STUDIES ON THE THERMAL SENSITIVITY OF MARINE BACTERIA[1]

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There are numerous reports (Benecke, 1933, Waksman, 1934, ZoBell, 1938) on the occurrence and importance of bacteria in the sea in different parts of the world but, unfortunately, the methods of investigation used by various workers have been so widely divergent that neither the qualitative nor the quantitative results are comparable. One of the greatest variables is the temperature to which the bacteria have been subjected, although Forster (1892), Drew (1910), Berkeley (1919), and others have emphasized the extreme thermal sensitivity of marine bacteria. In fact, due to a lack of appropriate refrigeration of water baths, incubators, and other facilities while working on a boat at sea, marine bacteria have been subjected to wide ranges of temperature. This paper is concerned with the effect that this may have upon the life processes and death of the bacteria.

Only those bacteria found in the sea which will grow in nutrient sea water media but not in corresponding freshwater media, or those which have been isolated from a marine environment at places remote from possibilities of terrigenous contamination, are regarded as marine species. This distinction is made to exclude bacteria of obviously terrestrial origin with which bays, estuaries and coastal waters are contaminated. While there may be an interchange of bacteria between the land and the sea (Burke,

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1934) with certain bacteria common to both environments, the majority of those occurring under truly oceanic conditions are believed to be distinctive marine species (ZoBell and Feltham 1933).

EXPERIMENTAL METHODS

Samples of sea water for analysis were obtained by means of the bacteriological sampler described by ZoBell and Feltham (1934) and bottom deposits were obtained with a Trask coring tube (Hough, 1939). Radially central portions of the mud core were dissected out aseptically for bacteriological purposes. Most of the samples were analyzed shortly after collection although sometimes the urgency of other activities, rough weather or other adverse field conditions necessitated storage of the samples. Since earlier studies have revealed that the total number of bacteria in sea water (ZoBell and Anderson, 1936) as well as in mud samples (ZoBell, 1938) increases rapidly with storage, a change which is accompanied by a decrease in the number of different species discernible, the stored samples were held in the refrigerator at near 0°C. At this temperature the changes in the bacterial population are minimized, though not entirely prevented. There is no evidence that any species are killed or overgrown even after several days storage at 0°C., but many heat-sensitive species are destroyed by a few minutes exposure to temperatures exceeding 25°C. and the fastidious ones are soon crowded out when samples are stored at temperatures higher than 5°C. Prescott and Winslow (1931) have discussed the effect of storage upon water bacteria with particular reference to temperature.

Sterile sea water was used for dilution blanks. The nutrient media contained 0.2 per cent each of Bacto-peptone, proteose-peptone and beef extract and 0.002 per cent iron citrate in sea water using either 1.2 per cent agar or 10 per cent gelatin as solidifying agents. The reaction was adjusted to pH 7.6 with N/10 NaOH after autoclave sterilization. Unless otherwise stated, 10 ml. of medium was used to pour the plates and the plates were incubated for two weeks at 22°C. The colonies were counted with a Stewart colony counter using a 3.5× engraver's lens.

POURING TEMPERATURE OF THE MEDIUM

Not infrequently nutrient agar is poured into inoculated plates before it has cooled to 42°C., below which temperature it begins to congeal. (Similar concentrations of agar congeal at a somewhat higher temperature in sea water than in freshwater.) In order to ascertain to what extent the temperature of melted agar influences plate counts, samples of sea water were plated with agar at temperatures ranging from 42° to 60°C. The medium was cooled to exactly the stated temperature in a water bath and then poured into 10 cm. Pyrex Petri dishes previously inoculated with 1.0 ml. of raw sea water.

TABLE 1

Relative number of colonies developing from sea water or marine mud when plated with nutrient agar at different temperatures, the plate counts being expressed as percentages of the average plate count on media poured at 42°C.

INOCULA	NUMBER OF SAMPLES	POURING TEMPERATURE OF AGAR				
		42°C.	45°C.	50°C.	55°C.	60°C.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Sea water.....	14	**100**	95.8	89.4	34.2	17.5
Marine mud.....	9	**100**	93.4	82.1	26.9	11.4

More bacteria developed on the medium which was poured at 42°C. than on that poured at higher temperatures. Moreover, the agreement between duplicates was better when the agar was poured at the lower temperatures. The results obtained with 14 different samples of sea water and 9 of marine bottom deposits which were plated in duplicate with nutrient agar at different temperatures are summarized in table 1. The average plate counts are expressed as ratios on a basis of the 42°C. count being 100.

The data reveal that about 95 per cent as many bacteria form colonies when the agar is plated at 45°C. as when it is plated at 42°C. and successively smaller percentages at higher temperatures. However, the experiment fails to show what percentage of the bacteria may be rendered incapable of multiplication by pouring the agar at 42°C., a temperature which is considerably

in excess of that of their environment. Since agar begins to congeal at 42°C., gelatin was used to prepare a solid medium which could be plated at lower temperatures. Gelatin is not entirely satisfactory as a solidifying agent because it is liquified by the actively proteolytic bacteria which are abundant in the sea. Therefore, gelatin plates must be counted before the slower-growing bacteria have had time to develop into macroscopically visible colonies.

The nutrient gelatin was poured at temperatures ranging from 30° to 50°C. The plates were incubated at 12°C. for a week. The relative numbers of bacteria which developed on the medium poured at each temperature are shown in table 2 which gives the

TABLE 2

Relative number of colonies developing from sea water or marine mud when plated with nutrient gelatin poured at different temperatures, the plate counts being expressed as percentages of the average plate count on media poured at 30°C.

INOCULA	NUMBER OF SAMPLES	POURING TEMPERATURE OF GELATIN				
		30°C.	35°C.	40°C.	45°C.	50°C.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Sea water	12	100	98.6	96.5	87.5	76.2
Marine mud	16	100	97.9	91.3	83.4	67.8

average of duplicate analyses on 12 samples of sea water and 16 of marine mud.

Approximately as many bacteria developed on the gelatin medium poured at 35°C. as on that poured at 30°C. and almost as many developed on the medium poured at 40°C. However, significantly fewer bacteria developed on the medium poured at higher temperatures. It is evident from these observations that while marine bacteria are extremely sensitive to heat, they are not sufficiently so to invalidate the use of nutrient agar poured at 40° to 42°C. for estimating the abundance of bacteria in marine materials since, at their best, plate counts detect only a small percentage of the bacterial population. It is obvious, though, that the medium should be cooled at least to 42°C. before pouring to insure comparable and maximum counts because even

at this temperature certain heat-sensitive species are inactivated. If, due to the exigencies of field conditions, media are poured at temperatures exceeding 50°C., more than half of the bacteria may fail to develop. Drew (1910) reports that the bacteria from tropical waters around the Bahamas are very sensitive to temperatures as high as 40°C. and exposure at 45°C. causes the death of a large proportion of them.

In the foregoing experiments the plates themselves were at room temperature (21° to 22°C.) at the time the media were introduced. It was found that when the plates were cooled on ice prior to the introduction of the medium, plating temperatures in excess of 42°C. were less injurious. This is what might be expected because the introduced medium is cooled faster, thus subjecting the bacteria therein to the higher temperature for a shorter period of time. As revealed by the results summarized in table 3, little or no practical advantage is gained by cooling the plates on ice when the medium itself is cooled to 42°C. before pouring but the beneficial effect of the use of ice is quite pronounced when the medium is poured at higher temperatures. The use of ice to hasten the cooling of the media might save a few heat-sensitive species from destruction but for practical purposes this procedure does not increase the plate count enough to offset the disadvantages of the extra work involved and the undesirable effects of the medium congealing in the cold plate before it has been evenly distributed (Green, 1936).

Other factors which influence the effect of the pouring temperature of the medium are the type of plate, the heat-conductivity of the table upon which the plate rests and the size of the inoculum. The media are cooled faster by thick-walled Pyrex Petri dishes of high heat-holding capacity than by thin-walled ones, assuming that the dishes themselves are at a low temperature when the medium is introduced. Similarly, the media are cooled faster when the plates are resting on a foundation of concrete, metal or soapstone than on one of wood. Such factors influence the magnitude of plate counts and the agreement between duplicates.

Prescription bottles are quite widely used as a substitute for

Petri dishes for field studies. One milliliter of the appropriately diluted sample is introduced directly into 15 ml. of melted nutrient agar in the bottles cooled to 40° to 42°C. The inoculated bottles are then placed on their sides until the agar has solidified. In the initial experiments less than half as many colonies developed in prescription bottles inoculated by this procedure as when a similar medium was used in Petri dishes, probably because

TABLE 3

Relative number of colonies developing from sea water plated with nutrient agar poured at different temperatures into plates at different temperatures, the plate counts being expressed as percentages of the average plate count on media poured at 42°C. into dishes having a temperature of 21–22°C.

TEMPERATURE OF PLATE	POURING TEMPERATURE OF AGAR				
	42°C.	45°C.	50°C.	55°C.	60°C.
°C.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Near 0	108	103	95	68	51
21–22	100	97	86	41	16
30	79	72	61	36	14

TABLE 4

Relative number of colonies which developed from samples of sea water or mud after being held at the stated temperature for 10 minutes, the plate counts being expressed as percentages of the plate count of material held for ten minutes at 20°C.

INOCULA	NUMBER OF SAMPLES	EXPOSURE TEMPERATURE						
		20°C.	30°C.	40°C.	50°C.	60°C.	80°C.	100°C.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Sea water	10	100	81.3	21.9	6.8	3.0	0.2	+
Marine mud	10	100	68.5	18.3	10.3	5.2	0.7	+

the prescription bottles cool so slowly. By immersing them to the neck for thirty seconds in ice water immediately after inoculating, almost as many colonies developed in prescription bottles as in Petri dishes.

Marine bacteria are not unique in their susceptibility to the pouring temperature of nutrient agar because the senior author found that the bacteria indigenous to Lake Mendota (a freshwater lake in Wisconsin) are similarly heat-sensitive. Using the pre-

scription bottle technique, two to five times as many bacteria developed when the bottles were quickly cooled after inoculation by immersion in cold water as when they were merely permitted to cool on the table top, although in both cases the medium was cooled to 40° to 42°C. before inoculation.

The use of pre-solidified agar as advocated by Anderson and Stuart (1935) obviates the necessity of exposing the bacteria to the temperature of melted agar. While, statistically, the counts obtained by this procedure compare favorably with plate counts obtained by the conventional technique, there is a greater divergence of duplicates and many time-consuming precautions must be exercised to prepare satisfactory plates.

THERMAL DEATH POINT

The temperature tolerance of marine bacteria was determined using thermal death point technique. For this purpose 2.0 ml. portions of recently collected sea water or appropriately diluted mud samples were placed in 6 ml. serological tubes. The use of the small thin-walled tubes reduced to a minimum the time required to change the temperature of the contents. Pairs were immersed in water baths ranging in temperature from 20° to 100°C. After exactly ten minutes the tubes were transferred to ice water and 1.0 ml. of the heated suspension was spread uniformly over the surface of pre-solidified nutrient agar in Petri dishes. The plates were incubated at 12°C. for two weeks. Table 4 shows the average number of colonies which developed from samples of sea water and marine mud treated in this manner.

There is no evidence to suggest that any of the bacteria are injured by ten minutes exposure to a temperature of 20°C. regardless of the temperature of the environment from which they were obtained. However, about one-fourth of the bacteria were rendered incapable of multiplication in ten minutes at 30°C. and only one-fifth of them survived after being held for this period of time at 40°C. Direct microscopic observations of the material showed that the heat treatment had not merely caused the bacteria to clump together or to adhere to the walls of the sero-

logical tubes which would have reduced the plate counts. Moreover, as will be discussed below, the respiration of the bacteria was impaired by the heat treatment.

In general, the bacteria from bottom deposits were found to be somewhat more heat-sensitive than those occurring in sea water. A few heat-tolerant spore-forming bacteria were found in nearly all of the samples, there being more of these in mud than in water. Most of the bacteria which survived temperatures higher than 40°C. proved to be spore formers. Not many of the spore formers survived at 80°C. and too few survived boiling for ten minutes to warrant the numerical expression of an average from the available data.

TABLE 5

Number of pure cultures which multiplied after being held at the stated temperature for 10 minutes

DESCRIPTION OF CULTURES	EXPOSURE TEMPERATURE						
	20°C.	30°C.	40°C.	50°C.	60°C.	80°C.	100°C.
"C_1" from water	25	24	9	3	0	0	0
"C_1" from mud	25	21	11	5	1	1	0
Stock cultures	78	78	36	14	8	6	2

The temperature tolerance of several pure cultures of marine bacteria was tested by noting their ability to reproduce after being heated. Nutrient sea-water broth was inoculated and distributed in serological tubes. Pairs of these were held in water baths at different temperatures for ten minutes and then cooled immediately in ice water, after which the bacteria were tested for viability. Table 5 shows the number of cultures which multiplied following this treatment.

The cultures designated "C_1", are colonies differing superficially from each other, fished directly from pre-solidified nutrient agar which had been inoculated with freshly collected samples of sea water or marine mud and incubated at 12°C. At no time were these organisms subjected to temperatures higher than 12°C. until they were tested for their temperature tolerance. The "stock" cultures, all differing either morphologically, culturally

or physiologically from each other, have been isolated over a period of years from sea water or other marine materials. They have been sub-cultured many times and maintained on sea-water agar slants in the refrigerator at 0° to 4°C.

Only five cultures out of the 128 tested failed to grow after being held at 30°C. for ten minutes but many of them multiplied less rapidly, indicating that while all of the individuals comprising any one culture had not been killed, many of the individuals were injured. This was confirmed later by plate counts on the cultures and observations on the respiration of heat-treated cultures. It is noteworthy that over half of the pure cultures were killed in 10 minutes at 40°C. Similarly Bedford

TABLE 6

Oxygen consumed by suspensions of marine bacteria in two hours at 20°C. after being heated to the stated temperature for ten minutes and the number of viable bacteria in the heated suspensions

	EXPOSURE TEMPERATURE			
	20°C.	30°C.	40°C.	50°C.
Oxygen uptake (mm.^{3})	0.92	0.54	0.19	0.04
Bacteria per ml. × 100,000	267	184	51	9

(1933) found that 37°C. was lethal for 40 of the 71 cultures of marine bacteria with which he was working.

According to Bronfenbrenner *et al.* (1939) the respiration of bacteria is a better criterion of their viability than their ability to reproduce in a given medium. Studies on the oxygen uptake of suspensions of marine bacteria demonstrated that many were rendered incapable of respiration by 10 minutes exposure at 30°C. The tests were made by pipetting 2.0 ml. of a heavy suspension of a 24-hour old enrichment culture of mixed marine microflora into Barcroft respirometer flasks. Duplicates of each were held in water baths at 20°, 30°, 40° and 50°C. for ten minutes, after which they were cooled immediately to 20°C. After placing 0.2 ml. of 10 per cent KOH solution in the inset the respirometer flasks were fitted to the manometers and the oxygen uptake of each suspension was noted after two hours at 20°C.

Appropriate dilutions of each suspension were plated on nutrient agar to determine the number of viable cells. The results are summarized in table 6.

It will be observed that the decrease in the number of viable bacteria as indicated by plate counts is proportional to the decrease in the oxygen uptake of the heat-treated bacteria. Similar observations were made on four different heat-sensitive pure cultures which failed to multiply after being held at 40°C. for ten minutes. The loss of the ability of heated cultures to consume oxygen indicates that the respiratory enzymes of the bacteria have been inactivated. According to Edwards and Rettger (1937) the maximum temperature tolerance of bacteria is related to the minimum temperature at which their respiratory enzymes are destroyed.

OPTIMUM TEMPERATURE OF INCUBATION

In spite of the fact that Standard Methods of Water Analysis (1933) is concerned primarily with the bacteria which are of sanitary significance and which may differ markedly from the autochthonous microflora of natural waters, many bacteriologists have taken literally the instructions to count agar plates at either 20° or 37°C. when analyzing ocean, lake or river water. Most frequently the plates have been incubated at some intermediate temperature, namely, 25° or 30°C. We have studied the effect of the temperature of incubation upon plate counts by inoculating Petri dishes in groups of 14 each with 1.0 ml. of sea water or marine mud. Duplicate plates from each sample were incubated at 4°, 12°, 18°, 22°, 25°, 30° and 37°C. After different periods of incubation the colonies were counted. Table 7 shows the average results obtained with ten samples of sea water and four of marine mud. The colony counts were calculated as percentages of the maximum colony count, assuming the latter to be the colony count on plates incubated at 18°C. for 18 days. As a matter of fact the maximum number of colonies appeared on only half of the plates incubated at 18°C. for 18 days. The maximum counts of four samples occurred on plates incubated at 12°C. and the maximum counts of the other three samples were on plates

incubated at 22°C. Results with water and mud samples were almost the same.

For the first few days of incubation the most colonies were found on plates incubated at 25° or 30°C., but after seven to ten days the most colonies were found on plates incubated at 12° to 22°C. The bacteria which multiply at the higher temperatures do so more rapidly and hence appear earlier as colonies than those incubated at lower temperatures. As a rule the colonies found on the plates incubated at the higher temperatures are larger than those growing at lower temperatures and give the former

TABLE 7

Relative number of colonies appearing on nutrient agar incubated for different periods of time at different temperatures, the plate counts being expressed as percentages of the average plate count after 18 days at 18°C.

INCUBATION TIME	INCUBATION TEMPERATURE						
	4°C.	12°C.	18°C.	22°C.	25°C.	30°C.	37°C.
days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	0	18	30	36	41	44	8
4	0	28	41	60	65	61	12
7	4	46	67	82	78	69	12
10	9	67	91	96	84	71	13
14	17	90	98	97	85	70	
18	26	97	100	95	82	63	
21	33	98	96	87	74	53	

plates the superficial appearance of having more colonies. The decrease in the counts after two weeks on plates incubated at 25°C. or higher is due to the merging of colonies and the liquefaction of the agar by certain bacteria, thereby obliterating the surrounding colonies. Very few colonies developed on the plates incubated at 37°C.

The results confirm previous experiments on the thermal sensitivity of marine bacteria and show that, in general, media inoculated with sea water or marine sediments should not be incubated at temperatures exceeding 22°C. The greatest number of different species can be detected on plates incubated at 12°C. or lower, presumably because fewer heat-sensitive species are

inactivated and apparently all of them can grow, though slowly, at 4° to 12°C. Moreover, the lower temperatures seem to favor pigment production (Hess, 1933a) and as pointed out by ZoBell and Feltham (1934) most marine bacteria are chromogenic under favorable conditions. However, due to the slowness with which colonies form at relatively low temperatures, the maximum number of visible colonies will be found on plates incubated at 18° to 22°C. for a week or two. Except for special purposes it is not feasible to incubate plates for several weeks before counting.

DISCUSSION

Since the temperature of the ocean is monotonously constant with over 80 per cent of the water and bottom perpetually colder than 5°C., it is not surprising to find that marine bacteria are extremely thermo-sensitive. It so happens that the critical point for the majority of them is near the temperature at which nutrient agar begins to congeal. While too few bacteria are killed when the medium is properly cooled to invalidate the use of plating procedures for estimating bacterial populations, it should be emphasized that prolonged exposure at 40° to 42°C., or instantaneous exposure at temperatures a few degrees higher, is lethal for a large percentage of the bacteria from the sea and perhaps from lakes also.

Anomalously the optimum temperature for the multiplication of marine bacteria in the laboratory is several degrees higher than the environment inhabited by them. Though coming from an environment which is for the most part considerably colder than 12°C., the optimum temperature for maximum plate counts of bacteria from the sea is between 12° and 22°C. The pure cultures which have been studied have temperature optima ranging from 18° to 37°C., the range for the majority being 18° to 25°C. This has been found to be true of cultures which were isolated from plates incubated at 12°C. and which had not been subjected to higher temperatures until the tests were made. Working with 71 species of bacteria from the northern Pacific Ocean, most of which were concerned with the spoilage of fish at refrigeration temperatures, Bedford (1933) found that none of them had

optima lower than 20°C. Too many intrinsic as well as extrinsic factors are involved which influence the temperature tolerance of bacteria to speculate at this time why certain bacteria have temperature optima which are several degrees higher than the environment inhabited by them.

Bacteria which are transferred directly from their native habitat to artificial media are subjected to many abrupt environmental changes besides temperature but the inimical effect of the adverse condition will probably be proportional to the temperature in accordance with the R.G.T. or van't Hoff rule. However, if the bacteria survive the shock of transplantation and start to multiply, the resulting cultures are more tolerant of laboratory conditions in general, including temperature extremes which may be because, as expounded by Sherman and Cameron (1934), physiologically old cells withstand adverse conditions better than young ones. Consequently the temperature tolerance seems to increase and the most tolerant individuals will soon predominate in the culture as the less tolerant ones fail to multiply or perish, or as stated by Reimann (1937), "an environment unfavorable to one of two mutants will cause the elimination of one and permit the growth of the other." It may be for this reason that Kluyver and Baars (1932) maintain that the longer a culture has been in the laboratory the less adaptive ability it possesses. Incidentally, like the experience of Casman and Rettger (1933) with members of the "*subtilis* group," our attempts to acclimate marine bacteria to temperatures higher than the maximum of 3- or 4-week old sub-cultures have been unsuccessful.

There is no evidence to indicate that temperatures as low as 0° to −5°C. injure marine bacteria although according to Hess (1933b), they slowly die at −16°C. Most of them multiply and are otherwise physiologically active until the water essential to chemical reactions is removed by solidification due to freezing. ZoBell (1934) reports that 76 out of 88 different species of marine bacteria multiplied slowly at 0° to −4°C. Bedford (1933) found that all of his 71 species except three grew at 0°C. and 23 of them grew at −5°C. Neither Bedford nor the senior author have

found true psychrophiles, or cultures which grow best at relatively low temperatures, but Hess (1933b) gives 5°C., as the optimum temperature for the multiplication of the marine bacteria which he has studied. Berry and Magoon (1934) who have been working with microorganisms which grow at sub-zero temperatures question the existence of a true cold-loving or psychrophilic flora.

SUMMARY

Many of the bacteria occurring in sea water and marine sediments are sensitive to the plating temperature of nutrient agar, there being significantly fewer which formed colonies on agar plated at 45°C. or above than on that plated at 42°C. There were only 81 to 83 per cent as many colonies which developed on nutrient gelatin plated at 45°C. as on that plated at 30°C.

Heating samples of sea water and mud to 30°C. for ten minutes killed around 25 per cent of the bacteria, and only 20 per cent of the bacteria survived 40°C. for ten minutes.

A diminution of the oxygen uptake of suspensions of heat-treated bacteria indicated that the respiratory enzymes of some forms are inactivated by temperatures as low as 30°C.

Nutrient agar inoculated with sea water or marine sediments yields maximum colony counts when incubated at 18° to 22°C. for a week or two. Very few colonies develop on plates incubated at 30° to 37°C.

Most of the bacteria isolated from the sea grow best at temperatures which are considerably higher than the marine environment inhabited by them. Although nearly all marine bacteria multiply slowly at near zero temperatures, true psychrophiles have not been found.

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REFERENCES

ANDERSON, E. H., AND STUART, C. A. 1935 A quantitative differential method for counting mixed cultures of bacteria. J. Bact., **30**, 207–209.

BENECKE, W. 1933 Bakteriologie des Meeres. Abderhalden's Handb. biol. Arbeitsmethoden, **404**, 717–872.

BEDFORD, R. H. 1933 Marine bacteria of the northern Pacific Ocean. The temperature range of growth. Contrib. Can. Biol. Fisheries N. S. **7**, 431–438.

BERKELEY, C. 1919 Study of marine bacteria. Strait of Georgia, B. C. Trans. Roy. Soc. Can., **13**, 15–43.

BERRY, J. A., AND MAGOON, C. A. 1934 Growth of microorganisms at and below 0°C. Phytopathology, **24**, 789–796.

BRONFENBRENNER, J., HERSHEY, A. D., AND DOUBLY, J. 1939 Evaluation of germicides by a manometric method. J. Bact., **37**, 583–597.

BURKE, V. 1934 The interchange of bacteria between fresh water and the sea. J. Bact., **27**, 201–205.

CASMAN, E. P., AND RETTGER, L. F. 1933 Limitation of bacterial growth at higher temperatures. J. Bact., **26**, 77–123.

DREW, G. H. 1910 On the precipitation of calcium carbonate in the sea by marine bacteria, and on the action of denitrifying bacteria in tropical and temperate seas. J. Marine Biol. Assoc. United Kingdom, **9**, 479–524.

EDWARDS, O. F., AND RETTGER, L. F. 1937 The relation of certain respiratory enzymes to the maximum growth temperatures of bacteria. J. Bact., 34, 489–515.

FORSTER, J. 1892 Ueber die Entwickelung von Bakterien bei niederen Temperaturen. Zentr. Bakt. Parasitenk., **12**, 431–436.

GREEN, G. 1936 Experiments to devise a better procedure for making bacterial counts than outlined in Standard Methods. Ohio Conf. Water Purification, **18**, 77–87.

HESS, E. 1933a Cultural characteristics of marine bacteria in relation to low temperatures and freezing. Contrib. Can. Biol. Fisheries, N. S. **8**, 459–474.

HESS, E. 1933b Effects of low temperatures on the growth of marine bacteria. Contrib. Can. Biol. Fisheries, N. S. **8**, 489–505.

HOUGH, J. L. 1939 Bottom-sampling apparatus. Recent Marine Sediments. Bull. Am. Assoc. Petroleum Geol., Tulsa, Okla., 629–664.

KLUYVER, A. J., AND BAARS, J. K. 1932 On some physiological artefacts. Proc. Acad. Sci. Amsterdam, **35**, 1–11.

PRESCOTT, S. C., AND WINSLOW, C.-E. A. 1931 Elements of Water Bacteriology. John Wiley and Sons, New York, pp. 6–37.

REIMANN, H. A. 1937 The significance of bacterial variation. J. Bact., **33**, 513–523.

SHERMAN, J. M., AND CAMERON, G. M. 1934 Lethal environmental factors within the natural range of growth. J. Bact., **27**, 341–348.

Standard Methods of Water Analysis. 1933 Standard Methods for the Examination of Water and Sewage. Am. Pub. Health Assoc., New York, 180 pp.

WAKSMAN, S. A. 1934 The distribution and conditions of existence of bacteria in the sea. Ecol. Monographs, **4**, 523–529.

ZOBELL, C. E. 1938 Studies on the bacterial flora of marine bottom sediments. J. Sedimentary Petrology, **8**, 10–18.

ZoBELL, C. E. 1934 Microbiological activities at low temperatures with particular reference to marine bacteria. Quart. Rev. Biol., **9**, 460–466.

ZoBELL, C. E., AND FELTHAM, C. B. 1933 Are there specific marine bacteria? Proc. Pacific Sci. Congr. 5th Congr., Can., **3**, 2097–2100.

ZoBELL, C. E., AND FELTHAM, C. B. 1934 Preliminary studies on the distribution and characteristics of marine bacteria. Bull. Scripps Inst. Oceanog., Univ. Calif., Tech. Ser., **3**, 279–296.

ZoBELL, C. E., AND ANDERSON, D. Q. 1936 Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surface. Biol. Bull., **71**, 324–342.