STUDIES ON MARINE BACTERIA. I. THE CULTURAL REQUIREMENTS OF HETEROTROPHIC AEROBES[1]

By

CLAUDE E. ZOBELL

Scripps Institution of Oceanography
University of California
La Jolla, California

The observations of Issatchenko (1914), Benecke (1933), Waksman (1934), ZoBell (1938) and others have established that bacteria are quite widely distributed in the sea where they probably play an important role influencing chemical, physico-chemical, geological and biological processes. However, due primarily to the widely divergent analytical procedures which have been used by various investigators studying the distribution and activities of marine bacteria, the quantitative results obtained by different workers in different parts of the world are not comparable.

The nutrient medium used to grow the bacteria has been one of the greatest variables. A large variety of nutrient ingredients in varying concentrations and combinations which yield markedly different results have been used and recommended for plate count media. Following literally Standard Methods (1933) of water analysis some workers have used fresh water to prepare their media either with or without the addition of salt, while others have used synthetic or natural sea water although, as will be pointed out below, most marine microorganisms have fairly specific salt requirements. Still other workers have regarded the bacteria of the sea as not unlike those in the soil and have treated them accordingly with discordant results. As pointed out by Lipman (1929), within relatively narrow limits the composition of the medium is an important determinant of the number and kinds of organisms found in the sea. At the present time there are almost as many kinds of nutrient media in use for estimating the marine bacterial population by plate count procedure as there are workers in the field and, as will be shown below, they yield very different results.

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The following report is concerned primarily with a systematic study of the cultural requirements of marine bacteria with special reference to the preparation of a nutrient medium for heterotrophic aerobes which will yield maximum and reproducible counts. While it is recognized that no one combination of nutrients or cultural conditions can provide for the growth of all kinds of bacteria which occur in the sea, the unquestioned value and extensive use of plating procedures for estimating the bacterial population of marine materials, for studying cultural characteristics and for obtaining pure cultures for physiological studies makes it highly desirable to have data which will permit the adoption of media which will yield maximum and reproducible counts. Moreover, such information on the nutritional requirements of marine bacteria will prove to be invaluable in studying their physiological activities and in estimating their importance as biochemical agents in the sea.

Observations during the last eight years on the occurrence and distribution of bacteria in the sea have afforded an excellent opportunity to study their cultural requirements. In analyzing sea water, bottom sediment or other marine materials it has required little additional effort to use two or more combinations of media rather than merely one and moreover, this procedure has served to check the results. Thus, over a period of years, it has been possible to compare the relative growth-promoting properties of several different media which have been proposed for the growth of marine bacteria. New formulae have been developed as indicated by the cumulative results and tried.

EXPERIMENTAL METHODS

Sea water for analysis was collected by means of a bacteriological sampler described by ZoBell and Feltham (1934) which insures exclusion of any extraneous contaminating material. Radially central portions of the cores of bottom deposits obtained with a Trask coring tube (Hough, 1939) were dissected out for analysis, using aseptic precautions in all manipulations. Except where otherwise designated, the samples were collected at places which are remote from possibilities of terrigenous contamination. Autoclaved sea water or a mixture of 75 per cent sea water with 25 per cent distilled water was used to dilute the samples, the samples being diluted sufficiently to provide for the growth of 30 to 300 colonies on the plates which were acceptable.

In the preliminary work a medium similar to that recommended by Standard Methods (1933) of water analysis was used:

Bacto-peptone	5.0 gm
Beef extract	2.0 gm
Bacto-agar	15.0 gm
Sea water	1000.0 ml

This will be referred to as Formula 1 and has been used as the standard for comparing many other media. The sea water used in the preparation of the media, unless otherwise stated, was collected and aged for several weeks in 5 gallon glass bottles to insure uniformity in the composition.* The reaction of Formula 1 is pH 7.6 without adjustment after sterilizing at 120° C. for 20 minutes. Special care was taken to cool the nutrient agar to 42° C. before pouring into the inoculated plates because, as has been pointed out by ZoBell and Conn (1940), most marine bacteria are thermo-sensitive, many of them being injured by even short exposure to temperatures exceding 42° C. The inoculated plates were incubated at 22° C., it having been shown that prolonged exposure at higher temperatures is lethal to some marine bacteria. The colonies which developed on the plates after five to ten days were counted, using a 3.5X engraver's lens and a Stewart counting chamber.

SALINITY REQUIREMENTS

Fresh water, either with or without the addition of salts, has been quite commonly used for estimating the bacterial population of marine materials. Lloyd (1930), Burke (1934) and others report that marine bacteria grow equally well in either fresh water or sea water media. Conversely, Berkeley (1919), Korinek (1926) and Lipman (1926) maintain that the mere dilution of sea water materially reduces the number of marine bacteria which will grow. According to Korinek (1927) marine bacteria can be distinguished from non-marine forms upon a basis of their specific salt tolerance.

*Matudaira (1939) and others have noted a marked difference in the growth-promoting properties of sea water collected at different depths, stations and dates on the growth of diatoms which is attributed to something organic in nature. Similarly we have noted that bacteria behave differently in media prepared with sea water collected from different stations. These differences in the growth-promoting properties of sea water disappear within a few weeks when the sea water is stored or "aged" at room temperature presumably because the organic fractions which are responsible for the differences are oxidized by the increased bacterial activity which accompanies the storage of sea water in glass bottles (ZoBell and Anderson, 1936a).

The validity of these antithetical views was tested by preparing nutrient media which were similar in all respects except salt content. For this purpose, Formula 1 was used with the sea water diluted with distilled water to give various concentrations. These media were inoculated with appropriately diluted samples of raw sea water and their relative growth-promoting properties determined. Table I gives the protocol of a representative experiment. To facilitate the comparison of results the "growth index" for each medium is recorded. The growth index is the expression of the average plate counts as ratios on a basis of the plate counts on the standard medium being 100.

TABLE I

RELATIVE NUMBERS OF BACTERIA IN A SAMPLE OF RAW SEA WATER WHICH FORMED COLONIES IN NUTRIENT MEDIA PREPARED WITH DIFFERENT CONCENTRATIONS OF SEA WATER

Per cent sea water	100	75	50	25	10	5	0
Per cent distilled water	0	25	50	75	90	95	100
Bacteria in sample 9A	218	196	139	87	38	24	13
Bacteria in sample 9B	231	204	143	82	35	28	21
Average number of bacteria	224	200	141	84	36	26	17
Growth index	**100**	89	63	41	16	12	8

Over thirty samples of sea water and nearly as many samples of marine bottom deposits collected from various stations off the coast of Southern California have been analyzed in this way. Without exception appreciably more bacteria grew in the media prepared with sea water than in corresponding fresh water media. Only 55 to 65 per cent as many colonies appeared on plates of media prepared with sea water diluted one half with fresh water, and in fresh water media only 5 to 20 per cent as many bacteria developed as in that prepared with undiluted sea water. Similar results were obtained regardless of whether tap water, distilled water or glass re-distilled water was used, thus ruling out the possibility of the fresh water carrying any toxic agent. From the data it is quite apparent that sea water and not distilled water or tap water should be used in the preparation of nutrient media for marine bacteria.

In the experimental procedure outlined above 1.0 ml. of raw sea water or mud diluted with sea water was used to inoculate approximately 10 ml. of nutrient agar. It follows, then, that upon the inoculation of the fresh water medium in which no sea water was used in its preparation, there is around 10 per cent of sea water in the medium, and the other media will actually contain a somewhat larger percentage of sea water than that designated in the table. Sea water

can be almost completely excluded from the fresh water media by the use of fresh water for diluting mud and other marine materials containing a rich microflora, but another complication is introduced: the viability of marine bacteria is rapidly weakened by such treatment. In a few experiments in which fresh water dilution water was used there were only 3 to 5 per cent as many bacteria developed in the fresh water medium as in that prepared with sea water.

Bacteria in bottom deposits are slightly less sensitive to salinity changes than are those which occur in water as shown by the composite data in Table II. Those obtained from terrestrially polluted

TABLE II

RELATIVE NUMBERS OF BACTERIA FROM MARINE AND TERRESTRIAL SOURCES WHICH DEVELOPED IN NUTRIENT MEDIA PREPARED WITH DIFFERENT CONCENTRATIONS OF SEA WATER

Source of Inocula	*Samples Analyzed*	*Per cent of sea water in medium*					
		100	75	50	25	10	0
		Average growth index					
Raw sea water	31	**100**	93	65	38	17	9
Marine mud	18	**100**	89	61	46	28	19
Mission Bay mud	4	**100**	107	117	142	156	106
San Diego Bay mud	3	**100**	108	131	142	139	114
Mission Bay water	5	**100**	103	115	108	118	97
Sewage	13	13	27	51	79	106	**100**
Tap water	8	4	10	28	58	105	**100**
Soil (near sea)	7	48	56	79	90	97	**100**
Soil (inland)	6	15	23	40	66	89	**100**
Mouth microflora	5	9	15	41	78	93	**100**

bays (Mission Bay and San Diego Bay) seem to grow almost equally well in fresh water or sea water nutrient media, or at least there are about as many bacteria capable of growing in fresh water as in sea water media. The growth of terrigenous or fresh water bacteria is inhibited by sea water. Several samples of sewage, tap water, soil and microflora from the buccal cavity of man were plated with media containing different concentrations of sea water and the results are summarized in Table II. The samples of microflora from the buccal cavity were obtained by having several laboratory workers rinse their mouths with sterile saline and pooling the washings.

It is noteworthy that soil bacteria collected from inland valleys were much more sensitive to sea water than those in soil samples collected on the Scripps Institution grounds adjacent to the sea. How-

ever, this is not surprising because ZoBell and Mathews (1936) have shown that from 70 to 95 per cent of the bacterial flora being transported by off-sea breezes are of marine origin and that marine bacteria occur in the atmosphere 30 miles inland. Most of the bacteria which are transported inland from their marine environment to which they are indigenous soon perish; more resistant ones may survive for a time and a few probably have become acclimatized to the soil with attendant changes in their properties (Burke and Baird, 1931; Burke, 1934). Consequently, one would expect to find more marine-like bacteria in the soil near the sea than farther inland. According to ZoBell and Mathews (1936) the microflora transported by off-sea breezes is predominately terrestrial in character, fewer than half of the bacteria being able to grow in sea water media. Thus, the wind plays an important role in the interchange of bacteria between the land and sea and helps to account for the fact that 5 to 20 per cent of the bacteria in the sea are capable of growing in fresh water media and a like proportion of the bacteria in fresh water or terrestrial materials are capable of growing in sea water media. However, as will be elaborated elsewhere, certain bacteria are euryhaline.

Although a large proportion of the bacteria in marine materials are definitely stenohaline upon initial isolation, they rapidly lose their saline specificity following laboratory cultivation. After a few weeks most cultures which initially would grow only in sea water media grow equally well in either sea water or fresh water media (ZoBell and Michener, 1938).

The specific salt requirements of bacteria from oceanic environments as compared with those from brackish water, polluted bays, fresh water or terrestrial sources may account for many of the contradictory opinions expressed in the literature concerning the characteristics of marine bacteria. It is astonishing to note how many kinds of water bacteriologists have designated "sea water" without any qualifying statements concerning the salinity, isolation from oceanic circulation or other properties. Since much of the early work was done on samples obtained from seas or surface water having a low salinity, bays, harbors, estuaries and other coastal waters which are subject to varying degrees of dilution with freshwater and terrigenous contamination, one does not know if the workers were dealing with either marine bacteria or sea water.

An attempt has been made to ascertain just what there is in sea water which makes it indispensable for the initial growth of most marine bacteria, but the complete explanation has not yet been found. As shown by the data in Table III, the addition of 3.0 per cent

TABLE III

RELATIVE NUMBER OF BACTERIA IN 8 SAMPLES OF RAW SEA WATER AND 6 OF MARINE MUD WHICH DEVELOPED IN COMPARABLE MEDIA PREPARED WITH VARIOUS SALT SOLUTIONS

	Natural sea water	*3.0% sodium chloride solution*	*Artificial sea water*	*Isotonic sea salt solution*	*Distilled water*
			Average growth index		
Raw sea water	**100**	56	77	89	7
Marine mud	**100**	61	74	94	16

sodium chloride to fresh water nutrient medium to render it isotonic with sea water materially improves results, but such a medium is not as good as sea water for the growth of marine bacteria. Synthetic or artificial sea water prepared with several salts including a trace of ferric phosphate in the hypothetical combinations in which they occur in sea water (Lyman and Fleming, 1940) is better than the sodium chloride solution, but it fails to provide for the growth of as many bacteria as natural sea water. There seems to be something in sea water besides its mineral constituents which is essential for the growth of marine bacteria because not even isotonic concentrations of sea salt were as good as natural sea water (Table III). The sea salt was prepared by evaporating sea water in vacuo and then adding distilled water to prepare nutrient media. Allen (1913) found that certain diatoms would not grow in synthetic media unless a little sea water was added, an observation often made on other organisms.

Contrary to popular conception, marine bacteria do not tolerate high concentrations of salts much better than they tolerate hypotonicity. This is illustrated by the results summarized in Table IV,

TABLE IV

RELATIVE NUMBERS OF BACTERIA IN 12 SAMPLES OF RAW SEA WATER WHICH DEVELOPED IN NUTRIENT MEDIA PREPARED WITH SEA WATER TO WHICH DIFFERENT SALTS WERE ADDED

			Per cent of salt added			
Salts added to sea water	0	1.5	3.0	6.0	12.0	18.0
			Average growth index			
Sodium chloride	**100**	96	88	41	12	3
Artificial sea water	**100**	90	72			
Sea salt	**100**	81	49			

which were obtained by inoculating media prepared with sea water to which different concentrations of sodium chloride, artificial sea water constituents or evaporated sea salt were added. Surprisingly enough, sea water rendered hypertonic with evaporated sea salt or artificial sea water constituents was more toxic than that rendered hypertonic by the addition of sodium chloride. This is probably because marine bacteria are more sensitive to large concentrations of certain specific salts found in sea water than they are to hypertonicity. The substitution of potassium chloride and sodium sulphate for sodium chloride revealed that sulphate and potassium are tolerated in concentrations two to three times higher than that in which they occur in sea water. Similar procedures showed that calcium and magnesium occur in sea water in concentrations which are near the limits of tolerance, double this amount inhibiting the growth of many marine bacteria. According to Korinek (1926, 1932) fresh water bacteria are much more resistant to changes in osmotic pressure than are marine bacteria. This was also recognized by Coupin (1915), Lipman (1926), Berkeley (1919) and others.

Further studies on the effect of diluted and concentrated sea water upon bacterial multiplication established that while marine bacteria are sensitive to salinity changes, they grow equally well in nutrient sea water throughout a salinity of 30 to 40‰. The growth of about ten per cent of them is inhibited if sea water is diluted until the salinity is 25‰ or if it is concentrated to a salinity of 45‰. Since the salinity of sea water the world over is well within these limits, comparable results should be obtained as far as salinity is concerned, regardless of where sea water is collected for the preparation of bacteriological media.

EFFECT OF NITRATE

Probably due primarily to the work of Gran (1902) and Drew (1911, 1914) on the abundance in the sea of denitrifying or nitrate-reducing bacteria whose demonstration requires the use of nitrate in the medium, many subsequent workers have continued to add nitrate in varying amounts to nutrient media used for the cultivation of marine bacteria. Though differing otherwise in composition, the media used by Berkeley (1919), Lipman (1926), Bavendamm (1931), Gee (1932) and many other leading marine microbiologists all have nitrate in common. The more or less general adoption of nitrate as a standard constituent of nutrient media recommended its use in our preliminary surveys on marine bacteria (ZoBell and Feltham, 1934) but controlled experiments with media without nitrate failed to confirm its merits.

The addition of 0.005 to 0.10 per cent of potassium nitrate to Formula 1 did not increase the plate counts and there was some evidence of the higher concentrations being inimical. This is illustrated by the results which are summarized in Table V. Similarly,

TABLE V

EFFECT OF DIFFERENT CONCENTRATIONS OF POTASSIUM NITRATE ON THE PLATE COUNTS OF MARINE BACTERIA. THE GROWTH INDICES RATHER THAN THE ABSOLUTE PLATE COUNTS ARE GIVEN

	Number of	*Concentration of Potassium Nitrate*				
Sample	*Samples*	0	0.005	0.01	0.05	0.10
		Average Growth Index				
Sea water	6	**100**	102	95	102	87
Sediment	5	**100**	99	101	96	84

the nitrate neither increased the size of the colonies nor the number of kinds of bacteria which developed. Sodium nitrate was not unlike potassium nitrate in its effect upon the growth and multiplication of marine bacteria. In the course of the investigations nitrate was added to several other combinations of nutrients which were tried but in no case did it prove to be beneficial.

According to Gran (1902), certain marine bacteria can use nitrate as the sole source of nitrogen. It is now recognized that many bacteria are endowed with this ability, but the author is aware of no bacterium for which nitrate is indispensable for its nitrogen nutrition. Nitrate-reducing, denitrifying or nitrate-assimilating bacteria have been found to grow just as well in the absence of nitrate as in its presence. This generalization applies to nearly a hundred pure cultures which have been tested and it substantiates the plate count observations that the addition of nitrate to nutrient media does not enhance the growth of marine bacteria. Moreover, just as many nitrate-reducing or denitrifying organisms are found on plates of nutrient media without nitrate as on plates of similar media containing 0.05 per cent potassium nitrate. Therefore, it would seem that there is no reason for adding nitrate to the medium unless the latter is to be used for a specific purpose for which nitrate is required; for example, testing bacteria for their ability to reduce nitrates.

PHOSPHATE

Lea and Nichols (1936) believe that a lack of sufficient phosphate in natural waters not infrequently limits the growth of bacteria, but

the work of Waksman and Carey (1935) and Renn (1937) indicates that it is doubtful if phosphate limits the growth of bacteria in the sea. Nevertheless, it may be desirable to supplement the phosphate content of sea water used in the preparation of nutrient media where, besides supplying the phosphorus requirements of bacteria, it might also function as a buffer and in other ways. Certain workers (Reuszer, 1933; Lipman, 1929; Bedford, 1933) recommend the addition of 0.005 to 0.10 per cent of potassium phosphate to nutrient sea water media while others make no provision for supplementing the phosphorus content. The effect of this variance in technique on the growth of marine bacteria has been investigated.

Dibasic potassium phosphate in concentrations ranging from 0.005 to 0.1 per cent were added to Formula 1. The reaction of each medium was adjusted to pH 7.6 after autoclave sterilization. The media were inoculated with 1.0 ml of appropriately diluted samples of raw sea water or marine mud. Table VI shows the relative numbers of colonies which developed on each medium. Included in Table VI are also the results obtained with and without the use of dibasic potassium phosphate in Reuszer's (1933) and PPP media:

Peptone	1.0 gm	Proteose peptone	2.0 gm
Glucose	1.0 gm	Bacto peptone	3.0 gm
K_2HPO_4	0.05 gm	Beef extract	2.0 gm
Agar	15.0 gm	Agar	15.0 gm
Sea Water	1000.0 ml	Sea Water	1000.0 ml

The three nutrient media were not used simultaneously so the data in Table VI should not be misconstrued as comparing their relative

TABLE VI

EFFECT OF DIBASIC POTASSIUM PHOSPHATE ON THE GROWTH OF MARINE BACTERIA

Nutrient Medium	*Samples Tested*	*Concentration of K_2HPO_4*				
		0	0.005	0.010	0.025	0.10
		Average growth index				
Formula 1	6	**100**	104	98	103	91
Reuszer's	6	**100**	105	102	101	84
PPP	8	**100**	97	104	98	96

merits. The data show how the addition of phosphate influenced the growth of bacteria in each medium. It will be observed that within the limits of experimental error bacterial development was indifferent to the presence of the added phosphate.

There is some evidence of the lower concentrations (0.005 to 0.01 per cent) of dibasic potassium phosphate improving the media slightly but the results are not conclusive. Definitely, it is not inimical to the growth of marine bacteria unless used in nutrient sea water media in concentrations exceeding 0.025 per cent. Since marine bacteria are quite sensitive to changes in the hydrogen-ion concentration, the use of phosphate might be indicated in media containing little or no peptone or other good buffers.

The apparent inhibitory effect of 0.1 per cent potassium phosphate is probably not due to any direct effect of the salt on the bacteria because, as shown by Mallman and Gallo (1931) the growth and metabolism of many bacteria is improved by the use of this much sodium or potassium phosphate in nutrient media. When added to sea water the phosphate tends to precipitate the calcium, iron and other salts from solution, and in large concentrations tends to materially affect the mineral composition of sea water. This complication can be largely obviated without diminishing the theoretical or real benefit of additional phosphate by the use of one which is relatively insoluble, such as tricalcium or ferric phosphate. Since iron salts have proved to be highly beneficial, the use of ferric phosphate is recommended.

IRON AND OTHER MINERALS

While testing the tolerance of marine bacteria for iron salts, it was observed that the addition of small quantities of either ferrous or ferric salts to nutrient sea water media materially increased the plate counts. Although sea water itself probably contains enough iron (Thompson and Robinson, 1932; Cooper, 1935) to support an extensive bacterial population and the peptone, beef extract and other media constituents also contain more iron than that required for the cell substance of the bacteria which grow in such media, the iron might not be in the right form or concentration to provide for optimal conditions. Evidence will be presented elsewhere which suggests that iron like aluminum compounds precipitate from solution or otherwise inactive toxic agents such as silver or copper, for example, which occur in sea water and which may have an unfavorable oligodynamic action on microorganisms. It is also probable, as suggested by Baudisch (1932), that iron in the proper form and concentration acts as a biocatalyst and otherwise stimulates bacterial growth. Allen (1913), Gran (1931) and others have observed that the addition of iron to sea water cultures stimulates the growth of diatoms. The compilation of culture media by Levine and Schoenlein (1930) shows that iron salts are widely used to promote the growth of fastidious microorganisms.

Preliminary experiments revealed that from a viewpoint of their availability and effect on bacterial development, ferric chloride, ferrous sulphate, ferric citrate and ferric phosphate were the best iron salts to use in nutrient sea water media. Table VII summarizes the

TABLE VII

RELATIVE NUMBER OF BACTERIAL COLONIES WHICH DEVELOPED ON NUTRIENT SEA WATER AGAR WITH DIFFERENT CONCENTRATIONS OF IRON. THE AVERAGE PLATE COUNTS OF SIX SAMPLES OF SEA WATER ARE GIVEN EXPRESSED AS RATIOS UPON A BASIS OF THE PLATE COUNT ON THE CONTROL MEDIUM SANS ADDITIONAL IRON BEING 100

Salt used	*Molecular Weight*	*Concentration in per cent* 0	0.001	0.01	0.10
			Growth Index		
Ferric citrate	356.58	**100**	143	163	107
$FeCl_3 \cdot 6\ H_2O$	270.31		132	154	82
$FeSO_4 \cdot 7\ H_2O$	278.02		134	141	90
$FePO_4 \cdot 4\ H_2O$	222.93		149	178	126

results obtained when using different concentrations of these salts in a medium prepared with 0.3 per cent Bacto-peptone, 0.2 per cent proteose peptone, 0.2 per cent beef extract and 1.5 per cent agar in sea water. The reaction of each medium was adjusted to pH 7.6 with the addition of N/1 NaOH. The media were inoculated in duplicate with six different samples of sea water and the colonies were counted after 7 days incubation at 22° C. The results are reported as ratios upon a basis of the plate count on the control medium to which no iron was added being 100.

It will be observed that the enrichment of the medium with 0.001 to 0.01 per cent of the iron salts increased the plate counts 32 to 78 per cent. One-tenth per cent of ferric citrate or ferric phosphate was less stimulating and this concentration of ferric chloride or ferrous sulphate was slightly toxic. Not only are the plate counts significantly higher in media enriched with favorable concentrations of iron salts; the colonies are larger and more kinds of bacteria develop on iron-enriched media than on control media to which no iron is added.

Further experiments with various nutrient combinations and different iron salts show that ferric salts are no better than ferrous salts for stimulating the growth of marine bacteria in nutrient sea water media. It has been noted that regardless of whether ferrous or ferric iron is added to the medium, both forms are present following its

sterilization and inoculation, the concentration of each depending upon the oxidation-reduction potential of the medium, its pH and the initial concentration of iron added. Being highly ionized and acidic, high concentrations of sulphates or chlorides of iron are more toxic than similar amounts of the poorly soluble and nearly neutral phosphates or citrates of iron. Moreover, since the addition of even low concentrations of iron chlorides or sulphates precipitate certain constituents from sea water necessitating the filtration of the media and making it difficult to adjust the pH recommends the use of citrates or phosphates of iron which do not present these difficulties. Statistically different nutrient combinations have yielded somewhat larger plate counts when ferric citrate or ferric phosphate has been used than when ferrous sulphate or ferric chloride has been used in the optimum concentration. It is much easier to prepare clear media of the proper pH when the iron is added as the citrate or phosphate rather than as the chloride or sulphate.

Although there are many species of marine bacteria which can utilize citrate as a sole source of carbon and energy, the addition of citrate to peptone medium does not increase the number of colonies which develop from sea water or sediment samples. Therefore, the beneficial effects from the addition of ferric citrate are to be ascribed to the iron and not the citrate. However, as pointed out above, additional phosphate in nutrient media might serve a useful function which would indicate the use of ferric phosphate as a source of iron in preference to ferric citrate. Further experiments with the use of these two iron salts alone and together in different concentrations in different combinations of nutrients show that nothing is gained by using both ferric citrate and ferric phosphate. There was little difference observed in plate counts, colony size or diversity of species, regardless of whether ferric citrate or ferric phosphate was added to the media, but under most conditions the use of either one increased appreciably the plate count on nutrient sea water agar.

Since both ferric citrate and ferric phosphate are only slightly soluble in sea water, an excess of either is not toxic. Almost equally good results were obtained from the use of any concentration of either salt between 0.001 and 0.025 per cent. For convenience and simplicity, 0.01 per cent, 0.1 gram per liter, has been selected for routine use. In no combination of nutrient sea water medium tried, except in acidic ones, has this concentration of ferric phosphate or ferric citrate exhibited any inimical effects, and in neutral or slightly alkaline media it has increased the plate counts by 20 to 80 per cent or more. The addition of 0.01 per cent of either salt to 0.5 per cent peptone sea water agar gives a clear medium without filtration having a re-

action of pH 7.5 to 7.6. The average plate counts of nearly a hundred samples of sea water and marine mud are between 3 and 4 per cent higher when ferric phosphate was used than when ferric citrate was used. Both yield equally reproducible results. This slight statistical advantage of ferric phosphate, together with the theoretical and possibly practical results from the phosphate enrichment of nutrient sea water media, recommends its use. Studies on a large number of pure cultures of marine bacteria to be reported elsewhere showed that the addition of ferric phosphate to various sea water media accelerated the growth and metabolism of the bacteria.

In testing the growth-promoting properties of iron in nutrient media of different pH values it was noted that iron is less stimulating as the pH decreases and is more beneficial with increasing pH, although as will be amplified below, maximum plate counts are obtained on media having a reaction of pH 7.5 to 7.8, irrespective of the presence of iron. This is illustrated by the data summarized in Table VIII which shows the average number of bacteria in twelve

TABLE VIII

Relative Number of Bacteria which Grew on Plates of Nutrient Sea Water Agar of Different pH Values with and without the Addition of 0.01 per cent Ferric Phosphate Expressed as Ratios on a Basis of the Number of Colonies which Developed on the Medium Sans Iron at pH 7.5–7.8 Being 100. The Increase or Decrease in the Plate Count Caused by the Iron in Media of Different pH Values Is also Given Expressed as per cent

pH of medium	5.8–6.0	6.4–6.6	7.0–7.2	7.5–7.8	8.1–8.3	8.4–8.7
Growth index without iron	38	53	71	**100**	82	26
Growth index with iron	21	54	99	158	142	58
Percentage difference with iron	−44.8	+1.9	+39.4	+58.0	+73.2	+123.0

samples of sea water which grew on plates of nutrient sea water agar of different pH values, with and without the addition of 0.01 per cent ferric phosphate, the plate counts being expressed as ratios on a basis of the number of colonies which developed on the medium sans iron at pH 7.5 to 7.8 being 100. Further analysis of these data reveal that whereas 0.01 ferric phosphate reduced the plate count by 44.8 per cent in media at pH 5.8 to 6.0, it augmented the plate count in less acidic media from 1.9 to 123.0 per cent, the greatest stimulation occurring in the more alkaline media.

Lower concentrations of iron were less toxic in acidic media which suggests that the increased solubility of iron in acidic media may

account for its increasing toxicity with decreasing pH. In view of the fact that 0.01 per cent of any soluble iron salt is from ten to a hundred times as much as Hotchkiss (1923) or Elvehjem (1931) have found to be tolerated by microorganisms, it is believed that marine bacteria seem to tolerate large concentrations of iron salts in sea water media only because virtually all of the iron is precipitated from solution by the phosphates, carbonates and other constituents of sea water. Additional studies will be required to elucidate the mechanism of the beneficial effects of iron salts in nutrient sea water media, but for the practical purpose of cultivating marine bacteria the use of concentrations which are theoretically toxic (0.001 to 0.01 per cent of ferric phosphate) are indicated. The foregoing observations emphasize that consistent or reproducible results are contingent more upon the pH of the sea water media than the amount of iron added.

When used in nutrient sea water media to which no iron was added, from 0.001 to 0.01 per cent of aluminum chloride stimulated the growth of marine bacteria, but not to the same extent as similar concentrations of iron salts. There was no synergetic action of aluminum and iron, the two used together producing results which were no better than when iron only was added. Low concentrations of manganese salts were also beneficial in media to which no iron salts were added, but less beneficial than either iron or aluminum. Manganese failed to fortify media which were enriched with iron or aluminum. Sea water probably contains more than enough aluminum and manganese to provide for the growth requirements of marine bacteria; the beneficial effect from the use of additional amounts of these elements probably being attributable to a catalytic or regulative function for which purpose iron is superior. According to Harvey (1939) small quantities of manganese stimulate the growth of marine diatoms.

Zinc is an indispensable nutrient for many microorganisms. It is always present in sea water although in some places only in traces (Thompson and Robinson, 1932). Since the enrichment of sea water media with varying concentrations of zinc salts failed to stimulate bacterial multiplication, it can be assumed that there is enough zinc in sea water to provide for the growth requirements of those bacteria which need it.

A lack of silica is believed by some workers to limit the growth of diatoms at certain places in the sea. It is still indeterminate if silica is required for the growth of marine bacteria but no beneficial effects followed the addition of small amounts of sodium silicate to nutrient media.

HYDROGEN ION CONCENTRATION

The pH of surface sea water ranges from 7.8 to 8.8 and decreases with depth. Very little is known concerning the hydrogen ion concentration of marine sediments but the pH of those which have been investigated at the S.I.O. ranged from 5.3 to 9.8. The pH of most of the samples of sea water whose bacteriological analysis is discussed in this paper ranged from 8.0 to 8.3; the sediments from pH 7.4 to 8.5.

If it can be assumed that the environment to which bacteria are indigenous provides conditions which are more or less optimal for

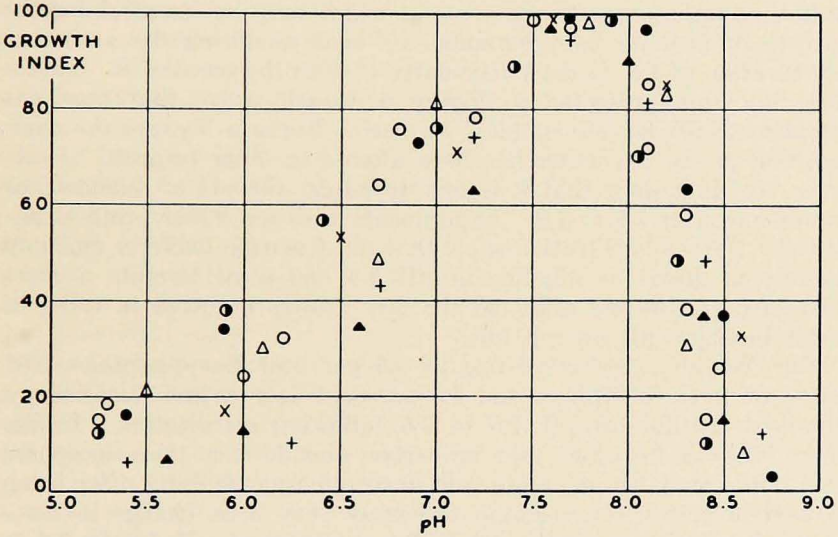

Figure 6. Effect of pH on the multiplication of marine bacteria. Circles show growth indices of bacteria in media not enriched with iron and triangles and crosses show growth indices of bacteria in media enriched with iron.

the bacteria, one would expect marine bacteria to grow best at pH 8.0 to 8.3. However, early in the work it was noted that when cultivated under laboratory conditions this pH is near the upper limit tolerated by the bacteria from the sea, the maximum development occurring in nutrient sea water media at pH 7.5 to 7.8. This is illustrated by the data summarized in Figure 6 on which the average results of seven different experiments are recorded. The pH of the media was determined with glass electrodes using a Beckman pH meter.

The pH of the nutrient sea water media is readily reduced to the desired level by the addition of HCl and it does not change much with autoclave sterilization, but considerable difficulty is encountered

in maintaining sea water media at a high pH without materially altering its composition due to the pronounced effect of carbon dioxide on the hydrogen ion concentration of sea water and other peculiarities of its buffer mechanism (Thompson and Robinson, 1932). By depressing the ionization of hydroxyl ions the addition of a little alkali to sea water medium may result in a decrease rather than an increase in pH. The pH is increased by the addition of more alkali but in the meantime much of the calcium, magnesium, iron and perhaps other cations are precipitated from solution along with the carbonates, phosphates and maybe other anions. The precipitation of these constituents, some of which may be essential for the growth of bacteria, is accentuated by heat as during the autoclave sterilization of the media particularly if the pH exceeds 7.8. Therefore the data presented in Figure 6 do not prove that reactions alkaline to pH 8.0 are inimical to marine bacteria because the composition of the sea water has been altered in other respects. However, the data show that it is best to adjust the pH of nutrient sea water media at 7.5 to 7.8. Experiments with sea water broth sterilized by Berkefeld filtration show that most marine bacteria multiply readily in media as alkaline as pH 8.5 and some tolerate a reaction of pH 10.0–10.3 although the best growth occurred in tubes of broth having a pH around 7.6.

Our Medium 2216 consisting of 0.5 per cent Bacto-peptone, 0.01 per cent ferric phosphate and 1.5 per cent agar in sea water has a terminal reaction of pH 7.5 to 7.6 following sterilization. Unlike more basic media which take up carbon dioxide from the atmosphere and thus slowly become more acid in reaction particularly after being poured in plates, this medium undergoes very little change in reaction after it is prepared. Its buffer capacity at pH 7.5 to 7.6 is adequate to prevent much change in the reaction even from the growth of bacteria. Many marine bacteria liberate ammonium from proteinaceous substrates which would have a tendency to increase the pH but ostensibly such a change is counterbalanced by the carbon dioxide which is produced by the respiring bacteria together with that which is taken up by the medium from the atmosphere.

SUCCESSIVE DILUTION METHOD vs. PLATE COUNTS

The recorded observations of Domogalla (1932), Waksman, et al. (1933), Bere (1933) and others that plate counts detect only a small percentage of the bacteria present in water suggested the advisability of examining the possibilities of applying the successive dilution method with liquid media to the enumeration of the marine bacterial

population. Theoretically, liquid medium is more suitable for the growth of water bacteria than solid medium and moreover, as elaborated by Butterfield (1933), the former is used in tubes which are easily manipulated aseptically and it makes some provisions for the simultaneous tests on the physiological characteristics of the bacteria. According to Butkewitsch (1932) the inoculation of tubes of liquid media with successive dilutions of material from the Barents Sea demonstrated the presence of from ten to a hundred times as many bacteria as plate counts on solid media.

Parallel analyses on a large number of samples of sea water and bottom sediments were made, using nutrient agar for plate counts and the successive dilution method with the identical medium without agar in test tubes. Plates were poured in duplicate, care being exercised to cool the nutrient agar to 42° C. before pouring into the plates inoculated with bacteria. The colonies which developed were counted after incubating the plates two weeks at 22° C. Following the procedure prescribed by Ziegler and Halvorson (1935) 1.0 ml. portions of each of three to five or more successive dilutions of the material differing by log one were used to inoculate the liquid medium, ten tubes being inoculated with each dilution. Thus, a minimum of thirty to fifty or more tubes were used for each sample analyzed, the number of dilutions required depending upon one's ability to estimate beforehand the order of magnitude of the bacterial population of the material being examined. With water from the open sea, which rarely contains more than a few hundred bacteria per ml. it is a matter of ease to select three effective dilutions, but with sedimentary material which may contain from less than a hundred bacteria to several million per ml., it is sometimes necessary to use eight or nine dilutions in order to insure the inoculation of the medium with three effective dilutions. Table IX shows the number of tubes inoculated with each dilution in which growth occurred within two weeks at 22° C. and the most probable number of bacteria indicated thereby according to the probability table of Halvorson and Ziegler (1933). The average number of bacteria indicated by plate counts on the same sample is also given. In order to simplify the comparison of results obtained by the two methods, only the significant figures are recorded.

A total of 38 samples were analyzed in this way, the dilution method demonstrating more bacteria in 23 of them and the plate counts being higher in the other 15 samples. The ratio of the dilution method counts to the plate counts ranged from 0.51 to 1.89; the average for the 38 samples being 1.12. In other words, the composite results show the dilution method counts to be an average of

TABLE IX

MOST PROBABLE NUMBER OF BACTERIA IN SAMPLES OF MARINE MATERIAL INDICATED BY THE SUCCESSIVE DILUTION METHOD AND THE PLATE COUNT OF THE SAME SAMPLE AND DILUTION

Sample No.	*Positive tubes in dilution*			*Successive dilution method count*	*Corresponding plate count*	*Ratio of dilution method to plate count*
	n_{10}	$n_{1.0}$	$n_{0.1}$			
1234	10	8	4	2210	1230	1.84
1235	10	10	2	3490	2120	1.64
1237	10	6	1	933	1590	0.59
1238	10	8	0	1300	1740	0.75
1314	10	7	0	1010	820	1.23
1315	10	4	2	700	370	1.89
1318	9	6	0	381	750	0.51
1319	9	8	3	499	580	0.86
1412	10	7	1	1160	690	1.68
1413	7	4	3	208	320	0.65
1432	7	4	0	171	260	0.66
1433	8	4	0	217	260	0.83
1443	10	6	0	792	630	1.26
1444	10	8	1	1500	830	1.81

12 per cent higher than the plate counts. Similarly, Butterfield (1933) found that when enough tubes were used under standardized conditions, plate counts are not markedly different from dilution method estimates of water bacteria. Ziegler and Halvorson (1935) note good agreement between plate counts and dilution method counts with some organisms but not with others, probably due to the predilection of some organisms for liquid media and others for solid media. It is believed that in most reported cases in which many times more water bacteria were demonstrated in liquid media by the dilution method than on plates of solid media, the investigators either (a) failed to use enough tubes or enough effective dilutions to get an accurate estimate of the bacterial population, (b) they were dealing with highly specialized organisms, (c) the solid medium was deficient in some property, or (d) the solid media were poured so hot that many of the thermosensitive bacteria were killed. According to Drew (1912), marine bacteria are very sensitive to temperatures as high as 40° C. and many are killed at 45° C. ZoBell and Conn (1940) report that pouring nutrient agar at temperatures exceeding 50° C. kills a large proportion of the bacteria from the sea. Working under field conditions or in an improvised laboratory on a rolling boat at sea, it is not always expeditious to cool the media to 42° C., although such treatment is prerequisite to reliable results.

During the last eight years, several different combinations of nutrients have been used to compare plate counts and successive dilution method counts. Whenever enough tubes of nearly any kind of medium tried have been inoculated with enough effective dilutions of inocula to yield reasonably reproducible results, this procedure has demonstrated the presence of very few, if any, more bacteria than equally carefully conducted plate counts. This generalization applies to the mixed heterotrophic bacterial population of sea water and marine sediments and does not apply to a few certain specific types of bacteria such as the nitrifiers, for example, which do not form colonies readily on solid media immediately following initial isolation.

In order to compare the reproducibility of results with plate counts and that of dilution method counts, a series of ten water samples were analyzed in duplicate by each method. Each of two plates of nutrient agar were inoculated with 1.0 ml. of a dilution selected to contain between 30 and 300 viable bacteria and two sets of thirty tubes of similar liquid medium were inoculated with 1.0 ml. of three effective dilutions differing by log one, each dilution being used to inoculate two sets of ten tubes. The sets of thirty were regarded as duplicates. The number of bacteria detected by both methods is recorded in Table X, which also shows the deviation of the duplicates.

TABLE X

NUMBER OF BACTERIA IN WANTER SAMPLES INDICATED BY DUPLICATE ANALYSIS BY THE SUCCESSIVE DILUTION METHOD AND PLATE COUNT PROCEDURES. THE DEVIATION OF DUPLICATES IS EXPRESSED IN PER CENT

	Successive Dilution Method				*Plate Count Procedure*			
Sample	*Number of bacteria*			*Per cent*	*Number of bacteria*			*Per cent*
No.	*A*	*B*	*Average*	*deviation*	*a*	*b*	*Average*	*deviation*
1701	239	310	274	25.9	263	231	247	12.9
1702	174	94	134	59.6	157	140	148	11.5
1703	197	115	156	52.5	113	120	116	6.0
1704	79	170	114	79.9	130	133	131	2.3
1705	275	334	304	19.4	263	228	245	14.3
1706	70	93	81	28.4	115	105	110	9.1
1707	101	231	166	78.3	194	163	178	17.4
1708	128	253	190	63.6	143	115	129	21.7
1709	381	116	248	106.7	225	241	233	6.8
1710	228	329	278	36.4	263	241	252	8.7

The successive dilution method indicated the presence of an average of 12.5 per cent more bacteria than the plate counts but the results

show that the reproducibility of the plate counts was five times as great as that of the dilution method counts. The plate counts deviated from the mean of the two duplicates by 2.3 to 21.7 per cent, the average for the ten samples being 11.1 per cent. The successive dilution method counts deviated from the mean of the two duplicates by 19.4 to 106.7 per cent, the average for the ten samples being 55.1 per cent. Halvorson and Ziegler (1933) present theoretical considerations which show that when three effective dilutions are used to inoculate ten tubes each, the results might deviate from the modal values by 50 to 130 per cent. They conclude that over a hundred tubes would have to be inoculated with three effective dilutions in order to have the reproducibility of the results approach that commonly obtained by plating procedures.

These observations seem to warrant the conclusion that the advantage of the slightly larger number of bacteria in marine materials indicated by the successive dilution method is more than offset by its poor reproducibility except when excessively large numbers of tubes are used. The relatively large amounts of time and material ordinarily required by the successive dilution method makes it impractical except for estimating the abundance of specific types of microorganisms with differential media.

CHOICE OF A SOLIDIFYING AGENT

Rendering sea water media sufficiently solid for plate counts and other cultural purposes presents special problems. If agar is used, the media commence to congeal at 42° C. at which temperature some of the thermosensitive bacteria from the sea are killed almost instantly (ZoBell and Conn, 1940). Nutrient gelatin can be prepared so that it does not congeal until its temperature has been reduced lower than the thermal death point of any known marine bacteria but the actively proteolytic bacteria which constitute a large portion of the marine flora liquefy the gelatin causing colonies to merge before many of the slower growing bacteria have time to form macroscopically visible colonies. Sea water media can be solidified with silica gel but plate counts thus obtained do not compare favorably with those obtained with media solidified with agar. Cognizant of these difficulties Bertel (1936) has recommended the use of the skeletal material of sponges which has certain applications but it is not suitable for general plate counts and other cultural purposes. Similarly bentonite, calcium sulphate, kieselguhr, cellulose and other solidifying agents have a strictly delimited usefulness. In our quest for a general utility medium for marine bacteria comparative tests have been made on different solidifying agents.

A few preliminary tests on the available materials with which we are familiar limited the choice of a solidifying agent for nutrient media to agar or gelatin although unexpectedly good results were obtained with silica gels. When prepared with freshwater by the method outlined by Hanks and Weintraub (1936) sufficiently firm silica gels contain around 3.5 per cent of sodium chloride which is formed when the sodium silicate is neutralized with hydrochloric acid. Since sea water contains nearly this much sodium chloride, it is not surprising to find that many marine bacteria will grow in properly enriched silica gel media without the necessity of dialyzing them to remove the sodium chloride as is commonly practiced when freshwater bacteria are to be grown on silica gels. Using a procedure similar to that described by Moore (1940) it has been possible to prepare and sterilize a solution of sodium silicate which produces a suitable gel which sets slowly when mixed with a second solution containing dilute hydrochloric acid and peptone or other nutrients. These two sterile solutions are mixed and poured immediately into a Petri dish inoculated with marine bacteria. The gel sets within a few minutes but not until there has been adequate time to permit the inoculum to be mixed with the sample.

About half as many colonies develop on nutrient silica gels prepared and inoculated in this manner as on plates of nutrient sea water agar (see Table XI). The media used in the experiments summar-

TABLE XI

Relative Numbers or Marine Bacteria which Formed Colonies on Nutrient Silica Gel Medium and Corresponding Nutrient Sea Water Agar and Nutrient Sea Water Gelatin after Different Period of Incubation at 22° C.

Period of incubation	*Nutrient silica gel medium*	*Nutrient sea water agar*	*Nutrient sea water gelatin*
4 days	13	38	42
7 days	22	71	63
10 days	30	89	68
14 days	37	97	—
18 days	46	100	—
24 days	54	95	—

ized in Table XI all contained 0.3 per cent Bacto-peptone, 0.2 per cent proteose peptone and 0.01 per cent ferric phosphate. The colonies which developed on the nutrient silica gel medium were smaller in size and exhibited less diversity of form than those which developed on nutrient sea water agar. The superiority of the sea

water agar is probably attributable to the specific salt requirements of the marine bacteria rather than being due to any preference of the bacteria for agar over silica gel because from the experiments summarized in Table III it will be recalled than only 56 to 61 per cent as many marine bacteria developed in a medium prepared with 3.0 per cent sodium chloride as in that prepared with sea water, agar being the solidifying agent in both cases. However, the attempts to improve the silica gel media by adding sea water to the solutions used to prepare the silica gel have failed to yield satisfactory results to date. A heavy precipitate forms when sodium silicate is added to sea water, and while the nutrient hydrochloric acid solution can be prepared with sea water, any advantages which accrue from the increased concentration of sea salts seem to be offset by the inimical effects of the salinity of the resultant medium. Though inferior to sea water agar for general plating procedures, silica gel media prepared by the method outlined above with appropriate nutrients have proved to be useful for isolating and studying the characteristics of certain marine microorganisms. Dialyzing plates of silica gel in sea water enriched with the proper mineral salts has given excellent media for the growth of marine nitrifiers, sulphur bacteria and diatoms.

The results summarized in Table XI show that marine bacteria develop somewhat faster in nutrient sea water gelatin than in a corresponding medium prepared with agar as the solidifying agent. A total of 24 samples of raw sea water and marine sediment have been analyzed in this way and after 4 days incubation there were 11.4 per cent more colonies on the gelatin plates than on the agar plates. However, after 7 days incubation when appreciably more colonies had appeared on both media, the agar excelled and the margin of its superiority increased progressively thereafter. Gelatin liquefaction by some colonies was evident in four days and after 7 days it had become so extensive that surrounding colonies were engulfed by expanding liquefied areas. The gelatin in some plates was quite completely liquefied after 10 days incubation at 22° C. and the colonies on only those plates which were inoculated with relatively small numbers of bacteria could be counted after 14 days.

Corroborating the observations of ZoBell and Conn (1940) it was found that the number of colonies on the nutrient agar continued to increase until the maximum was reached in about 18 days although there was little difference between the counts made on the fourteenth to the twenty-fourth day. The decreasing colony count observed after the eighteenth day is due to the obliteration of small colonies by rapidly growing ones.

Three or four per cent of the bacteria from the sea on the average

digest agar; a much larger proportion being found in plankton tows than in other marine materials. As a rule they do not liquefy agar as rapidly or as extensively as the proteolytic bacteria liquefy gelatin. Agar digesting bacteria usually form depressions in the nutrient agar, the diameter of the craters thus formed slowly increasing in diameter. Only infrequently is a plate of agar rendered completely uncountable by the activities of agar digesting bacteria. Angst (1929), Lundestad (1928), Waksman and Bavendamm (1931) and others have discussed the occurrence and activity of agar decomposing bacteria in the sea.

The fact that bacteria develop a little faster on nutrient gelatin than on nutrient agar during the first few days of incubation suggests that gelatin might supply some essential nutrient. According to Koser et al. (1938) gelatin is a good source of growth-promoting substances for bacteria. However, the addition of concentrations of gelatin ranging from 0.1 per cent to 1.0 per cent to nutrient agar failed to increase the plate counts or to improve results in any way.

From these experiments it is concluded that while agar is not a perfect solidifying agent for media for marine bacteria, it is better than anything else which is available. Vast differences were noted in the ease of dissolving in sea water and geling powers of different brands of agar as well as in the degree of clarity of the resulting medium. In all three respects Bacto-agar was superior. As little as 1.0 per cent of Bacto-agar is enough to render sea water medium solid enough for plate count purposes although it sets more firmly if 1.2 to 1.5 per cent is used. The concentration of agar from 1.0 to 2.0 per cent does not influence the number of colonies which develop.

ORGANIC NUTRIENTS

A survey of the literature reveals that scores of different concentrations and combinations of organic nutrients have been used for investigating the occurrence and distribution of bacteria in the sea. Peptone, beef extract, fish infusions, nutrose, gelatin, starch, dextrose and organic acids have been most extensively used constituents. The growth promoting properties of these constituents alone or in combinations including several dozen combinations specifically recommended for the multiplication of marine bacteria have been compared with our Medium 2216 consisting of 0.5 per cent Bacto-peptone, 0.01 per cent ferric phosphate and 1.5 per cent agar dissolved in sea water. Most of the media recommended by Russell (1893), Fischer (1894), Gran (1902), Drew (1911), Korinek (1926), Lipman (1926), Lloyd (1930), Levine and Schoenlein (1930), Bavendamm (1932),

Gee (1932), Bedford (1933), Benecke (1933), Reuszer (1933) and Waksman et al. (1933) for the growth of marine bacteria as well as various modifications have been tested. In the interests of the economy of space may it suffice to state that without exception all were found to be inferior to Medium 2216, there being only from 2 to 86 per cent as many bacteria growing on them as on Medium 2216 and, in general, fewer species.

One of the best media tried was one recommended by Reuszer (1933) and used by Waksman et al. (1933), Renn (1940) and others at the Woods Hole Oceanographic Institution. It consists of 0.1 per cent each of peptone and dextrose, 0.005 per cent K_2HPO_4 and 1.5 per cent agar dissolved in sea water. In a large number of comparisons it was found that only from 53 to 78 per cent as many colonies developed on this medium after different periods of incubations as on our formula 2216. Upon the addition of 0.01 per cent ferric phosphate to Reuszer's formula it gave plate counts which were an average of 86 per cent as high as those obtained on Medium 2216.

The effect of dextrose and the concentration of peptone on plate counts is illustrated by the data summarized in Table XII. The

TABLE XII

COMPARATIVE PLATE COUNTS ON FERRIC PHOSPHATE SEA WATER AGAR PREPARED WITH DIFFERENT CONCENTRATIONS OF BACTO-PEPTONE WITHOUT AND WITH 0.1 PER CENT DEXTROSE

Medium	*Plate count without dextrose*	*Plate count with dextrose*
0.10 per cent peptone	72	86
0.25 per cent peptone	87	90
0.50 per cent peptone	**100**	102
1.0 per cent peptone	93	94
2.0 per cent peptone	96	91
Reuszer's medium (No Fe)	—	69

best results were obtained on media containing around 0.5 per cent Bacto-peptone. Dextrose is beneficial if the concentration of peptone is low but no advantages were noted for the use of dextrose in media containing as much as 0.5 per cent peptone. There is much more tendency for the colonies to be mucoid or to spread with subsequent merging on media containing more than 0.5 per cent peptone or on that enriched with glucose.

Some of the media used in the work of ZoBell et al. (1933, 1936, 1940) have been enriched with proteose-peptone, neopeptone, tryptone

or beef extract on the theory that these substances might favor the growth of certain fastidious organisms. While either alone or in combination with Bacto-peptone these substances have proved to be useful for the cultivation of pure cultures, our extensive experiments indicate the use of none of them for obtaining the maximum development of colonies (total numbers as well as kinds). In concentrations not exceeding 1.0 per cent they are not inimical except that beef extract imparts an undesirable brownish color to the medium.

Starch, glycerol, mannitol, citrates, lactates, succinates and malates have been recommended for the enrichment of media for the growth of marine bacteria by certain workers. The addition of from 0.1 to 1.0 per cent of the compounds to Medium 2216 has not improved it although studies on pure cultures to be reported elsewhere show that these compounds are utilized by certain bacteria and they are most useful constituents of differential media.

The work of Ostroff and Henry (1939) with pure cultures proves that marine bacteria are not fastidious in their carbon or nitrogen requirements. Some of their cultures could utilize as many as 14 of 21 different nitrogen compounds investigated and most of the bacteria grew luxuriantly on amino acids; glutamic acid, aspartic acid and asparagine being best in the order named. It was not within the scope of their work to try peptone which is a mixture of many amino acids and other nitrogen compounds. We find that neither glutamic acid, aspartic acid nor asparagine is as good as peptone for the cultivation of the general marine bacterial population. The enrichment of peptone media with 0.01 per cent of these amino acids either alone or in combination did not improve it for plate count purposes.

Snow and Fred (1926) found sodium caseinate agar to be superior to any other medium they tried for the cultivation of freshwater bacteria. Other workers have recommended the use of nutrose or sodium caseinate either with or without additional nutrients for the cultivation of marine or fresh water bacteria. The results which we obtained with the use of sodium caseinate and Bacto-nutritive caseinate are summarized in Table XIII. The average plate counts of four samples of sea water and three of marine sediments are given expressed as ratios on a basis of the plate count on Medium 2216 being 100. From the table it will be noted that only about half as many colonies appeared on the sodium caseinate media as on the peptone medium and those which did appear were much smaller and developed slower. The Bacto-nutritive caseinate was inferior to sodium caseinate in these tests. Many colonies on the caseinate medium might have escaped detection due to their small size and the opaque-

TABLE XIII

COMPARATIVE NUMBER OF MARINE BACTERIA WHICH FORMED COLONIES ON SEA WATER IRON AGAR ENRICHED WITH VARIOUS NUTRIENTS AFTER DIFFERENT PERIODS OF INCUBATION AT 22° C.

Nutrients	*Comparative plate count after* 7 *days*	14 *days*
0.2% sodium caseinate	19	46
0.5% sodium caseinate	32	53
1.0% sodium caseinate	37	54
0.2% sodium caseinate + 0.5% peptone	51	68
0.5% sodium caseinate + 0.5% peptone	56	71
1.0% sodium caseinate + 0.5% peptone	38	49
0.2% nutritive caseinate	15	23
0.5% nutritive caseinate	19	32
1.0% nutritive caseinate	20	38
0.5% peptone (Medium 2216)	86	**100**

ness of the media. The opaqueness of the media is also largely responsible for the fact that not as many colonies were found on the caseinate media enriched with peptone as on the peptone medium although it is not improbable that the mineral balance of the sea water was disrupted by the caseinate precipitating certain ions. The plate counts on the peptone medium were much more closely reproducible than on the caseinate media as indicated by the agreement of duplicates.

Considerable difficulty was encountered in trying to obtain reproducible results with various fish and seaweed infusions. In no case did any of four different fish and mollusk infusions or extracts either with or without peptone yield plate counts which were as large as those obtained without the infusions or extracts. Fish bouillon agar prepared with sea water according to the methods of Fischer (1894) was decidedly inferior to our standard peptone agar. Negative results were likewise obtained with the extracts prepared from two different red algae and three brown ones. Similarly after trying the extracts from marine organisms Lloyd (1930) concluded that they are not essential for the growth of marine bacteria in spite of the claims of many earlier workers.

The recent reviews of Koser and Saunders (1938) and Janke (1939) stress the importance of accessory growth factors, many of which are organic in nature, for the multiplication and metabolism of microorganisms. Harmsen and Verweel (1936) and others have recommended the enrichment of plating media with growth-promoting substances such as yeast extract or soil extract. The unpublished ob-

servations of Michener and others in the S.I.O. laboratory indicate that marine bacteria require certain accessory growth substances similar to those required by terrestrial and fresh water bacteria but apparently there is enough in sea water to initiate the growth of marine bacteria. Traces of nicotinic acid, thiamin chloride, pantothenic acid, indolacetic acid and yeast extract added to our ferric phosphate peptone Medium 2216 have not increased the plate counts although as will be reported elsewhere, some of these substances are beneficial for the growth of pure cultures in synthetic media.

SUMMARY

Most of the bacteria recovered from sea water as well as bottom sediments at places remote from possibilities of terrigenous contamination have specific salt requirements which are satisfied best by natural sea water. Neither synthetic sea water nor other isotonic salt solutions are satisfactory substitutes. Merely diluting sea water with fresh water materially reduces the number of marine bacteria which will grow in it. Conversely very few terrestrial or freshwater bacteria are able to grow in nutrient sea water media upon initial isolation.

There is no evidence that the addition of nitrate to nutrient sea water media as has been recommended by others is beneficial, and concentrations exceeding 0.1 per cent potassium nitrate are inhibitory.

There are indications for the addition of 0.01 per cent dibasic potassium phosphate to peptone sea water media although it normally contains sufficient phosphate to provide for the multiplication of marine bacteria.

The enrichment of nutrient sea water media with a trace of iron increases plate counts 18 to 76 per cent, 0.01 per cent ferric citrate or ferric phosphate being almost equally beneficial. The use of the latter is recommended because it provides for the phosphate requirements as well as the iron. This concentration of iron in sea water is toxic only when the medium is acid in reaction.

Marine bacteria appear to grow best in media having a pH of 7.5 to 7.8 although sea water *in situ* is usually somewhat more alkaline than this.

Successive dilution method counts using tubes of nutrient sea water broth demonstrated the presence of 12 per cent more bacteria than plate counts on comparable nutrient sea water agar but the latter yielded much more reproducible results and require much less medium and time.

A comparative study of the merits of different solidifying agents

recommends the use of 1.2 to 1.5 per cent Bacto-agar as the best for plating media. Gelatin is liquefied too rapidly by marine bacteria to be satisfactory and although silica gel prepared by a simplified method has certain advantages, it does not give results as good as agar for the demonstration of the heterotrophic bacterial population.

The use of various algae and fish infusions, carbohydrates, organic acids, nitrogen compounds and other organic nutrients in different concentrations and combinations have not proved to be as good as Bacto-peptone for the growth of marine bacteria as indicated by the number and kinds of bacteria on plates as well as the reproducibility of plate counts.

Although special media are required for certain groups of organisms and for special purposes, for maximum plate counts and for the cultivation of most aerobic heterotrophic marine bacteria Medium 2216 is recommended. It contains 0.5 per cent Bacto-peptone, 0.01 per cent ferric phosphate and 1.5 per cent Bacto-agar dissolved in aged sea water, the pH being around 7.6 after autoclave sterilization.

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