the refrigerator, or in the ocean at depths of 200 or 1,000 feet. In all instances, there were probably insufficient nutrients; and the bacteria died off at rates which would leave few or none living in 1 or 2 weeks.

Survival times might have varied more had the media contained more nutrients. It is likely that the populations of bacteria would have increased initially. The nutrients would probably have been used up more rapidly at some temperatures than at others, and the subsequent decline in numbers might then have been more markedly influenced by temperature.

Since the E. coli in this experiment were enclosed in dialyzer bags, it is possible that the bacteria would die even more rapidly if discharged directly into the ocean. It seems unlikely that they would have survived for a longer period of time, unless they were protected in some manner, such as inside a grease ball or inside a fecal mass.

During the summer in temperate latitudes, and year around in lower latitudes, near-shore surface waters circulate and mix as a layer. Water below the thermocline, which develops in these waters at depths of 100 feet or so, mixes only slightly with the water above the thermocline

[4]. Under these conditions, sewage discharged at a depth of 1,000 feet would be less likely to contaminate surface waters than would sewage discharged near or above the thermocline. Bacterial survival time would be a less important consideration at the greater depth, so far as man is concerned.

The brief survival period of E. coli cells in containers floated on the surface of the ocean and exposed to sunlight (0-, 1-, and 4-hour samples, Table 2) was not surprising. It is well known that sunlight kills E. coli in shallow dishes of water [5,6].

The apparent germicidal properties of sunlight on the samples exposed several feet below the ocean surface for 24 and 48 hours (Tables 1 and 2) was somewhat unexpected. These samples were tethered to the nylon line at a distance of approximately 10 feet from the surface. The short cord by which they were attached to the line may have permitted the samples to rise a few feet but not to the surface. The samples, no doubt, oscillated in depth with the waves; but, in any event, the sunlight had to penetrate several feet of water to reach the E. coli cultures.

It is generally believed that, at the most, the germicidal radiation in sunlight penetrates 1 meter in seawater [5]. Zobel [6] gives 3 meters as the limiting depth for the penetration of abiotic radiation, but he concludes that at greater depths than 10 to 20 centimeters the radiation is too feeble to sterilize seawater.

In the last of the sunlight experiments at CEL, germicidal radiations were able to penetrate both pyrex glass and polyethylene plastic. Pyrex glass is opaque to wavelengths shorter than 2,800 Angstroms. Its transparency gradually increases with longer wavelengths, and it transmits greater than 90% of radiation from 3,600 to 7,000 Angstroms [7]. Polyethylene is opaque to wavelengths shorter than 2,270 Angstroms. Its translucency gradually increases with longer wavelengths, and it transmits a relatively high percentage of ultraviolet and visible radiation longer than 2,800 Angstroms [8]. Finally, that part of the sun's radiation that is composed of wavelengths shorter than 2,920 Angstroms is cut off completely by the atmospheric ozone layer and by oxygen [9]. The results of the CEL experiment indicate that the intensity of sunlight radiation with wavelengths greater than 2,920 Angstroms must be great enough to kill E. coli cells in seawater.

An extensive investigation by Lukiesh [7] at General Electric Laboratories, Cleveland, OH, indicated that the maximum germicidal effectiveness in the killing of E. coli in shallow dishes of water is exhibited by radiant energy with wavelengths of 2,537 to 2,575 Angstroms. However, with high enough intensities and prolonged exposures, all wavelengths in the ultraviolet, and even in the visible spectrum, were germicidal. At a wavelength of 4,000 Angstroms, 10,000 times as much radiant energy, and at a wavelength of 7,000 Angstroms, 100,000 times as much radiant energy were required to kill E. coli cells as was required at a wavelength of 2,540 Angstroms. Hence, even though surface sunlight contains no radiation shorter than 2,920 Angstroms, it is germicidal, apparently because it contains some ultraviolet radiation with wavelengths

from 2,920 to 4,000 Angstroms and very intense visible radiation from 5,000 to 6,000 Angstroms. The intensity of the ultraviolet radiation in sunlight varies considerably from day-to-day and hour-to-hour.

The transmission of ultraviolet light in seawater also varies considerably. The concentrations of microorganisms, nitrate ions, sediments, and organic matter all help to determine the transparency of seawater to ultraviolet radiation [10]. As depth increases, the intensity of ultraviolet light decreases. The shorter the wavelength, the greater is the decrease in intensity with depth. In consequence, the average wavelength of sunlight radiation increases with depth of penetration.

If there were radiations in sunlight with a wavelength of 2,540 Angstroms, the most highly germicidal wavelength, they would penetrate no more than 1/2 meter of seawater. Ultraviolet radiation with a wavelength of 2,920 Angstroms, the shortest in sunlight, penetrates no more than approximately 3 meters of seawater [6]. Ultraviolet sunlight with a wavelength of 4,000 Angstroms may penetrate 10 to 20 meters, and visible light may penetrate to a depth of 100 meters [8].

The maximum depth at which the detrimental effects of sunlight are great enough to overcome the ability of E. coli cells to resist and multiply has not been precisely determined. Beyond the depth of sunlight penetration, E. coli cells survived for nearly 2 weeks in seawater containing insufficient nutrients for growth. Whether they would survive for shorter periods when exposed to sunlight radiation penetrating to depths of 10, 25, 50, or 100 feet is not known. This information is part of that needed for a thorough assessment of the hazards of disposing of sewage at sea.

CONCLUSIONS

Escherichia coli, the principal bacterial species of sewage, is very sensitive to sunlight. Unless protected inside a grease ball or a fecal mass, microorganisms of this species in sewage discharged near the ocean surface would surely perish within a few days. There would be relatively small differences in the mortality rates of E. coli in sewage discharged at ocean depths of 200 and 1,000 feet. Very little sunlight penetrates to these depths, and sewage bacteria of this species would survive for an estimated week or two. If the sewage were discharged at a depth of 1,000 feet, there would be no danger of contaminating surface waters because the cold deep water does not mix with the warmer surface waters. If the sewage were discharged at a depth of 200 feet, there would probably also be no danger of contaminating surface waters unless the thermocline was deeper than that.

ACKNOWLEDGMENTS

LT Anthony M. Parisi and LTJG James E. Halwachs designed the assembly for anchoring the water samples in position in the ocean; and they were in command of the diving vessel and all operations at sea. Members of the crew included Navy Divers Larry Hecht, Larry Wenban, Donald Forster, Larry Stowers, Joe Hierholzer, and Robert Hurt. Mr. Kirk Kingsbury of the CEL Riggers and Mechanics Shop operated the drum winches used to raise and lower the ocean-anchored lines. The invaluable contribution of these men is greatly appreciated.

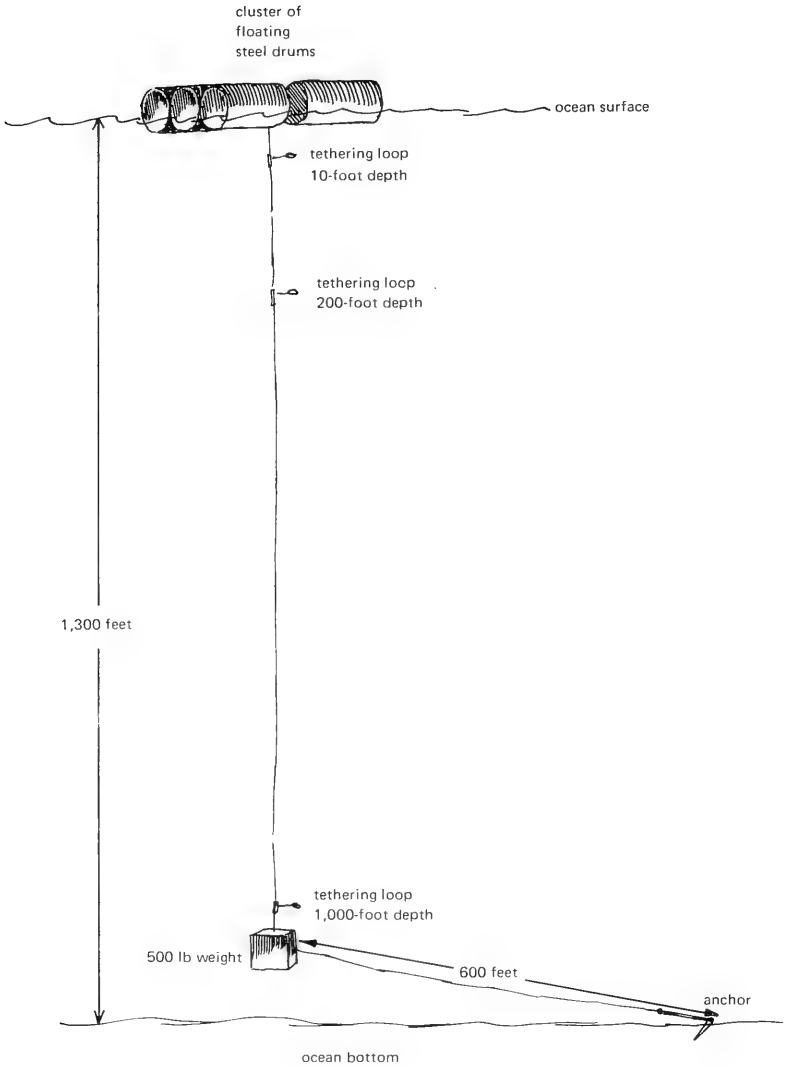

Figure 1. Diagram of ocean exposure station.

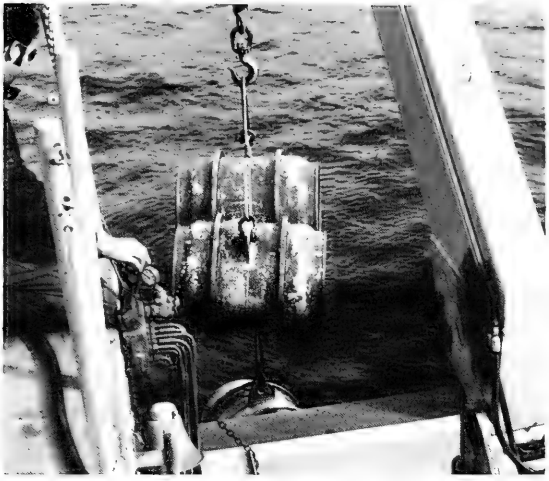

Figure 2. Lowering steel drums from which sample line will be suspended in the ocean.

Figure 3. Weight for holding sample line taut in the ocean.

Figure 4. Winch for lifting and dropping sample line and anchor in the ocean.

Figure 5. Serial dilution of samples for bacterial counts.

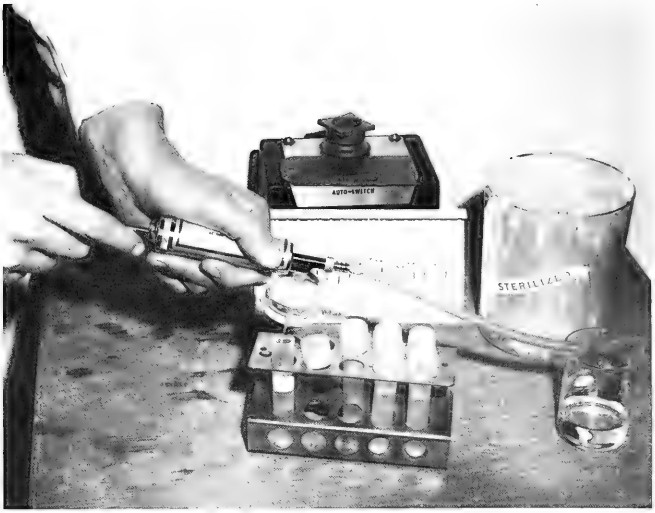

Figure 6. Filtering bacteria from diluted fraction of water sample.

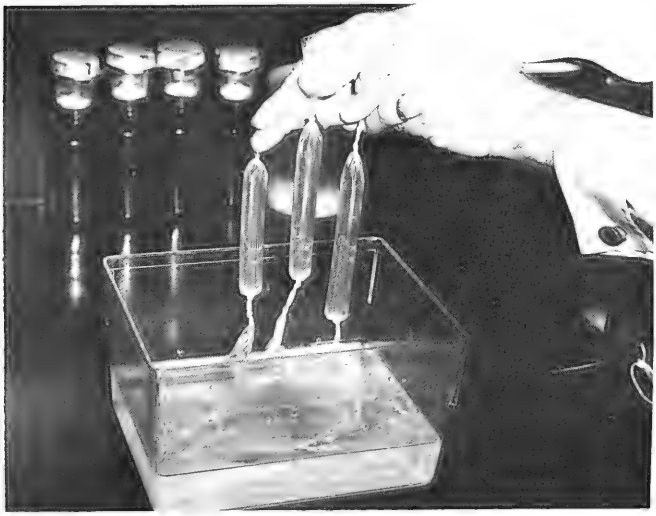

Figure 7. Dialyzer bags filled with cultures of E. coli in seawater.

Figure 8. Translucent and opaque cages for holding bagged water samples.

Figure 9. Emptying dialyzer bag into sterile test tube.

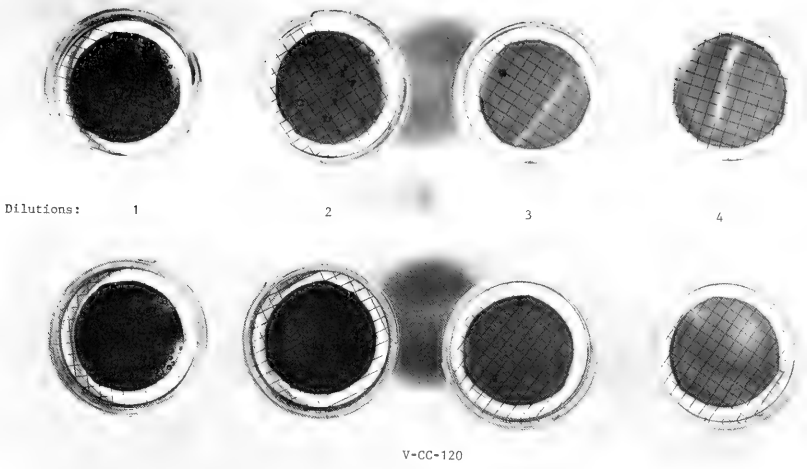

Figure 10. Bacterial colonies on duplicate sets of membranes for 120-hour samples from translucent cage at 200-foot depth.

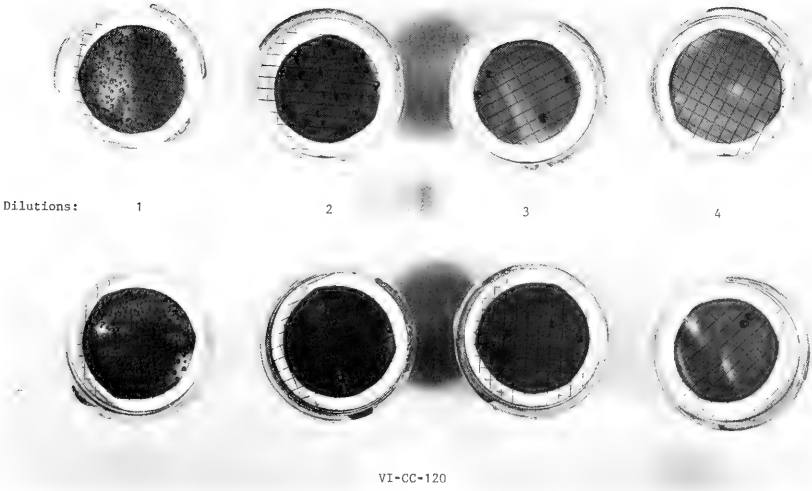

Figure 11. Bacterial colonies on duplicate sets of membranes for 120-hour samples from opaque cage at 200-foot depth.

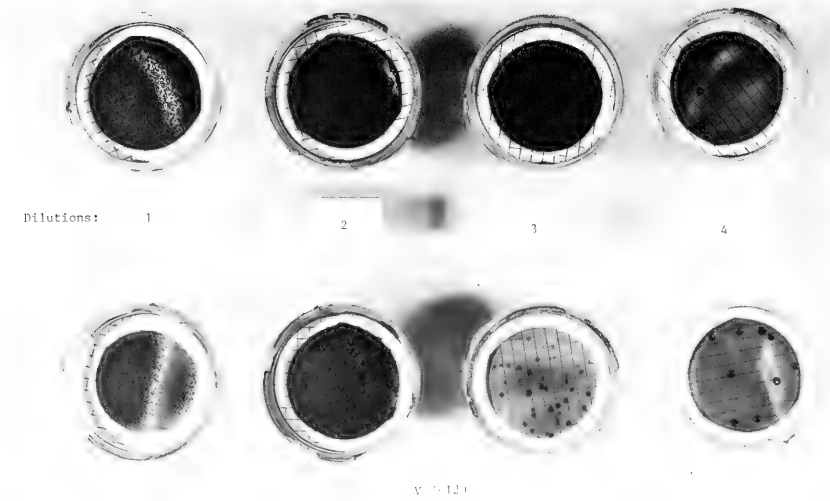

Figure 12. Bacterial colonies on duplicate sets of membranes for 120-hour samples from translucent cage at 1,000-foot depth.

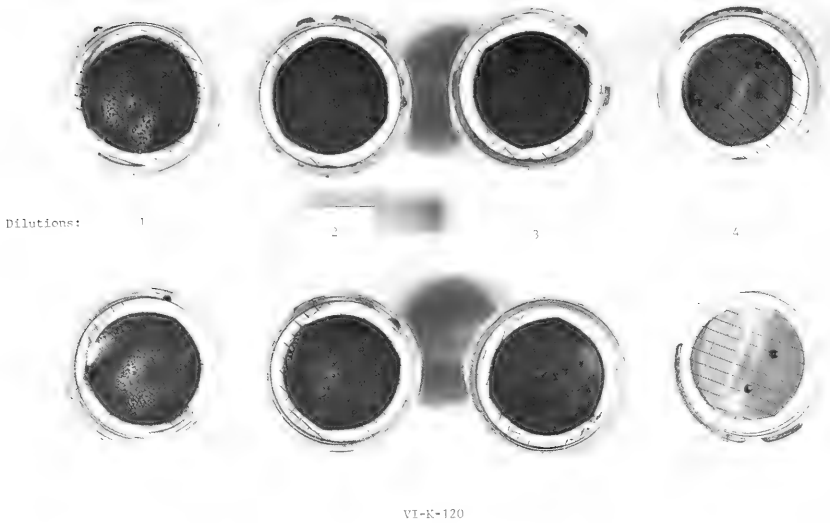

Figure 13. Bacterial colonies on duplicate sets of membranes for 120-hour samples from opaque cage at 1,000-foot depth.

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