A Survey of the Marine Bacteria of the Clyde Area.

Thesis presented to the University of Glasgow for the Degree of Doctor of Philosophy.

by

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PREFACE.

The work embodied in these pages comprises an introductory survey of the marine bacteria of the Clyde area. The major part of the work was carried out in the Department of Botany and Bacteriology, Royal Technical College, under the supervision of Professor D. Ellis, D.Sc.,Ph.D.,F.R.S.E., for whose guidance and encouragement the writer wishes to express grateful acknowledgments.

Thanks are also due to the Scottish Marine Biological Association for permitting the frequent use of their boat the 'Nautilus'; to Mr. Elmhirst, the Superintendent of their laboratory at Millport for readily affording facilities for work there; to Mr.R.J. Nairn,B.Sc.,PH.C., for frequently deputising for the writer in the collecting of samples and for preparing the photographs on Plate 1; and to Dr.J.A.Cranston for much helpful criticism and advice.

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INTRODUCTION.

Marine bacteria have not in the past occupied the attention of bacteriologists to any considerable extent. This may be because the economic significance of the subject has not been appreciated, and thus no special technique evolved. It is noteworthy that among the variety of bacteriological handbooks on the technique of water-examination, none that has come to the writer's notice gives consideration to the special problems of marine bacteriology.

In the sea,,as on land, one may expect the planktonic bacterial population to consist largely of saprophytes, and, to a lesser extent, of prototrophic forms. One of the most significant roles bacteria may play is in the nitrogen-cycle in seawater. The normal terrestrial macrophyte assimilates its nitrogen as nitrate; and this holds good as far as is known for most water-plants whether phanerogamic or cryptogamic, though some algae appear to be able so to utilise ammonium compounds. Now both the littoral macrophytic algae and the microphytic plankton are continuously using up the nitrate in the sea , which is present in minimal amounts and therefore acts as a limiting factor in the summer season. The sources from which the nitrate supply is replenished do not appear to have been adequately discussed in the literature. Two obvious ones are land drainage and rain, but the relative importance of these and their sufficiency are

unknown. Nitrogen-fixing organisms, comparable to those which are symbionts with the Leguminosae on land, have been reported, but ~~to what extent they contribute~~ the extent of their contribution to the nitrate supply of the sea is also unknown.

The question of nitrogen fixation, nitrification, and denitrification by bacterial action in the sea is a matter of some economic importance, and, accordingly, this aspect of marine bacteriology has been considered in the following pages.

There is similarly a sulphur-cycle in the sea; sulphate-reducing species, and a number of marine organisms storing sulphur as anabolyte within the cell are known. The study of sulphur-bacteria is a matter of some difficulty. No method of making pure cultures of these has been established beyond doubt, and physiological work has therefore to be done on natural samples possibly contaminated with other organisms. Both sulphur and nitrogen bacteria are grouped as prototrophic forms, but the latter and probably the former, live also as ordinary saprophytes.

In the sea then, one may look to find both prototrophic and saprophytic forms as natural inhabitants, and in the neritic areas, i.e., those subject to terrestrial influence, will be found a cosmopolitan bacterial population of varied origins. There we may meet with not only the true

marine denizens, but also soil forms washed down from land by rivers, and intestinal forms resulting from the drainage of sewage into the sea. It is in such an area that the work to be described has been done.

Historical.

The first work on marine bacteria was chiefly descriptive. In 1889, Sanfelice described the bacteria of the Gulf of Naples (33); in the same year, de Giaxa (17) worked on pathogenic micro-organisms from seawater, and found experimentally that whereas pathogenic forms flourished in vitro in sterile seawater, they failed to live long when true marine bacteria were also present. Russel described bacteria from the Gulf of Naples in 1892 (31), and from the Atlantic in 1893 (32). B.Fischer gave numerical bacterial analyses for the water samples collected on the German Plankton Expedition of 1889, and furthermore named and described the microscopic and cultural characteristics of a number of organisms. Most of the species were assigned to a new genus, Halibacter, characterised by short rods, frequently phosphorescent ; it is difficult to recognise any of these species today, as the data given would be regarded as insufficient for the identification of any form.

At the beginning of this century attention was turned to the physiology of marine bacteria. In 1901, Schmidt-Neilsen worked on those species which might affect the curing of herring. More particularly was the part played

by bacteria in the nitrogen-cycle studied; Baur (2) and Gran (18) isolated and described several species which under certain conditions reduce nitrogen compounds. Some years later Keding (23) and Keutner (24) isolated free nitrogen-fixing species and also forms symbiotic with certain algae. In 1910, Thomsen made a further contribution to the study of nitrogen-bacteria in the sea (36). From 1911, Drew made extensive studies of nitrate reduction in tropical waters and isolated one such denitrifying species (10,11,12,13).

The work of the last decade on marine micro-organisms has been largely concerned with nitrogen bacteria. Issatchenko (21), Lipman (26,27) and Berkeley (4) have in recent years worked with mixed cultures, which gave conditions approximating more to those of the sea. Their results do not conform with one another; they are discussed under the appropiate section in the following pages.

SECTION II. QUANTITATIVE INVESTIGATIONS.

For qualitative work, samples were taken at various parts of the Clyde at intervals: for the quantitative work, three stations were selected, and samples taken from approximately the same place, at regular intervals.

Stations Selected.

The three stations selected for regular sampling were as follows:

I. LOCH STRIVEN. This loch is notably free from steamer traffic, and the adjoining land is sparsely populated. It serves therefore to represent a sea area remarkably free from industrial or human contamination in view of its close proximity to land. See map on P. II.

2. LOCH LONG. Thornbank Station was selected for regular sampling. This station is moderately free from contamination, but there are greater possibilities of influences from the land manifesting themselves than in Loch Striven, since there are habitations along the shores of the loch, and there is a certain amount of boat traffic.

3. GREENOCK. Here the waters are estuarine in character, and highly polluted with sewage, industrial waste from sugar refineries, and effluents generally, which are brought down by the river Clyde.

At the above stations, samples were taken each month. Samples of water and mud were also obtained from other areas.

Sampling.

In order to determine the vertical distribution of bacteria at any one station, it was necessary to employ a sampling apparatus with which it would be possible to collect water at any desired depth without at the same time collecting water from other depths when the apparatus was being hauled up. The Nansen water-bottle, and similar closing water-bottles employed in marine work generally are unsuitable for bacteriological purposes, since they are made of metal, and most metals are known to have a marked bactericidal effect (IO). A second objection to the use of such a water-bottle is that the sample has to be siphoned into sterile bottles, and, while this is being carried out on deck, there is a possibility of contamination from organisms like Pseudomonas liquefaciens. A third objection is that it is unwieldy to sterilise such a piece of apparatus efficiently between the taking of ~~each~~ samples.

Several bacteriological water-samplers have been described; a sampling apparatus similar to that described by Birge (7) and Wilson (37) was selected for use.

It consists of a sampling tube and tube-holder. The tube is an ordinary combustion tube I5 cm. x 3 cm., fitted with a one-holed cork from which projects a glass tube bent at right angles. The end is drawn out and sealed at a point about I2 cm. from the bend. The glass parts are sterilised in the usual way in a hot-air oven for 30 minutes

at 160° C; and the rubber corks in a Koch steriliser. The parts are then fitted together, and again sterilised by steam for half-an-hour.

The tube-holder consists of an iron sinker, with a clamp for the sampling-tube. At the top of the sinker is a projecting lever arm, at ~~whose~~ the free end of which is a small brass breaking pin. When the tube is in position, the tip of the inlet tube lies immediately above the breaking pin. The apparatus was sent down on a Kelvin sounding wire worked from a hand winch fitted with a depth-recorder indicating fathoms. When the tube reached the required depth a brass messenger was sent down the lowering wire; this operated the lever arm, thus breaking the tip of the capillary tube.

The above apparatus was originally described by Wilson, and has been used by Birge for limnological work in shallow waters. The sampling tubes were evacuated. See P. I

For the work described in the following pages, it was not found necessary to evacuate all the tubes intended for sampling. At a depth of 5 fathoms, a tube filled with air at atmospheric pressure would take a sample whose volume would be half that of the tube; at greater depths, the increased pressure caused proportionally greater filling of the tube. Surface-sampling tubes were evacuated to a pressure of 30 to 60 mm., and made airtight with paraffin wax. This apparatus was used for depths down to 62 fathoms; its advantages lay chiefly in its simplicity, the extreme rapidity with which a series of samples could be

taken at any one station, and the fact that the same tube served both for taking the sample, and for transporting it to land. If used for greater depths, the tube must be provided with a well fitting cork to prevent it from being forced inwards, or else the inlet arm and tube must be blown in one piece; the latter type was used by Drew for sampling at depths of about 70 fathoms.

During preliminary trials at depths greater than 50 fathoms, the cork was displaced 4 times out of 6, but in the course of this investigation, the greatest normal working depth was 40 fathoms, and only two tubes were broken out of about 130 samples taken.

While the sampler is being hauled up, the tip is of course open, and it might be thought that the sample would undergo admixture with water from the upper layers through which it is passing. However, as the apparatus is being raised, the pressure in the tube is being continuously reduced and the compressed air is flowing out of the tube at the narrow orifice, thus preventing any admixture of water.

For quantitative work, a series of samples were taken from the surface waters, and at intervals of 10 fathoms to the bottom; the sampling tubes were sealed off in the flame, and packed in sterilised cotton wool for transit ashore.

Transport of Samples.

Two of the most significant factors in making total counts of the bacterial content of water are the time which elapses between the taking of the sample and its examination in the laboratory, and the temperature at which the sample is kept during such an interval. It was found that when samples were transported in a padded box, the temperature of the water on arriving at the laboratory was not raised more than 2° C., so that multiplication of the bacteria contained in the sample would not be appreciably hastened by a rise in temperature.

The samples were taken at the same time of day, and the same time interval between sampling and inoculating elapsed for the different hauls at any one station, as follows:

	Time of Sampling.	Time of Inoculating.
Loch Striven.	I2.30-I.0 p.m.	5.0 p.m.
Loch Long.	I.0 p.m.	5.30-6.0 p.m.
Greenock.	4.0 p.m.	5.30-6.0 p.m.

Laboratory Technique.

The procedure recommended by the American Society of Bacteriologists (I) for the standard examination of water-samples was adopted as far as possible for routine work, but the regular bacteriological examination of seawater does not appear to have called for attention previously, and

certain aspects evoked the use of special media and special methods.

The following routine media were employed (see Appendix): Standard Agar(Medium A); Standard Gelatine (Medium E); Conradi-Drigalski Agar (Medium F); McConkey Agar (Medium O); litmus-lactose-broth. The three last served for the detection of sewage contamination: the two first for ordinary quantitative work.

For each sample, the following were plated in the usual way with varying amounts of inoculum as under :-

Agar: I.O cc., I.O cc.(duplicate), 0.5 cc., O.I cc., uninoculated control.

Gelatine: the same as agar.

Conradi: I.O cc.

McConkey: I.O cc.

The two last were incubated at 37° C. and counted at the end of 24 hours for possible coliform and typhoid organisms, but these of course were not included in the routine numerical work. The agar and gelatine plates were incubated in dark containers at room temperature, and counted with the naked eye at the fifth day, by which time the number of colonies was fairly constant.

It will be noted that I.O cc. serves as the standard unit for inoculation, even for the Greenock area where the bacterial content is relatively high. For Greenock, the dilution-method was tried experimentally, but the greater

ease with which the colonies could be counted by this means did not compensate for the further margin of error introduced. For a similar reason, the writer did not adopt the sector-method of plate counting; instead, the whole plate was counted, the disadvantage being in the tedium of counting when the number of colonies exceeds 200.

The American Society of Bacteriologists recommends that, in order to avoid factitious accuracy, approximate numbers be given as under:-

I-50 recorded as found.

5I - I00 recorded to the nearest 5.

I0I - 250 recorded to the nearest I0.

25I - 500 recorded to the nearest 25.

50I - I000 recorded to the nearest 50.

Since, however, eight plates were made from each sample, and an average made from the figures so obtained, this procedure was not adopted.

RESULTS.

Loch Striven.

This is the type area, since it is one remarkably free from land contamination. The number of bacteria per c.c. was low compared with the numbers obtained for other stations in the Clyde area. Among those organisms present, the greater number appear to be moderately inactive water or soil forms. McConkey plates gave low values, and lactose broth tubes usually gave negative results: these were the two routine methods employed for detection of coliform species in the samples examined.

The numerical results obtained from the monthly samplings are given in Table I. From the total volume of water-sample plated out (usually 5.2 cc.), the average number of bacteria per cc. is estimated for each sample. These averages are given in the last column, and are used for the graphs in Figs. I and 2. In Fig.I, the bacterial content is plotted against time for the depths taken:- surface, IO fathoms, 20 fathoms, 30 fathoms, and bottom. The bottom sample was taken well above the underlying mud, and varied from 37 to 40 fathoms, as the sides of the loch are steeply sloping. The following conclusions are drawn from the results noted.

Vertical variation.

I. The number of surface bacteria is greater than that

TABLE I LOCH STRIVEN.

No. of bacteria present, determined by colony counts of plate cultures incubated 5 days at room temperature.

Depth.	Agar Cultures. 1.0	1.0	0.5	0.1 cc.	Gelatine Cultures. 1.0	1.0	0,5	0.1	Total.	Average per c.c.
18/5/28.										
Surface.	28	34	31	36	26	-	-	-	155	43
10 fath.	8	47	1	1	17	-	-	-	74	20
20 fath.	4	4	0	0	3	-	-	-	11	3
30 fath.	6	2	0	1	10	-	-	-	19	5
Bottom.	1	3	1	0	2	-	-	-	7	2
15/6/28.										
Surface.	8	3	0	-	2	11	-	-	26	5
10 fath.	5	3	6	-	10	13	6	-	43	8
20 fath.	1	2	0	-	4	8	6	-	21	4
30 fath.	0	0	0	-	6	7	5	-	18	3
Bottom.	2	2	2	-	13	11	3	-	33	6
24/8/28.										
Surface.	43	30	23	16	36	34	7	13	204	39
10 fath.	31	43	1	4	1	5	4	5	94	18
20 fath.	9	9	3	1	9	11	1	5	51	10
30 fath.	15	21	13	0	6	12	11	4	87	17
Bottom.	20	16	4	4	19	22	29	7	121	23
5/10/28.										
Surface.	15	24	2	0	21	17	7	1	87	17
10 fath.	4	6	5	0	7	9	2	0	33	6
20 fath,	9	8	-	-	3	2	-	-	22	5
30 fath.	0	0	2	0	2	0	0	0	4	1
Bottom.	0	1	0	2	2	3	1	0	9	2
2/11/28.										
Surface.	73	118	63	23	113	185	89	47	711	136
10 fath.	9	13	6	1	11	5	3	1	54	10
20 fath.	1	8	3	0	4	4	0	0	20	4
30 fath.	6	2	1	1	3	3	0	0	16	3
Bottom.	3	8	5	0	4	4	2	0	26	5

TABLE I. (contd.)

1/12/28.										
Surface.	27	29	5	0	33	27	12	1	134	26
10 fath.	11	1	3	0	15	12	5	1	48	9
20 fath.	3	8	1	0	3	1	1	1	18	3
30 fath.	2	2	0	0	0	3	0	0	7	1
Bottom.	4	7	2	0	0	1	4	0	18	3
18/1/29.										
Surface.	17	21	4	2	90	81	12	1	228	44
10 fath.	2	8	4	1	10	13	7	0	45	9
20 fath.	3	5	3	0	2	7	1	1	22	4
30 fath.	6	6	7	0	0	7	0	0	26	5
Bottom.	7	6	1	3	2	5	1	0	25	5
15/2/29.										
Surface.	117	159	22	15	109	161	41	5	629	120
10 fath.	40	42	20	3	29	9	6	4	153	29
20 fath.	12	15	8	2	0	15	13	11	76	15
30 fath.	8	7	5	3	6	11	2	0	42	8
Bottom.	5	9	1	1	10	3	3	0	32	6
7/3/29.										
Surface.	24	1	-	-	-	-	-	-	25	12
10 fath.	10	7	-	-	-	-	-	-	17	3
20 fath.	12	13	-	-	-	-	-	-	25	12
30 fath.	7	7	-	-	-	-	-	-	14	7
Bottom.	7	10	-	-	-	-	-	-	17	8

SUMMARY of TABLES I, II, and III, LOCH STRIVEN.

Depth.	Total no. of bacteria counted.	Average per c.c.
Surface.	4565	30
10 fathoms.	825	15
20 fathoms.	453	8
30 fathoms.	361	7
Bottom.	417	8

Fig. 1:
Loch Striven - Average number of Bacteria per cc.
Seasonal variation at different depths

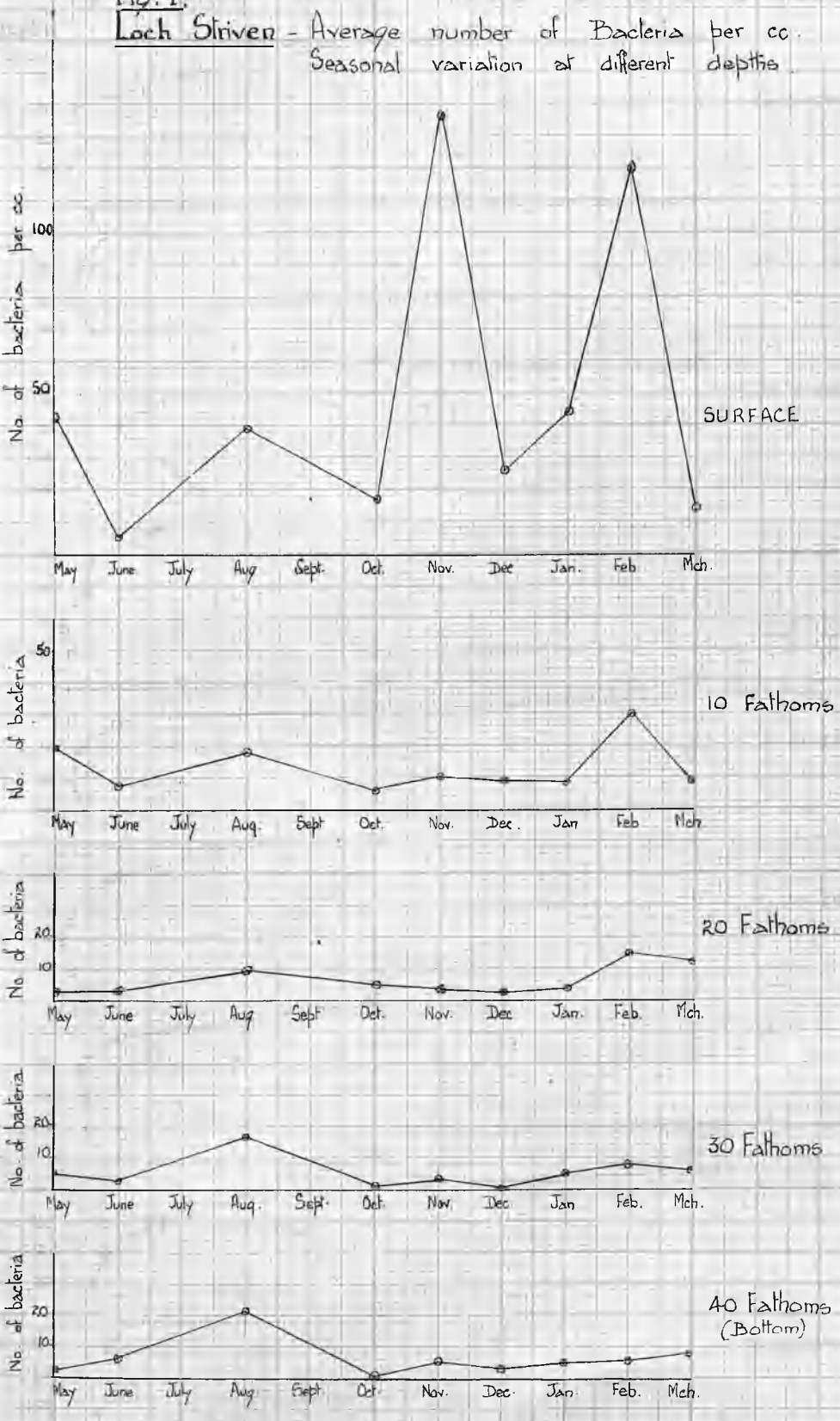
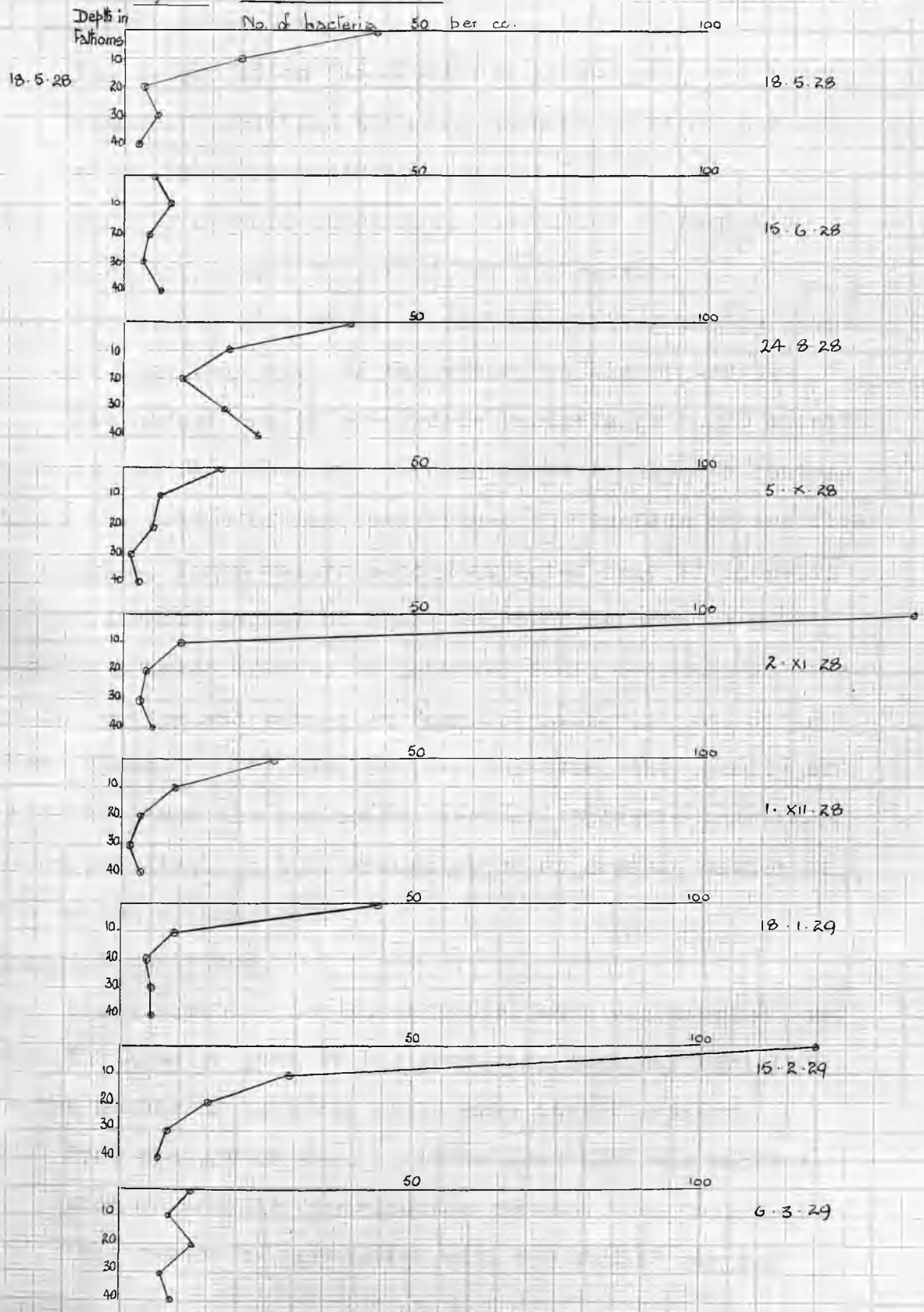


Fig: 2. Loch Striven - Vertical Distribution of Bacteria



of water at other levels.

2. The number of surface bacteria is subject to a wider range of variation than the numbers at other levels, which are comparatively constant.

3. From 10 fathoms downwards, the number of bacteria does not exceed 30 per cc. at any season.

4. The number of bacteria from Loch Striven bottom samples is less than that of the waters at higher levels.

The higher number of surface bacteria is to be attributed to the fact that any circumstances tending to contaminate the waters of the loch affect the surface waters first. In general, lower waters become affected only if there is a considerable amount of shore detritus of organic nature, such as bracken leaves, or detached fuci; this accumulates at the bottom and serves as food for bacterial and other saprophytes. In Loch Striven, however, the paucity of bacteria above the mud-bottom serves to indicate that there is comparatively little accumulation of organic matter at the bottom of the loch.

Seasonal variation.

Since sampling for quantitative work was carried out over the greater part of the year, any seasonal variation in the number of bacteria would make itself evident.

1. From a study of Fig. 1, it is seen that the bacteria show an erratic distribution through time, and that this cannot be correlated with any factor varying

TABLE II. LOCH STRIVEN.

Winter Series. 19/12/28-20/12/28.

Depth.	1 p.m.	4 p.m.	7 p.m.	10p.m.	1 a.m.	4 a.m.	7 a.m.	10 a.m.
Surface.	79	131	493	123	106	97	45	53
	123	172	560	103	181	181	38	75
(Average)	101	151	526	117	104	139	41	64
10 fath.	43	1	23	19	9	10	8	19
	18	27	29	18	13	9	9	16
(Average)	30	14	26	18	11	9	8	17
20 fath.	13	19	17	11	12	4	9	8
	10	22	21	18	9	12	7	-
(Average)	11	20	19	14	10	8	8	8
30 fath.	9	13	12	14	11	6	2	2
	1	10	9	14	6	11	12	10
(Average)	5	11	10	14	8	8	7	6
Bottom.	9	3	15	15	-	5	9	4
	3	13	13	11	20	8	8	2
(Average)	6	10	16	13	20	6	8	3

seasonally.

2. As previously stated, the surface bacteria vary from time to time over a wide range. Such fluctations may be due to sporadic factors, for example, the sudden increase of bacteria in November coincides with the arrival of herring in the loch and the consequent winter activities of herring fishing.

3. It is interesting to note that the August values are high for each level; this is the season when phytoplankton is at its minimal midsummer development. Whether these are inter-dependent, or whether both result from other causative factors, has not been determined.

Diurnal Variation.

The figures given in Table 1 were obtained from samples taken at different dates, but always at about the same time of day. In order to determine what diurnal variation, if any, took place in the course of the day, samples were taken in vertical series at 3-hour intervals over a period of 24 hours.

The conditions of plating differed from those of the monthly samples. Plating was done on the boat immediately after sampling; from each sample two agar plates were made, using 1.0 cc. as inoculum. Gelatine was not employed, its lower solidifying point rendering it less suitable than agar for work on a moving boat.

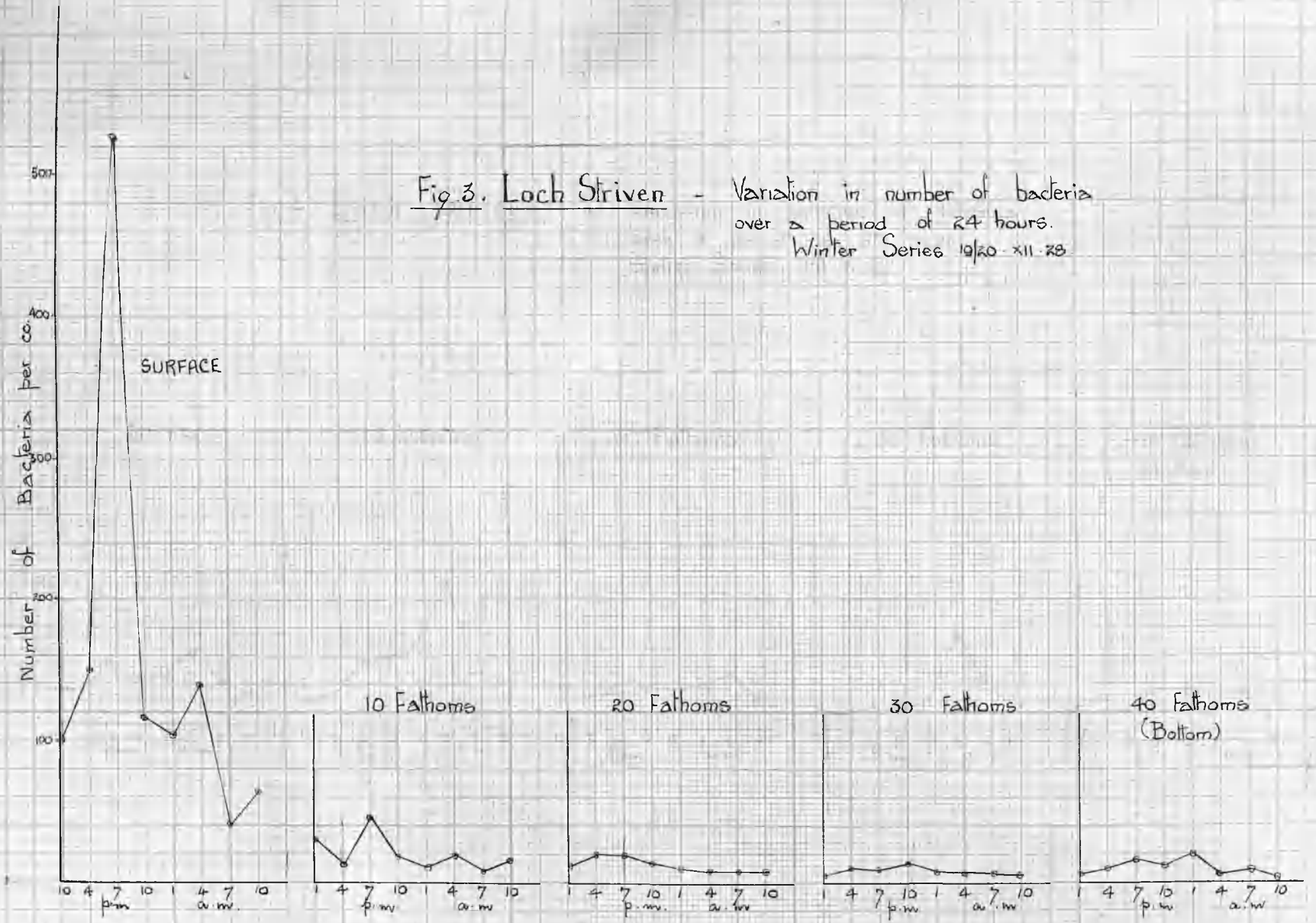
Two such 24-hours series were made. The results are given in Tables II and III, and summarised in Figs. 3 and 4.

TABLE III. LOCH STRIVEN.

Spring Series. 7/3/29 - 8/3/29.

Depth.	Noon	3 p.m.	6 p.m.	9 p.m.	Midnt.	3 a.m.	6 a.m.	9 a.m.
Surface.	24	14	24	18	12	22	14	7
	1	10	21	17	26	29	17	16
(Average)	12	12	22	17	18	26	15	11
10 fath.	10	20	11	22	29	45	22	23
	7	3	33	23	31	57	19	12
(Average)	8	11	22	25	30	51	20	17
20 fath.	12	12	11	13	16	10	17	12
	13	11	13	15	12	20	22	9
(Average)	12	11	12	14	14	15	19	10
30 fath.	7	3	10	4	9	33	17	6
	7	7	6	11	11	35	17	9
(Average)	7	5	8	7	10	34	17	7
Bottom.	7	9	7	15	21	14	11	4
	10	15	9	15	10	9	18	6
(Average)	8	12	8	15	15	12	14	5

Fig 3. Loch Striven - Variation in number of bacteria over a period of 24 hours.
Winter Series 19/20 XII 28



Winter Series, Dec. 19/20, 1928.

The following results were observed:-

1. The general vertical distribution over the 24-hour period agrees with that of the monthly samplings, i.e., there is a progressive decrease in the number of bacteria from the surface to the bottom, and the surface samples show a wider range in quantitative variation.

2. It was noted that at any given depth the numbers were higher at night, with a gradual decrease from 7.0 p.m. till the early hours of the morning.

3. With increasing depth, the maximum is delayed to a later hour, but the difference is only slight at 30 and 40 fathoms.

4. The surface numbers are unusually high: this may be correlated with the fact that the herring fishing was still in progress.

Spring Series, March 7/8, 1929.

A series of samples similar to the above was taken in the spring season. From an examination of Table III, Fig. 4 was constructed, and the following conclusions drawn:-

1. The number of surface bacteria is unusually low. This may be due to the low temperatures then prevailing, or to the fact that some weeks earlier the herring fishing had ceased.

2. The general tendency at all water-levels is for the numbers to increase during the night hours, with a maximum

TABLE IV. LOCH LONG.

No. of bacteria present, determined by colony counts of plate cultures incubated 5 days at room temperature.

Depth.	Agar Cultures. 1.0	1.0	0.5	0.1cc.	Gelatine Cultures. 1.0	1.0	0.5	0.1	Total.	Average per c.c.
23/5/28.										
Surface.	39	37	8	-	40	-	18	-	142	36
10 fath.	157	216	143	-	184	-	76	-	776	194
20 fath.	1	0	1	-	3	-	3	-	8	2
Bottom.	3	7	2	-	0	-	1	-	13	3
22/6/28.										
Surface.	39	10	0	-	53	48	37	-	187	37
10 fath.	21	10	2	-	40	18	14	-	105	21
20 fath.	5	7	0	-	9	6	4	-	31	6
Bottom.	1	0	1	-	8	6	6	-	22	4
27/8/28.										
Surface.	35	49	0	4	10	0	1	3	102	20
10 fath.	16	6	1	1	11	15	0	3	53	10
20 fath.	0	1	0	0	1	4	1	0	7	1
Bottom.	1	3	0	1	4	4	4	5	22	4
21/9/28.										
Surface.	38	70	33	3	123	39	83	5	449	86
10 fath.	9	7	5	1	23	20	4	3	72	14
20 fath.	9	11	3	3	1	12	6	11	56	11
Bottom.	19	11	4	1	34	19	11	1	100	19
12/10/28.										
Surface.	39	46	33	1	41	43	3	5	211	40
10 fath.	1	3	3	1	7	4	0	0	19	4
20 fath.	1	5	1	0	2	3	4	0	16	3
Bottom.	28	59	3	0	1	1	0	0	92	18
16/11/28.										
Surface.	129	107	51	5	50	43	14	12	411	79
10 fath.	20	21	7	6	19	17	2	0	92	18
20 fath.	3	13	4	1	0	2	5	1	29	6
Bottom.	27	24	15	2	12	10	1	1	92	18

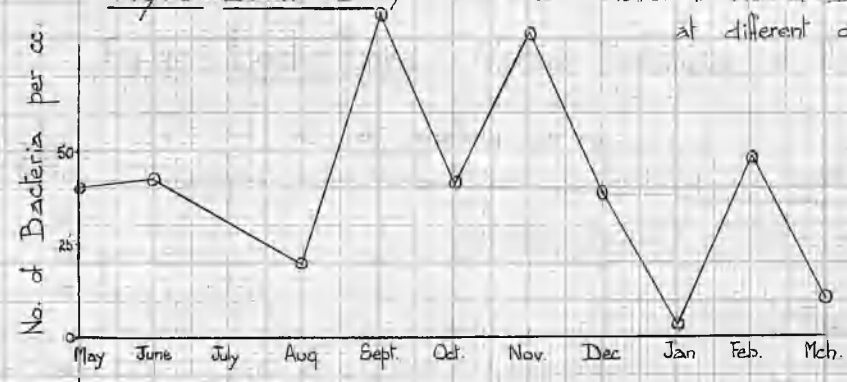
TABLE IV. (contd.)

17/12/28.										
Surface.	90	21	8	0	29	15	7	11	181	35
10 fath.	17	3	2	0	12	11	12	1	58	11
20 fath.	11	13	1	2	19	18	8	1	73	14
Bottom.	5	11	1	1	17	4	0	0	39	8
25/1/29.										
Surface.	3	3	0	0	0	4	1	1	12	2
10 fath.	1	0	1	0	5	2	1	0	10	2
20 fath.	3	2	0	0	3	2	2	0	17	3
Bottom.	6	2	3	1	5	2	1	0	25	5
22/2/29.										
Surface.	81	32	41	30	23	30	3	4	254	48
10 fath.	7	2	0	0	1	0	0	0	10	2
20 fath.	13	12	2	6	2	9	0	0	44	8
Bottom.	17	24	4	1	23	19	1	0	89	17
1/3/29.										
Surface.	7	24	5	4	2	3	0	0	45	9
10 fath.	3	1	7	1	1	0	0	0	13	2
20 fath.	5	4	1	0	5	9	2	1	27	5
Bottom.	10	9	4	2	11	5	1	1	43	8

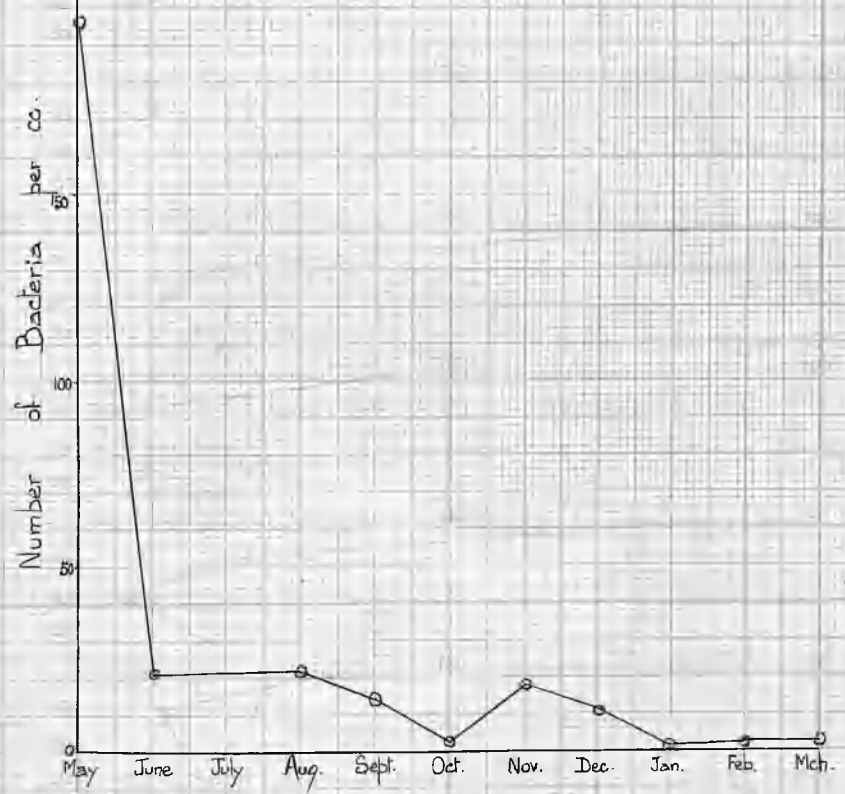
SUMMARY of TABLE IV. LOCH LONG.

Depth.	Total no. of bacteria counted.	Average per c.c.
Surface.	1740	39
10 fathom.	1198	27
20 fathom.	264	6
Bottom.	448	10

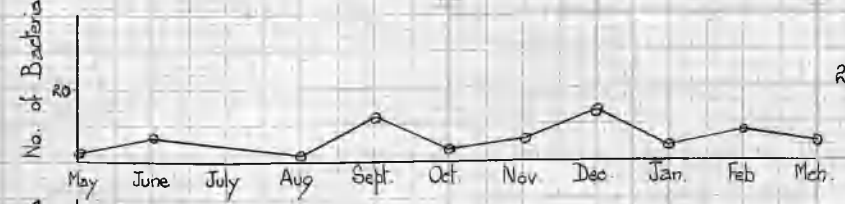
Fig. 5 : Loch Long - Seasonal Variation in No. of Bacteria at different depths



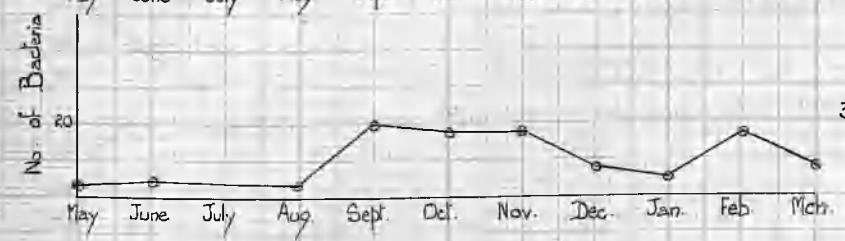
SURFACE



10 Fathoms

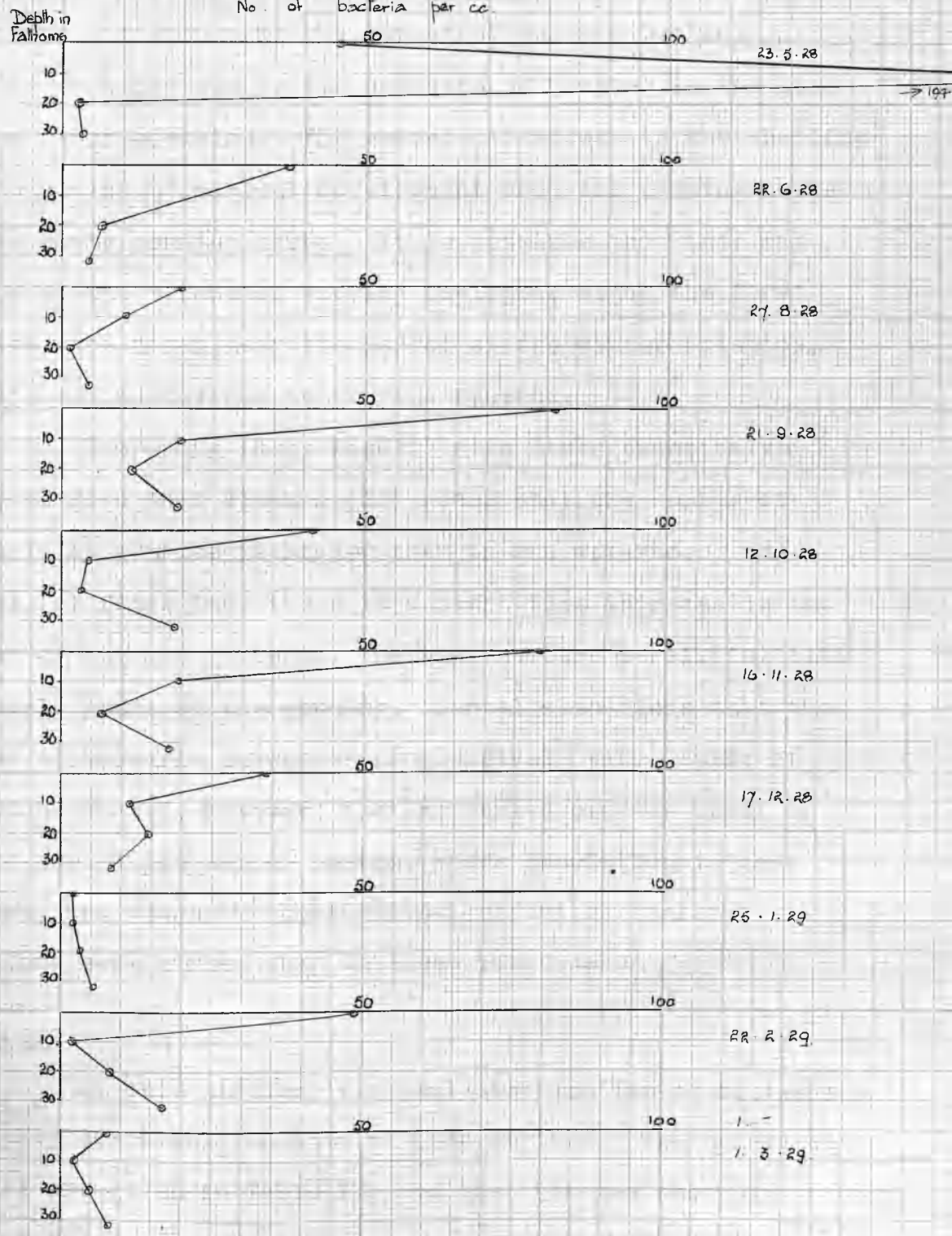


20 Fathoms



32 Fathoms (Bottom)

Fig. 6 : Loch Long - Vertical Distribution of Bacteria



at 3 a.m. in all but the bottom samples.

Comparing these two series of samples, we note firstly a difference in the number of surface bacteria; this is probably due to the presence of herring in the loch on the first occasion. The second difference is the shifting of the period of maximal development from the evening hours to the early morning hours. It is possible that this may be due to the effect of varying daylight, since the first samples were taken near the period of the Winter Solstice, and the second towards the Spring Equinox.

Comparing these results with other quantitaive data, we find that Fischer (15) notes that the number of bacteria at sunrise is higher than in the afternoon; and Bertel (6) finds that there is a night-time increase in the number of surface bacteria, the high numbers persisting till the early hours of the morning. Bertel also finds that the number of bacteria <u>increases</u> progressively with depth; it should be stated, however, that he worked off the coast of Monaco during the summer mpnths, where insolation is more intense, and where the bactericidal effect of sunlight is therefore more marked than in these latitudes.

<u>LOCH LONG</u>.

At this station, vertical distribution of bacteria is highly ? closely comparable to that of Loch Striven. The numerical results are given in Table IV, and the averages shown in Figs, 5 and 6.

The bacterial content is in general higher than that of Loch Striven, a fact to be connected with its greater boat traffic, and consequent greater liability to contamination.

Here again there is little evidence of seasonal variation; Fig. 5 shows the surface numbers to vary widely and apparently erratically, but the samples taken at other levels show a midsummer minimum with an autumnal increase, followed by low numbers in the months of January and February.

A comparison of Fig. 6 (Loch Long) with Fig. 2 (Loch Striven) shows that , whereas in the latter the number of bottom bacteria is low, in the former there is in most cases an increase in the number of bacteria from the bottom samples (32 fathoms) as compared with those taken at 20 fathoms.

It will be noted that very few bacteria were present in the January samples when the temperature was so low that the waters at the head of the adjoining loch were frozen. This seems to bear out the statement widely made that marine bacteria (and water bacteria generally) are sensitive to changes in temperature.

A noteworthy feature is that the May samples showed a much higher bacterial content at 10 fathoms than at the surface: this appears to be an exceptional case, and one for which no suitable explanation is at hand, unless we have regard to the fact that the number of coliform organisms

TABLE V. GREENOCK.

Np. pf bacteria present, determined by colony counts of plate cultures incubated 5 days at room temperature.

Depth.	Agar Cultures. 1.0	1.0	0,5	0,1	Gelatine Cultures. 1.0	1.0	0.5	0.1	Total.	Average per c.c.
8/6/28.										
Surface.	103	189	-	5	409	261	-	1	968	230
10 fath.	71	13	-	0	77	50	-	0	211	50
Bottom.	12	40	-	10	56	72	-	1	191	45
28/8/28.										
Surface.	123	80	6	28	119	71	31	63	521	100
10 fath.	8	17	3	6	88	93	25	34	274	52
Bottom.	153	61	6	5	113	51	35	17	441	85
22/9/28.										
Surface.	274	502	197	112	637	531	345	149	2747	530
10 fath.	62	33	11	2	102	73	12	2	297	57
Bottom.	36	48	63	3	110	143	79	51	533	103
12/10/28.										
Surface.	544	476	207	90	341	191	132	103	2084	400
10 fath.	71	112	39	13	29	41	67	20	442	85
Bottom.	51	107	28	11	13	23	13	6	262	50
16/11/28.										
Surface.	206	217	120	49	273	233	191	53	1342	257
10 fath.	39	20	24	13	29	35	12	3	173	33
Bottom.	38	31	31	2	44	47	16	4	213	41
17/12/28.										
Surface.	121	70	51	12	326	131	49	21	781	150
10 fath.	67	51	21	5	47	41	33	4	269	52
Bottom.	113	70	51	9	71	29	12	11	367	71
25/1/29.										
Surface.	49	46	36	5	88	139	29	1	393	75
10 fath.	69	119	69	19	63	130	39	13	521	100
Bottom.	18	17	4	0	8	11	6	0	64	12

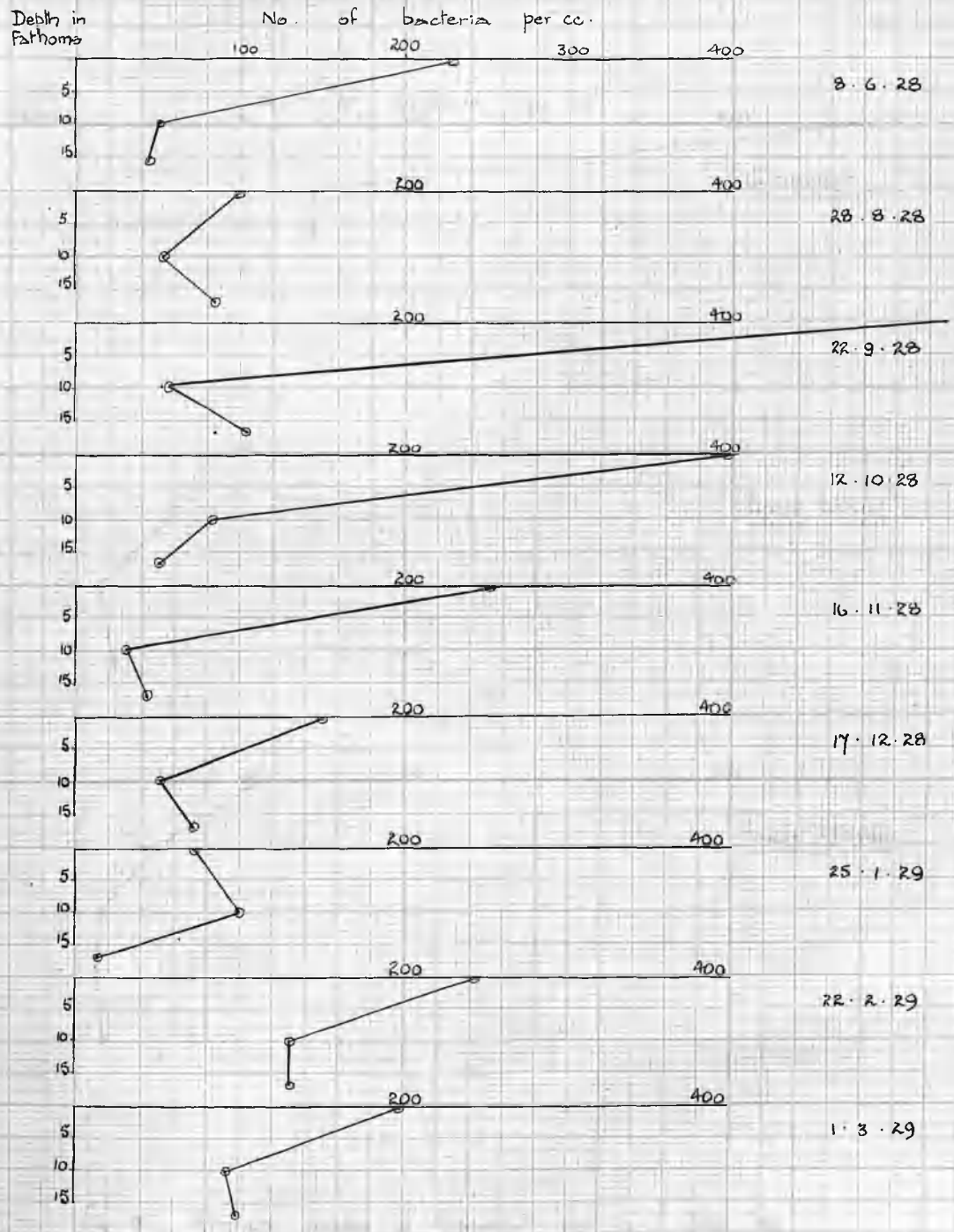
TABLE V. (contd.)

22/2/29.										
Surface.	401	317	91	35	177	210	23	7	1261	243
10 fath.	79	91	40	9	154	115	70	0	558	130
Bottom.	94	61	24	10	81	207	73	9	559	130
1/3/29.										
Surface.	76	201	153	71	89	331	71	41	1033	198
10 fath.	36	119	139	25	18	17	101	24	480	92
Bottom.	101	91	28	2	30	27	91	141	511	98

SUMMARY of TABLE V. GREENOCK.

Depth.	Total no, of bacteria counted.	Average per c.c.
Surface.	10162	240
10 fathom.	3014	72
Bottom.	2940	71

Fig. 8 Greenock - Vertical Distribution of Bacteria



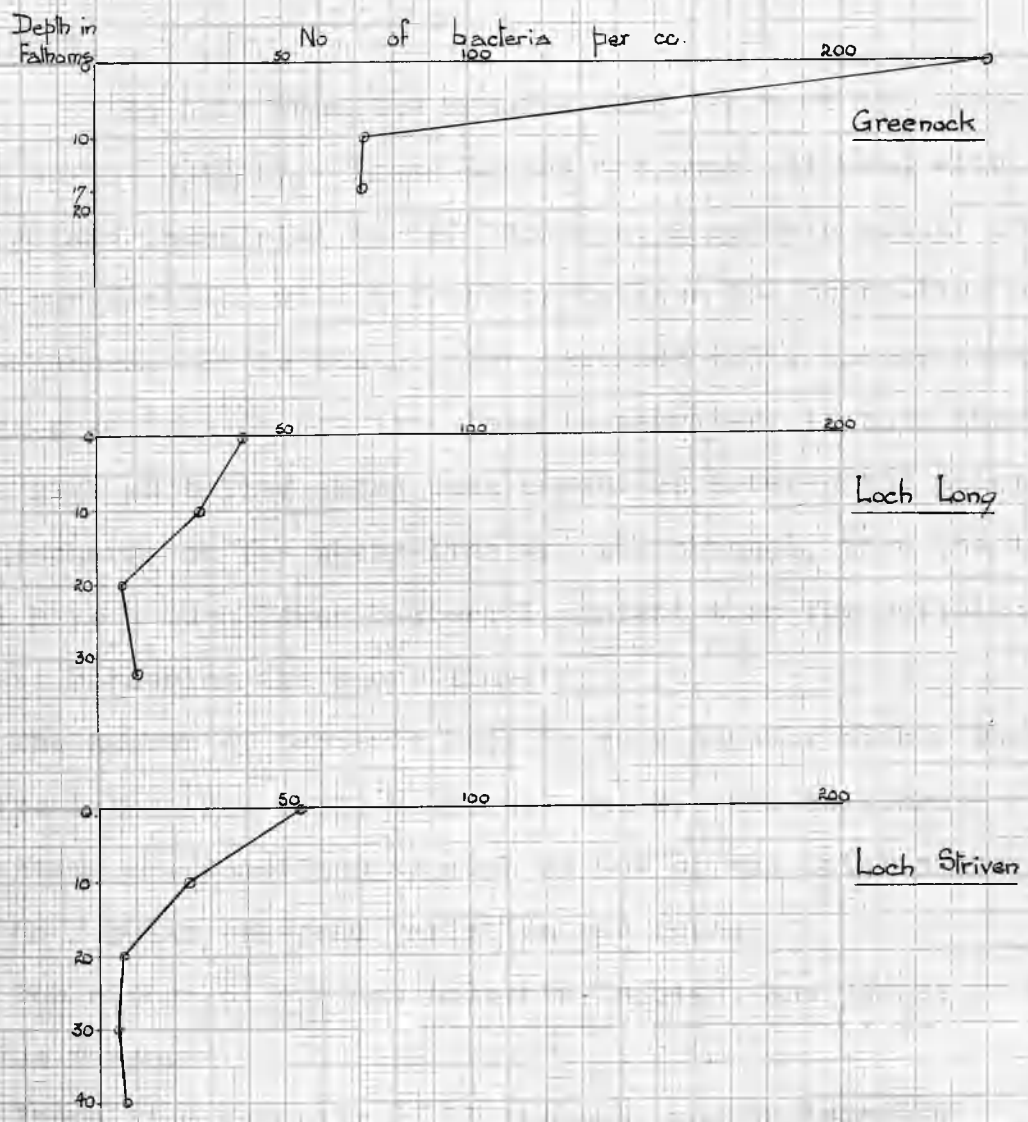


Fig: 9: Average number of bacteria per cc. over the period May 1928 - March 1929.

obtained on McConkey plates on that date was specially high, and three out of four lactose-broth cultures showed gas evolution.

GREENOCK.

In this area the samples were taken at the deepest part of the estuary. These waters are much polluted with sewage and industrial waste. However, a certain amount of self-purification will take place between the shore-line and mid-channel. It is stated, for instance (25), that pathogenic micro-organisms do not live long in seawater; if then there is a high mortality among such organisms between the point of discharge on the shore-line and mid-channel, then the latter will have a much lower bacterial content than the polluted waters bordering the shore-line.

1. The number of bacteria here is considerably higher than in the lochs (see Table V, Figs.7 and 8), and coliform organisms are sometimes as much as 50% of the total number.
2. No regular seasonal variation was found.
3. The number of surface bacteria is high, and varies over a wide range.
4. There is a decrease at 10 fathoms, and an increase again at the bottom.

CUMBRAE DEEP. (Off Arran. See Map.)

Ordinarily, samples were not taken at depths greater than 40 fathoms, but here a depth of 62 fathoms is

TABLE VI. CUMBRAE DEEP.

1/9/28.

Depth.	Agar Cultures. 1.0 cc.	0.5 cc.	0.1	Gelatine Cultures. 1.0	0.5	0.1	Total.	Average per c.c.
Surface.	204	56	22	193	103	29	607	100
10 fathom.	11	10	1	6	0	0	28	9
20 fathom.	40	5	3	-	1	0	49	23
30 fathom.	20	5	2	2	0	1	30	9
40 fathom.	6	3	0	3	1	0	13	4
50 fathom.	11	1	0	3	0	0	15	5
62 f.(bottom)	21	13	0	-	0	0	34	16

TABLE VII.

Quantitative bacterial analyses of littoral surface samples taken in the Fucus zone, CRAN BIGHT, outside the Millport Marine Biological Station.

Date of Sample.	Agar Cultures. 1.0 cc	1.0cc	0.1 cc	0.01 cc	Total	Average per c.c.
31/3/28.	442	457	91	0	990	470
2/4/28.	∞	∞	181	227	408	3000
3/4/28.	238	104	101	8	451	210
4/4/28.	201	-	15	5	221	220
5/4/28.	130	-	44	0	174	170
6/4/28.	521	479	71	1	1072	550
7/4/28.	89	198	21	0	308	150
8/4/28.	514	347	66	-	927	440
9/4/28.	193	85	10	10	298	140
10/4/28.	503	200	-	-	703	350
11/4/28.	371	366	-	-	737	370

Approximate mean average no. of bacteria per c.c. = 500.

TABLE VIII.

Quantitative bacterial analyses of surface samples taken from an Enteromorpha pool outside the Millport Marine Biological Station.

	0.1 cc	0.1 cc	0.01 cc	0.01 cc		
31/3/28.	494	593	61	27	1175	5300
2/4/28.	731	448	59	73	1311	6000
3/4/28.	728	568	81	35	1412	6400
4/4/28.	381	473	51	92	997	4600
5/4/28.	640	341	49	50	1080	4900
6/4/28.	760	451	60	81	1352	6100
7/4/28.	331	307	52	70	760	3500
8/4/28.	800	488	60	23	1371	6200
9/4/28.	411	571	21	82	1085	4900
10/4/28.	566	498	20	27	1111	5000
11/4/28.	470	403	21	72	966	4400

Approximate mean average = 4700

to be found. One series of samples was taken. An examination of Table VI shows the vertical distribution to be in general agreement with that of the lochs.

MILLPORT.

For purposes of comparison, Tables VII and VIII are appended, showing the bacterial content of the surface waters at the intertidal zone, and in a high water rock-pool respectively.

In both cases the numbers are very much higher than that of open water: this serves as an indication of the congestion of living organisms to be found in the narrow intertidal zone. Although, however, there was a larger number of individual bacteria, most of them could be classed as one of four or five very common species, and the total number of different species was less in number than that found in the open water.

Descriptive accounts of the different species will be found in the following section.

III. MORPHOLOGY AND CULTURAL CHARACTERISTICS OF SOME MARINE MICRO-ORGANISMS.

This work does not extend beyond the neritic zone, that is, the sea area bordering the land and subject therefore to land influences. Among the bacteria, as among other forms of life in this zone, there may be not only true marine species, but also stray organisms from the rivers and the land; the persistence of such species in the sea depends largely on their adaptability to salinity and marine conditions generally.

Soil organisms. When a vegetative bacterial cell is washed from the land by way of rivers into the sea, it suffers from a change in salinity of the surrounding medium. A sudden change would cause plasmolysis, but if any species can accommodate itself to the new conditions, then, other things being favorable, it would be able to establish itself in a marine habitat.

It is noteworthy that most of the undoubted soil organisms found in the Clyde sea area were spore-forming species; it is possible therefore that these do not live in the sea in a vegetative condition , but that their spores are present in seawater. Such soil organisms were found less frequently in the seawater itself than in the mud of

the sea bottom, where spores might remain in a resting condition over a long period.

The following viability test was carried out to determine the resistance to sea water of an ordinary soil organism in both the vegetative and the sporing state.

An agar-slope was inoculated with Bacillus megatherium and incubated at 32° C. Similar slopes were made at intervals of 12 hours for three days, and the series examined microscopically for spores. From each culture a suspension of the organism was prepared in sterile sea water. At the time intervals given below, the suspensions were well shaken and a loopful from each plated out on agar. After two days' incubation at 30° C the plates were examined for colonies of B. megatherium. The results are given below:-

Culture No:-	I	II	III	IV	V	VI	VII
Age of Culture:-	1hr.	18hrs.	30	42	54	66	78
Period of suspension							
12 hours	g	g	g	g	g	g	g
24 "	_	g	_	g	g	g	g
36 "	_	g	_	_	g	g	g
48 "	_	_	_	_	g	g	g
4 days	_	_	_	_	g	g	g
8 "	_	_	_	_	g	g	g
18 "	_	_	_	_	g	g	g
Spores present:-	0	0	0	few	sp.	sp.	sp.

g= growth of B.megatherium; _ = no growth; sp. =spores present.

A control experiment carried out by making suspensions in sterile tap water and plating out in the same manner, gave positive results.

From the above it may be concluded that vegetative cells of B. megatherium do not live longer than 36 hours in pure culture in sea water. It should be noted however, that its viability under natural conditions may be different. In the sea other factors intervene, as for example the presence of other micro-organisms which may favour or inhibit the growth of any one species.

Marine Organisms. In a sea area such as the Clyde it is a matter of some difficulty to decide which of its constituent organisms are truly native, and which are temporary inhabitants brought from the land, the river, or the estuarine zone. As far as they could be identified with known species, known soil organisms were present in comparatively small numbers in the sea water itself. On one exceptional occasion a large number of Actinomyces colonies were obtained from a surface sample in Loch Long. At Greenock the high bacterial content of the water is due in great measure to the coliform organisms present as a result of sewage contamination, and to Pseudomonas fluorescens (B. fluorescens liquefaciens).

In pure waters, notably those of Loch Striven, this organism is not of frequent occurrence, but whenever a great increase in the number of bacteria was observed, the dominant species was Ps. fluorescens. Samples taken at ten fathoms in

Loch Long in May 1928, and surface samples taken in Loch Striven in November of that year showed a high bacterial content which was largely due to the presence of this organism. It does not appear to be a true free-floating water species, but it occurs commonly as a saprophyte on dead fish, boat decks, and decaying algae, and will multiply in water only when there is an increase in the organic content. This was found to be of practical use, since Ps. fluorescens could thus serve as an indicator of the amount of organic matter present, in much the same manner as B. coli is used as an indicator of sewage pollution.

Apart from these species, there exist in the water organisms which are not normally found in other habitats. Many of these are readily killed and do not grow on ordinary culture media. If a water sample is centrifuged a number of forms are found, especially spirilla, which cannot be isolated on plate cultures in the usual way. The hardier species can however be isolated and their cultural and physiological characteristics worked out in the usual way.

It has not been found possible to identify all the forms isolated with known species, but as far as possible the unknown organisms have been assigned to a genus and classified near any species which appeared to be closely related. The classification of Bergey(3) has been adopted.

For each organism so treated, the chief morphological, cultural and physiological characteristics

are given below. With the exception of one organism, characterised by a distinctive blackening on agar, these marine species have all been assigned to the following genera:-

Family Coccaceae:	Genus Micrococcus.
Family Spirillaceae:	Vibrio.
Family Chromobacteriaceae:	Serratia.
	Flavobacterium.
	Achromobacter.
Family Bacillaceae.	Bacillus (sensu strictu).

Genus Micrococcus. Under this genus Bergey includes those forms which are normally spherical and which form aggregates of cells. Cells are rarely motile, and never form spores. Three of the marine species isolated from Clyde waters have been assigned to this genus.

1. Micrococcus sp. (laboratory type No. XVII).

   Numerical diagnosis: U 3031-52119-1111.(Society of American Bacteriologists).

   Morphology. Cells spherical, diameter 0.8 to 2.0μ, adhering to one another in loose and irregular masses of two to twenty individuals. The smaller cells form clusters, while the larger cells occur singly. When mounted in tap water they agglutinate very readily. Cell division may take place in any plane, but it rarely happens that more than two successive divisions take place in the same plane; as a result, one rarely finds in this organism chains of more than 4 cells. (See Plate III, fig. 9). Capsules, spores and zoogloeae

are absent, and the only variation displayed by the cells is a variation in size. The organism is Gram-negative.

Agar slope. Growth moderate, slightly raised, glistening, surface finely wrinkled and margin denticulate. Colour varies from a very faint pink to a very faint yellow, but apart from the colour variations, the cultural characteristics on agar are constant.

Agar colonies. Growth as for agar slopes, the colonies attaining a diameter up to eight mm. after seven days at 22° C. Colonies are irregular in outline, with a flat denticulate margin surrounding the central rugose zone. Figs. 6 and 7, Pl. III show two such colonies. The centre of old colonies frequently becomes raised and radially wrinkled.

Gelatine stab. Growth is best at the top, with only a faintly visible filiform growth along the line of puncture. The surface of the stab resembles an agar colony, until slow liquefaction sets in, and the colony sinks slightly into the medium. A ten-day culture incubated at 22° C shows a saccate liquefaction to a depth of four mm.

Gelatine colonies show the same characteristics as the surface growth of a gelatine stab. On ordinary meat-extract gelatine the rate of liquefaction is slow, but takes place more rapidly on a fish-extract gelatine (see Medium D, Appendix 1).

Nutrient Broth. Liquid becomes slightly turbid at first, and at ten days at 22° C a thick white or faintly pink pellicle develops on the surface; this is finely wrinkled like the

surface of the agar colonies. From the fact that gelatine stabs and broth cultures show the best growth at the surface, it may be concluded that this organism is strongly aerobic.

Litmus Milk. Acid and an acid curd occur after three weeks at 18° C; the curd is homogeneous without included gas bubbles.

Sugar reactions. Acid and gas are produced in two days from dextrose, saccharose, maltose and galactose; and in three days from lactose. Mannite is not fermented.

Chromogenesis. A faint pink colour is visible on most media, varying to yellow on agar. On potato and glycerine-potato the coloration is not more marked, though growth is good on both media.

Peptone water. This medium alone does not favour growth of this organism if no other nutrient substance is present. A small amount of ammonia is produced from peptone, but no indol and no hydrogen sulphide.

Denitrifying properties. This species of Micrococcus under normal cultural conditions grows best as an aerobe, but in the presence of a nitrate it flourishes under conditions of low oxygen supply, and reduces the nitrate with production of gas. Pl. III. figs. 4 and 5 show gas bubbles developed in the lower part of a nitrate gelatine stab, while in the upper part ordinary aerobic growth is in progress.

Occurrence. This organism has been found frequently in water samples at all depths from the lochs and the open part of the Clyde estuary. It has never been found in the Greenock

samples, nor in water collected from the shore-line and from rock pools at Millport.

Affinities. This organism is readily classed as a member of Bergey's genus Micrococcus, but it cannot be completely identified with any of the species described by him, as there is not total agreement in morphological, cultural and physiological characteristics.

2. Micrococcus sp. Laboratory type No. XIV.

Numerical diagnosis: U 3031-50229-1121.

Another species of Micrococcus isolated from the waters of the two lochs resembles the preceding type fairly closely, except in the following points:-

Agar colonies. These do not attain a diameter of more than 3mm. They are transparent, and develop a pink or opal colour, but no varieties showing a yellow colouration have been observed in this species. A concentric series of wrinkles is developed around the periphery of old colonies; the centre does not become raised and radially wrinkled, but remains transparent even in old cultures.

Gelatine stab. There is no liquefaction, and no growth deeper than 5 mm. after 5 days' incubation at 18° C.

Potato cultures show no growth.

Nitrates are reduced to nitrites, but no gas is formed.

Sugar reactions. Acid but no gas is formed from lactose.

Peptone water. Nitrites are formed from peptone.

3. Micrococcus sp. Laboratory type No. XXX.

Numerical diagnosis: U 3032-51130-1111.

Morphology. Cells spherical, diameter 0.5 to 1.5μ, forming irregular masses of from two to seven individual cells. These are non-motile and Gram-negative, and never form chains of cells like the two preceding species.

Agar slope. Growth on agar is white and hard, of a greyish-yellow colour. After about 12 days the centre becomes raised and assumes a chalky colour and consistency. The margin is entire and the surface smooth and shining in the early stages.

Agar colonies. These are circular in outline, dome-shaped, greyish in the early stages and chalky white later as on the slope cultures. The colonies are very rigid and appear to sink into the agar medium, although there is no liquefaction.

Gelatine stab. This organism is a strict aerobe, and growth therefore takes place at the surface only. Liquefaction does not take place.

Gelatine colonies. These resemble agar colonies.

Nutrient Broth. Medium becomes slightly turbid, with no formation of surface pellicle. A thick white sediment accumulates at the bottom of the tube after 5 days at 18° C.

Litmus milk. Acid is produced, but there is no reduction of the litmus, nor coagulation or peptonisation of the milk.

Sugar reactions. Acid and gas is produced in three days from dextrose, lactose, saccharose and mannite, but not from

maltose or galactose.

Chromogenesis is not found in this organism. Potato cultures show poor growth, greyish white in colour.

Peptone water. Ammonia and nitrites are produced from peptone; no indol nor hydrogen sulphide are formed.

Nitrates are not reduced.

Optimum temperature. 32° C.

Occurrence. Greenock estuarine water and Millport rock pools.

Affinities. There does not appear to be in the literature a description of a Micrococcus characterised by such colonies as are formed by this organism; physiologically, it resembles the preceding type, the chief difference being in its sugar reactions and the fact that it does not attack nitrates.

Family Spirillaceae: Genus Vibrio.

1. Vibrio sp. Laboratory type No. XVIII.

Bergey includes in this genus elongated curved cells, non-sporing, usually motile and dividing by transverse fission. Cells are short, bent rods which are rigid in shape, and may occur singly or in short chains. Typically these are Gram-negative water forms.

Morphology of Type XVIII. U5009-59130-3333.

Small, slender rods measuring 0.5 to 2.0μ in length and 0.2 to 0.5μ in breadth. These are slightly curved and generally occur singly, though pairs of cells are frequently found in young broth cultures. Cell-contents are very densely granular.

Agar colonies. Circular, greyish white colonies attain a diameter of about 2mm. after 5 days' incubation at 32° C. These are transparent, slightly raised above the surface of the medium, with an entire margin and a smooth and shining surface. Subsurface colonies do not develop well, but remain as small clear shining dots in the agar.

Agar slope. Growth on this is as for the above. The line of inoculation is visible as a clear transparent zone.

Gelatine stab. A filiform growth marks the line of the stab; the organism is a facultative anaerobe but grows best in the upper and more oxygenated layers of the medium. Liquefaction of the gelatine takes place with rapidity.

Gelatine colonies. On gelatine plates the medium is liquefied even more rapidly than in a stab culture; at 5 days the colonies lie in spreading saucer-shaped depressions filled with a clear liquid and free from sediment.

Nutrient Broth. Growth is marked by a clouding of the medium. There is no surface pellicle and no bottom sediment.

Litmus Milk. No reaction.

Sugar reactions. In the following sugars tried, there was no production of acid or gas in 14 days:- dextrose, lactose, saccharose, inulin, mannite, maltose and galactose.

Chromogenesis. No colour production was observed in the ordinary routine media tried: no growth was found on potato.

Peptone water. Nitrites, hydrogen sulphide and ammonia are

not formed from peptone; indol is found after 8 days' incubation at 32° C.

Nitrates are not reduced in the presence of peptone.

Optimum temperature. 32° C.

Occurrence. This organism occurs frequently in all surface samples from the open waters of the Clyde; it does not appear to thrive in the water of the Greenock area.

Affinities. This species appears to be related to Vibrio comma (Koch) and Vibrio strictus (Kutscher), but it cannot be identified completely with either of these, as it differs in some four or five cultural and physiological characteristics.

Family Chromobacteriaceae.

In this family are included, according to the classification of Bergey, rod-shaped cells without endospores producing a red, yellow, violet, green or blue pigment on solid media. Most of the marine forms isolated belong to this group, or to the following family, the Achromobactereae.

1. Serratia. This genus includes those forms which produce a red or pink pigment on solid media.

Serratia sp. Laboratory type No. VI.

Numerical diagnosis: U 5302-5219-1111.

Three varieties have been distinguished, differing chiefly in their cultural characteristics; such differences have remained constant after long sub-culturing, so they

appear to be distinct.

Variety (a).

Morphology. Cells small rods, 2μ in length and 0.5μ in breadth, occurring singly and in pairs, but rarely in long chains. Young cultures are actively motile; flagellation was not observed. The organism is Gram-negative.

Agar slope. Growth is slow on ordinary agar, but proceeds more rapidly on a medium to which a carbohydrate has been added. Mannite agar favours growth and pigmentation. The line of inoculation on this medium is visible after two days as a raised and shining viscid mass, with an entire margin and a smooth contour. There is a fetid smell, more pronounced when the culture is incubated at a temperature of 37°C. At a high temperature, the slope is of a creamy yellow colour, but incubation at room temperature gives a growth of a shell pink colour (see Pl. IV. fig. 2).

Agar colonies. These are circular, raised, smooth and shining, with a regular outline and an entire margin. Sub-surface colonies are smaller and often lenticular in shape; they are usually of a lighter colour.

Gelatine stab. Growth takes place along the whole line of the stab, but is best marked at the surface, where slow funnel-shaped liquefaction, from above downwards, occurs. A slight pink colour is developed.

Gelatine colonies. Slow downward liquefaction occurs, with production of a light pink colour.

Potato slope. Growth abundant, creamy and viscid. The culture is at first white, but if incubated at temperatures below 22° C the characteristic pink colour is developed.

Nutrient Broth. A pink surface pellicle is formed in two weeks at 22° C; the medium becomes turbid, and a brown sediment collects at the bottom of the tube.

Litmus Milk. In old cultures a homogeneous acid curd is formed.

Sugar reactions. Gas and acid are produced from dextrose, lactose saccharose; mannite, maltose and galactose are not fermented in 21 days at 18° C.

Bile salt agar. No growth.

Peptone water. Ammonia is formed from peptone, but no indol, no nitrite and no hydrogen sulphide.

Nitrates are reduced to nitrites in the presence of peptone.

Occurrence. This organism occurs in surface samples from all the areas studied. It is found more frequently in estuarine and littoral samples, so possibly it is not a true native of sea water.

Affinities. The type species in the genus Serratia is S.marcescens (B.prodigiosus). The organism now being described differs from S. marcescens in the following points:-

It liquefies gelatine only slowly, has smaller colonies on agar, and produces acid and gas in lactose and sucrose as well as in

dextrose. Of the species enumerated by Bergey, it appears to be most nearly related to S. mineacea.

Variety (b). This differed from variety (a) in the following points:-

Agar colonies do not exceed a diameter of 1 mm; they are compact with an entire margin and a hard, dull surface. In colour they are a deep red. This variety grows more slowly than the preceding.

Mannite agar slopes show a deep red growth. (Pl.IV. fig. 3).

Sugar reactions. Gas is produced in dextrose, and acid but no gas in sucrose and lactose.

Occurrence. As for the preceding variety.

Variety (c).Numerical diagnosis: U 5302- 52120- 1822.

This resembles variety (b) in all points except pigmentation. This white form is found far more frequently than the pigment-producing varieties. Such chromogenic organisms are known to vary greatly in their pigment-production and a difference in colour is not sufficient evidence for separating two varieties unless the difference under all conditions is constant. Here the non-chromogenic form cannot be induced to form colour under ordinary cultural conditions; it appears therefore to be a distinct variety.

Occurrence. This organism has been found in most of the water samples examined, and appears to be one of the most common forms, particularly in surface waters and in shore samples.

Genus Flavobacterium.

In this Bergey includes aerobic rods which are indifferent to carbohydrates, and which form a yellow or orange pigment on solid media. They are usually Gram-negative, and may be motile or non-motile. This genus consists of species which are typically water or soil organisms.

1. Flavobacterium sp. Laboratory type No. XXVIII.

Numerical diagnosis: U 5301-52115-1313.

Morphology. Small, broad rods, measuring 1.0 by 1.4 μ, occurring singly and in pairs. Cells appear clear and no plasmatic granules are distinguishable. In a young broth the cells are actively motile, but the method of attachment of flagella, if any, was not observed. Agar cultures and gelatine cultures produced non-motile individuals. The organism is Gram-negative.

Agar slope. Growth is abundant, spreading and moist. Young cultures have a smooth and shining surface, but after a few days they become wrinkled and folded, and of a yellow colour. (Pl. V. fig. 2). Minute irregularities on the surface are also developed and the growth becomes dull, with a brittle consistency when broken with a needle. There is little growth in the condensation water at the bottom of the tube.

Agar colonies. Colonies develop best on the surface; sub-surface colonies are small in size and die off very readily. The surface colonies grow rapidly, attaining a diameter of 4 to

10 mm. in 5 days at 18° C. They are circular, with entire margin as seen under a lens. A higher magnification shows the margin to be finely crenate in an old colony. Young cultures have a smooth and shining surface, but after some days concentric folds develop around the periphery, and the centre becomes raised. Between these, radiating wrinkles may arise, and the colony may present an appearance as is shown in Plate V. fig.5. These are usually of a pronounced yellow colour, but atypical white colonies with these characteristic markings sometimes occur in pure cultures of this form.

Gelatine stab. The line of puncture is marked by a filiform growth, but this is best developed at the top. Liquefaction is napiform, and takes place only slowly. The liquid so formed is a lighter yellow than the gelatine medium itself.

Gelatine colonies. As for the gelatine stab; liquefaction takes place slowly. The colonies do not show the characteristic markings of the agar colonies.

Nutrient Broth. The medium becomes clouded and flocculi occur throughout the liquid. There is also a white bottom sediment and old cultures show a surface pellicle which is white and wrinkled.

Potato slope. Growth is good on this medium; it is more abundant than on an agar culture of the same age, is of a dark yellow-brown colour, and becomes thrown into fleshy folds.

Litmus Milk. The top of the medium becomes alkaline, while the

bottom becomes acid and an acid curd is developed after 4 weeks.

Sugar reactions. In peptone water or in broth with an added sugar abundant growth is induced, but there is little evidence of fermentation. Maltose is fermented with gas production in 2 days and lactose in 7 days at 18° C. Neither acid nor gas is formed from dextrose, saccharose, mannite or galactose.

Chromogenesis. Under most cultural conditions a yellow or brown pigment is formed. It has already been noted that white colonies sometimes occur on agar, but in general even at a comparatively high incubation temperature, the yellow colour is produced.

Peptone water. Nitrites and ammonia are formed from peptone, but neither indol nor hydrogen sulphide. are formed.

Nitrates are reduced to nitrites and gas in the presence of peptone. This species is an active denitrifier, but does so more rapidly ~~if~~ when mixed with other micro-organisms than in pure culture.

Optimum temperature. 30° C.

Occurrence. In sea water samples at all depths individuals of this species have been found. It appears to be a true water form, as it is rare in Greenock, but is present in almost all the water samples taken from Loch Striven and Loch Long.

Affinities. This organism falls readily into the genus Flavobacterium, but it does not agree in cultural or physiological characteristics with any of the species described in the

literature. It is markedly different from those species enumerated by Bergey.

2. Flavobacterium sp. Laboratory type No. XXI.

Numerical diagnosis: U 5301-52115-1311.

This is similar to the preceding form except in the following points:-

Agar colonies. These have the characteristic concentric folds, but radial wrinkles do not develop. The margin is entire, smooth, irridescent and transparent.

Gelatine liquefaction. This takes place more rapidly than in the former.

Sugar reactions. Gas is produced from saccharose as well as from maltose and lactose.

3. Flavobacterium sp. Laboratory type No. XXII.

Numerical diagnosis: U 5302-52038-1111.

Morphology. Non-motile rods measuring 0.5μ by 1.5μ, occurring singly or occasionally in pairs. Gram-negative.

Agar slope. Growth moderate, beaded, convex and very much wrinkled so that the surface of the culture may be raised some millimetres above the agar. The growth is smooth and shining in the young state, with an entire margin and an orange-pink colour. In old cultures the medium becomes brown.

Agar colonies. These have the same characteristics as the agar slope, the colonies attaining a diameter of 2mm. after 2 days'

incubation at 1⅞° C. They are irregular in shape, and have a raised and much corrugated surface.

Gelatine stab. Growth is best developed at the upper part of the stab, though a filiform growth is found along the whole line of the puncture; the organism is thus a facultative anaerobe. There is no liquefaction.

Gelatine colonies. These are small, pink and circular. Subsurface colonies are of a lighter colour.

Nutrient Broth. Medium becomes clouded, with formation of a thin membranous pellicle on the surface. Orange flocculi occur throughout the broth of an old culture.

Potato slope. Growth moderate, orange-pink.

Litmus Milk. No reaction.

Sugar reactions. Gas and acid are produced from dextrose, lactose and saccharose, but not from mannite, galactose or maltose.

Peptone water. Ammonia is formed from peptone, but no indol, hydrogen sulphide or nitrite.

Chromogenesis. All cultures of this organism showed some colour ranging from pink to orange.

Nitrates are not reduced.

Occurrence. Found in Loch Striven water in surface, 10 fathom and 20 fathom samples. It has not been found in bottom samples.

Affinities. This organism resembles F.aurantiacum (Frankland) except that it is non-motile and that agar colonies are raised and irregular in shape and size.

4. Flavobacterium sp. Laboratory type No. XXIII.

Numerical diagnosis: U 5302-52116-2111.

Morphology. Small rods, 1.0μ by 1.2μ, motile, and Gram-negative.

Agar colonies. Orange yellow, shining, smooth, circular, with a diameter of 2 mm. after 5 days' incubation at 18° C. Young colonies are transparent and spreading; older colonies are opaque and somewhat raised.

Agar slope. Growth resembles agar colonies; a viscid orange mass develops in the condensation water at the foot of the tube.

Gelatine colonies. As agar colonies, except that slow liquefaction of the medium takes place.

Gelatine stab. Liquefaction funnel-shaped, with a faint growth along the line of puncture.

Nutrient Broth. Medium becomes clouded, with formation of a yellow sediment. There is no surface pellicle.

Potato slope. Growth moderate, of a deep orange colour.

Litmus Milk. No reaction.

Sugar reactions. Acid and gas are produced in dextrose, lactose and saccharose; there is no fermentation in mannite, maltose, or galactose.

Peptone water. ammonia and nitrites are formed from peptone, but no indol or hydrogen sulphide is formed.

Nitrates are reduced to nitrites and ammonia in the presence of peptone.

Optimum temperature. 22° C.

Occurrence. Found in small numbers in water from the lochs, and from rock pools, but not in estuarine polluted water.

Affinities. This species may be identical with F.caudatum (Wright). The main differences are that the latter may occur in pairs and in chains; that litmus milk is slightly reduced, and that starch is digested.

5. Flavobacterium sp. Laboratory type No. II.

Numerical diagnosis: U 5302-51126-1111.

Morphology. Small motile rods, measuring 0.5μ by 1.0μ to 1.5μ. These occur singly, and have somewhat square ends. Cells appear clear and are Gram-negative.

Agar slope. Growth on agar is scanty, orange yellow, smooth, shining and slightly raised above the surface of the medium. There is no spreading beyond the actual line of inoculation. A viscid orange sediment is formed in the condensation water at the foot of the tube. In old cultures the medium is often coloured a deep brown.

Agar colonies. These are slow in development, and do not attain a diameter of more than 1mm. after 5 days' incubation at 22° C. Colonies are circular, with the same general characteristics as on an agar slope. (See Pl. VI. fig. 3).

Gelatine stab. Growth takes place along the whole line of

the stab, and slow liquefaction proceeds from the surface downwards in the manner shown in Pl. VI. fig. 1. The liquid in the funnel-shaped zone of liquefaction is a bright orange colour.

Gelatine colonies. As for agar colonies, except that they sink into the medium as it becomes liquefied.

Nutrient Broth. The broth becomes slightly clouded, and a brown sediment is formed at the bottom of the tube. There is no surface pellicle.

Potato slope. Growth good, of an orange-brown colour.

Litmus Milk. No reaction.

Sugar reactions. Gas and acid are produced in 24 hours from dextrose, saccharose, mannite, maltose and galactose, and in 3 days from lactose at 18° C. It thus is exceptional in that it ferments the common sugars readily; the genus Flavobacterium is characterised in general by feeble powers of carbohydrate fermentation.

Peptone water. Ammonia and nitrites are produced from peptone, but neither indol nor hydrogen sulphide is formed.

Nitrates are reduced to nitrites in the presence of peptone.

Optimum temperature. 18° C.

Occurrence. Found frequently in samples taken at a depth of 20 fathoms in Loch Long and Loch Striven.

Affinities. This species does not agree closely in both cultural and morphological characteristics with any of the non-sporing rods producing an orange pigment. It is therefore classified as a previously unknown member of the genus Flavo-

bacterium.

These five species are the most common members of this genus found in the water samples studied. A number of other forms were encountered from time to time, but it was found impossible to work out even the general characteristics of the large number of apparently dfferent species. The principal forms are these described in this portion of the work.

Genus Achromobacter.

In this genus Bergey includes non-sporing rods, motile or non-motile, Gram-negative, not forming pigment on agar or gelatine. They are chiefly water or soil organisms.

1. Achromobacter sp. Laboratory type No.1.

Numerical diagnosis: U 5332-53210-2101.

Morphology. Rods measuring 0.8μ by 1.0μ, occurring singly and in pairs. They are non-motile and Gram-negative.

Agar slope. A white smooth growth, raised and shining, with an entire margin is formed on agar. Old cultures are opaque, but a 24-hour culture is translucent.

Agar colonies. Growth rapid, the colonies attaining a diameter of 5 mm. after 3 days' incubation at 18°C. Colonies are circular with an entire margin and a smooth raised surface. Sub-surface colonies assume a lenticular shape and are frequently of a dark cream colour.

Gelatine stab. Growth takes place along the whole line of the stab, but there is no liquefaction, and the greatest development is at the surface.

Gelatine colonies. Growth is as on agar.

Nutrient Broth. The medium becomes slightly clouded, and a surface pellicle develops, with pendent flocculi spreading from it downwards through the medium. There is no sediment in a sugar-free broth; if a sugar is added, a viscid white sediment is formed.

Potato slope. Growth moderate, white in colour.

Litmus milk. Acid is formed in 10 days at 18° C ; there is no curd and no peptonisation.

Sugar reactions. Acid and gas are produced in dextrose, saccharose and galactose; there is no fermentation of lactose, mannite or maltose. In a mannite-broth the medium becomes alkaline.

Peptone water. Ammonia and a small amount of gas are produced from peptone, but no indol, no nitrite, no hydrogen sulphide.

Nitrates are reduced to ammonia, nitrites and gas. This takes place very rapidly, as gas formation is evident in 3 days. This organism is probably largely responsible for any nitrate-reduction that may occur in the waters of the Clyde area.

Occurrence. This is the commonest organism of general distribution in the Clyde area. It is to be found in most samples taken at all depths, though it is more frequent in surface and bottom samples than in water taken from intermediate depths.

Affinities. This organism resembles A. ubiquitum (Jordan);

the only differences being that the latter has agar colonies with an entire to irregular margin, that it coagulates milk, and that although nitrates are reduced to nitrites there is no gas formation.

2. Achromobacter sp. Laboratory type No. VII.

Numerical diagnosis: U 5332-52210-2101.

Morphology. Small diplobacilli resembling the cells of the preceding species.

Agar colonies. These attain a diameter of 2 mm. after 5 days' incubation at 22° C, and vary in colour from pure white to cream or light brown. Sub-surface colonies are frequently lenticular in shape.

Occurrence. This organism is especially common in most samples and is next in order of frequency to the preceding species, which it resembles. It is particularly abundant in water samples taken from the shore-line and from rock-pools.

Affinities. The appearance of agar cultures of this organism is markedly different from the last-named species, but other cultural and physiological characteristics are similar. One morphological or cultural character does not suffice to separate one organism from a closely related form, so that possibly this second species of the genus Achromobacter is simply a variety of the first. The differences in appearance were constant, and the distribution of the two organisms does not exactly coincide. (See Pl. VII).

It resembles Achromobacter venosum (Vaughan), except that the latter does not reduce nitrates.

3. Achromobacter sp. Laboratory type No. III.

Numerical diagnosis: U 5032-51230-2333.

Morphology. Small oval diplobacilli, measuring 0.8 by 1.0 microns. They are non-motile and Gram-negative.

Agar slope. Growth scant, filiform, flat and transparent. The margin of the culture is finely serrate, and the surface smooth and glistening.

Agar colonies. The organism does not grow on ordinary standard agar, but gives somewhat better results on fish agar. (See Medium C, Appendix 1). Colonies are small, attaining a diameter of 0.5 to 1.0 mm. They appear as clear glistening transparent drops.

Gelatine stab. The organism is a strict aerobe, for growth takes place at the surface of the stab only. There is no liquefaction.

Gelatine colonies. As for agar colonies.

Nutrient Broth. Medium shows a slight clouding, with no surface pellicle and no bottom sediment.

Potato slope. No growth.

Litmus Milk. No reaction.

Sugar reactions. Acid is formed in dextrose, but there is no fermentation of saccharose, lactose, mannite, maltose or galactose.

Peptone water. The organism does not grow well in this medium; it produces from peptone ammonia, but no indol, no nitrite, and no hydrogen sulphide.

Nitrates are not reduced to nitrites.

Optimum temperature. 18° C.

Occurrence. Found in bottom samples and at other depths, but not in surface samples. The organism occurs frequently, but in small numbers. It grows with difficulty on ordinary culture media, so it is possible that the laboratory records of its frequency, made from agar and gelatine plates, do not represent its actual frequency in the sea.

Affinities. Bergey includes fifty species in his genus Flavobacterium, but this form does not resemble any among this number, although it falls readily into the group.

4. Achromobacter sp. Laboratory type No. IV.

Numerical diagnosis: U 5012-51230-2133.

Morphology. Motile rods, measuring 1.0 by 1.0 to 1.5 microns, occurring singly and occasionally in pairs. In old cultures the cells frequently divided without increasing their size much between each cell-division; this resulted in the presence of very much smaller rods measuring 0.4 by 0.6 microns. The cells are Gram-negative.

Agar slope. A transparent filiform line of growth is formed, but development takes place at a very slow rate and the culture dies off very readily.

Agar cultures. Colonies on an agar plate are small, transparent, colourless and slightly raised. A young colony is dome-shaped smooth and shining; at 8 days a colony usually has a central opaque dot and a series of concentric markings around the periphery.

Gelatine stab. The organism is a strict aerobe, and does not grow below the surface in a stab culture. There is no liquefaction.

Gelatine colonies. As on agar.

Nutrient Broth. The medium becomes somewhat cloudy, but there is no growth at the surface or sediment at the bottom.

Litmus Milk. Growth takes place, but there is no reaction with either the milk or the indicator.

Fermentations. Gas is produced in dextrose after 3 days at 18° C; growth but no fermentation takes place with lactose, saccharose, mannite, maltose or galactose.

Peptone water. Ammonia is formed from peptone.

Nitrates are reduced to nitrites in the presence of peptone.

Occurrence. Found in mud samples and in bottom samples, but not in surface samples.

Affinities. This species resembles A. guttatum (Zimmerman); the only points of difference being that the latter gives a yellowish growth on potato and does not reduce nitrates.

5. Achromobacter sp. Laboratory type No. VIII.

Numerical disgnosis: U 5333-52210-2101.

Morphology. Small non-motile rods measuring 1.0 by 1.5 microns.

The cells are usually associated in pairs, and are Gram-negative.

Agar slope. Growth abundant, spreading, with an undulating margin and irregular shining surface. Sediment in the condensation water at the foot of the tube is white and slimy.

Agar colonies. These are large and under favourable conditions, as e.g., where there are not many colonies on any one plate, they may attain a diameter of 15 mm. The edge of the colony is deeply lobate and the surface raised and undulating. (See Pl. VIII, figs 4 and 5).

Gelatine colonies. As agar colonies, except that growth is not so luxuriant and the margin is frequently entire.

Gelatine stab. Growth takes place along the whole of the line of puncture; there is no liquefaction.

Potato slope. A creamy white fleshy growth similar to that on agar is formed.

Nutrient Broth. The medium becomes very turbid, with a slimy grey sediment at the bottom of the tube, and a thin greyish pellicle on the surface.

Litmus Milk. Acid is formed, but not in sufficient amount to form a curd.

Sugar reactions. Acid and gas are formed in dextrose, saccharose, and galactose; there is no fermentation in lactose, mannite or maltose.

Peptone water. Ammonia is formed, but no indol.

Nitrates are reduced to nitrites and Ammonia in the presence of peptone.

Occurrence. In mud samples from loch Striven and from the firth opposite Largs.

Affinities. This organism closely resembles Type No. VII. except for its cultural characteristics (see Pl. VII and Pl. VIII); it may therefore be classed near Achromobacter venosum (Vaughan), although it differs in some of its physiological reactions.

Genus Bacillus.(sensu strictu).

This genus includes -sporing saprophytic aerobic rods which may be motile or non-motile. They generally liquefy gelatine and often grow in long chains, forming rhizoid colonies. The rod is not swollen at sporulation. (Bergey).

1. Bacillus sp. Laboratory type No. XXXV.

Numerical disgnosis: U 5801-52218-2131.

Morphology. Rods measuring 0.8 to 1.0 by 3.0 to 4.0 microns. These usually occur singly. Spores central and rounded (Pl. IX). Cells are Gram-positive, and contain densely granular cytoplasm.

Agar slope. Growth abundant, membranous becoming fleshy. In old cultures the surface is thrown into deep folds of a dark brown colour with a smooth and shining appearance.

Agar colonies. These attain a diameter of 7 mm. in 7 days at 18° C. They are circular with an irregular margin and a fleshy surface growth thrown into radial folds as shown in the photographs on Pl. IX. The centre is much raised.

Gelatine stab. The surface growth is abundant, but little or no growth takes place in the lower part of the tube, and

there is no liquefaction.

Gelatine colonies. These resemble agar colonies, or sometimes show no surface corrugations.

Nutrient Broth. The medium becomes turbid, with formation of a surface film but no bottom deposit.

Potato slope. Good growth occurs on this medium, resembling that on agar.

Litmus Milk. Acid is formed in 6 days at 18°C, and an acid curd in 20 days.

Sugar reactions. Acid and gas are formed from dextrose and saccharose; acid alone from dulcite; neither gas nor acid from lactose, mannite, maltose or galactose.

Peptone water. Indol and ammonia are produced in this medium, but no hydrogen sulphide nor nitrite.

Nitrates are reduced to nitrites in the presence of peptone.

Occurrence. Found in mud samples from Loch Striven, Cumbrae Deep and off Largs.

Affinities. This organism resembles Bacillus sphaericus (Neide) but is not identical with it, since the latter differs in the following points:- Growth on agar is slow, clear and transparent; On potato a thin greyish film is formed, and indol is not produced from peptone.

9. Bacillus sp. Laboratory type No. XXXII.

Numerical diagnosis: U 5231-53229-1101.

Morphology. Slender rods measuring 0.6 by 4 microns; these

occur singly, are motile, and Gram-negative. Cell contents are densely granular. Spores are oval and terminal or less frequently sub-terminal.

Agar slope. Growth thin and transparent. At 7 days the culture is a pale pink wrinkled membranous mass.

Agar colonies. These are small, circular, transparent and slightly wrinkled. They grow slowly, attaining a diameter of not more than 1 mm. after 5 days' incubation at 32° C.

Gelatine stab. Growth takes place along the whole line of puncture, but is best developed at the top. There is no liquefaction.

Gelatine colonies. As on agar.

Nutrient Broth. The medium remains clear, but there is a surface membrane and a botton sediment.

Potato slope. No growth.

Litmus Milk. There is no curdling of the milk, but it is slowly peptonised.

Sugar reactions. Acid and gas are formed from dextrose and saccharose; lactose, mannite, maltose and galactose are not fermented.

Peptone.water. Indol and ammonia are formed from peptone.

Nitrates are reduced to nitrites in the presence of peptone.

Occurrence. This occurs in surface and 10 fathom samples taken in Loch Long and Loch Striven.

Affinities. This organism does not agree with any of the species

described by Bergey in his manual.

3. Bacillus sp. Laboratory type No. V.

Numerical diagnosis: U 5101-5101-2101.

Morphology. Large rods, measuring 1.0 to 2.0 by 4.0 to 5.0 microns. These cells may occur in short chains, and are densely filled with granular contents. They are motile and Gram-positive. Spores are oval and central.

Agar slope. Growth moderate, slightly raised, glistening and clear white; the surface is finely rugose and the margin appears entire to the naked eye.

Agar colonies. Growth rapid, the colonies assuming a circular or irregularly lobate shape (see Pl. X. fig.3). The centre becomes raised in old colonies, and the margin at a higher magnification shows numerous irregular outgrowths formed by protruding chains of cells.

Gelatine stab. Growth is strictly aerobic, for the lower part of the stab shows no development of the organism. Liquefaction takes place slowly.

Gelatine colonies. As on agar, the colonies sinking slowly into the medium as liquefaction proceeds.

Nutrient Broth. The medium becomes turbid, with formation of a compact bottom sediment but no surface film.

Potato slope. No growth.

Litmus milk. Acid is formed in 3 days at 37° C, and an acid curd in 3 weeks at that temperature.

Sugar reactions. Acid and gas are formed from dextrose and

saccharose ; there is no fermentation in lactose, mannite, maltose or galactose.

Peptone water. Ammonia is formed from peptone, but no indol, nitrite or hydrogen sulphide.

Nitrates are vigorously reduced to nitrites and gas in the presence of peptone; denitrification takes place also in a peptone-free medium (see Medium N, Appendix 1), provided that other micro-organisms are present in the culture.

Occurrence. This form is found in small numbers in most water samples and mud samples.

Affinities. This does not resemble the species listed by Bergey or the denitrifying species described by Baur (2) and Gran (18).

4. Bacillus sp. Laboratory type No. XIII.

Numerical diagnosis: 5131-52110-1311.

Morphology. Rods forming long chains, each cell measuring 1.0 by 3.0 to 4.0 microns. Spores oval and equatorial, often smaller than the parent cell, and lying loose within it. They are Gram-positive and non-motile.

Agar colonies. Greyish-white, spreading and slightly raised in the centre. Colonies usually rhizoid with much branched margins, but rounded colonies without the characteristic shape sometimes occur in pure cultures. Several varieties differing only in their cultural characters were found; these are shown in Pl. XI.

Gelatine stab. Growth rapid, arborescent, with stratiform lique-

faction.

Gelatine colonies. As on agar.

Nutrient Broth. Culture clear, with starry flocculi scattered throughout the medium. There is no bottom sediment, but a surface pellicle develops after 2 weeks at 37° C.

Potato slope. Growth whitish to yellow, moderate.

Litmus milk. The medium becomes slightly alkaline and slow peptonisation takes place.

Sugar reactions. Acid and gas are formed from lactrose, saccharose, and maltose; there is no fermentation of dextrose, mannite or galactose.

Peptone water. Ammonia and nitrites are formed , but no indol nor hydrogen sulphide.

Nitrates are reduced to nitrites in the presence of peptone if the organism is in pure culture; in the presence of other micro-organisms gas is also produced.

Occurrence. Found rarely in surface samples, but it is a frequent component of the bacterial flora of muds.

Affinities. This species resembles an ordinary soil bacillus, and is morphologically like B. mycoides (Flugge). The only points of difference are that the latter causes turbidity in broth, does not reduce nitrates, and produces acid only in dextrose, and saccharose.

The foregoing organisms have been described because they are the most common forms in the Clyde area. About 60 other types of less frequent occurrence have been isolated;

further study may show that many of these are identical and that the number is in reality much lower, but the cultural and physiological characteristics of each of these have not been fully worked out.

One organism which cannot be classified with the foregoing, and which presented many features of interest, particularly its agar-blackening power, was studied in greater detail. The work done so far on this organism is described in the following pages taken from the current issue of the Journal of the Royal Technical College, vol. II No. 5.

EXTRACT

from the

JOURNAL OF THE ROYAL TECHNICAL COLLEGE.

Vol. II. No. 5.

Preliminary Note on a Marine Chromogenic Micro-Organism

By Miss B. Lloyd, M.Sc.

ABSTRACT.

A preliminary account is given of a hitherto undescribed bacillus isolated from a sea-water sample. Its morphology and its cultural characteristics are described; the conditions affecting its distinctive chromogenesis are noted; and its viability in water is considered.

The opinion is expressed here that it is genetically related to *Bacillus salmonicida*.

The following is a preliminary account of a micro-organism isolated from sea-water in the course of a quantitative bacteriological investigation of the Clyde sea area. Samples have been taken at depths down to 62 fathoms, using a water-sampler similar to that described by Wilson.[1] The original apparatus was designed for shallow-water limnological bacterial work, but it has been found to serve excellently for marine sampling down to the depths indicated.

The apparatus was worked from the "Nautilus," the boat of the Scottish Marine Biological Association, and the work was carried out in part at their laboratory at Millport. I am greatly indebted to the Superintendent, Mr. Elmhirst, for very readily placing at my disposal the facilities of the Marine Station and its boats.

The organism about to be described was isolated from a water-sample taken in Loch Striven at a depth of 37 fathoms. This was from the water immediately above the muddy bottom; the organism may therefore be a planktonic form or a mud inhabitant, living on the organic detritus which accumulates on the sea floor.

Morphology.—Microscopically, the organism was seen to be a short broad bacillus with rounded ends, an average breadth of 0·8μ and an average length of 1·2μ on solid nutrient media. In liquid media longer forms were sometimes encountered; on no occasion were involution forms observed.

[1] Wilson, Journ. Bact., 1920, p. 103.

At a magnification of 1,200 diameters it was possible to distinguish in optical section only the peripheral membrane and finely granular protoplasm; no other cell-contents were observed. Its life-history is of the simplest type known even among bacteria, as it consists only of serial stages in vegetative fission. No spores were observed, neither were zoogloeae or encapsulated or other resting stages found under the experimental conditions investigated. It is possible, however, that the cell itself may without visible change become resistant to unfavourable external conditions, because cultures of this bacillus were frequently found viable after subjection to such treatment as would normally kill any non-sporing micro-organism.

It is non-motile under laboratory conditions of culture in bouillon, peptone-water, agar, and gelatine.

Staining-reactions.—This organism is Gram-negative. Dry films prepared in the usual way and treated with carbol-fuchsin did not show to any marked degree the shrinkage that frequently follows the treatment of ordinary vegetative bacterial cells with this reagent. This is evidence of the possession of a resistant cell-membrane.

Cultural Characteristics.

Agar Slope.—A 24-hour culture incubated at room temperature shows a thin white transparent growth which becomes thereafter progressively more opaque. At all stages a marked violet fluorescence is shown, and the increasing opacity of an older culture is accompanied by a brown coloration of the growth itself. In the water in the butt of the tube a viscid fine white bacterial sediment is formed. The agar growth itself has an entire margin; the surface is slightly raised, moist, smooth, and glistening.

Pigmentation on Meat-agar.—A remarkable feature of cultures on ordinary meat-extract agar is the development of a dark pigment. At about the 15th day at room temperature the medium immediately below the bacterial growth becomes brown, the colour deepening with age. This colour diffuses through the medium, so that in four weeks' time the whole of the agar becomes uniformly a very dark brown. A chromogenesis of this nature is very distinctive, and the pigment-producing bacteria are usually readily classified and named. Such pigment-production is characteristic of members of the Chrombactereae (Bergey);[2] but this organism cannot be assigned to any known member of that group. See Plate XII.

Agar Colonies.—Agar plate cultures exhibit characteristics similar to those enumerated above for agar slopes. Colonies are round, only

[2] Bergey, Manual of Determinative Bacteriology, Baltimore, 1923.

slightly raised above the surface of the medium, and attain a maximum diameter of 2·0 mm. These colonies, like the agar streak culture, induce pigmentation in the agar, so that on old plates there is a zone of discoloration around each colony. The colour is not developed when the colonies are crowded, in pure or mixed culture.

Fish-agar.—This medium is prepared as follows:—Two lb. of cod flesh freed from skin are heated in one litre of sea-water for four hours at a temperature of 60—70°C. The extract is then decanted, filtered, and made up to one litre with distilled water. This serves as standard fish extract.

Procedure from this point is as for ordinary meat-extract agar, except that NaCl is omitted. 1 per cent. peptone and 1·5 per cent. agar are added; the solution is neutralized, cleared, and autoclaved in the usual way.

Growth on this medium is very similar to that on ordinary bouillon-agar; it is somewhat more luxuriant, as is to be expected of a marine organism growing on a fish-medium. Pigmentation takes place much more rapidly on fish-agar, chromogenesis being first noted on the fifth day, some 10 days earlier than on standard agar. There is some initial difference in the degree of coloration; on fish-agar the first evidence is a brown colour, whereas on meat-extract agar early coloration is so dark as to appear black by contrast with the unaffected portions of the medium. However, the final colour, a very dark brown, is the same in both cases.

Gelatine Colonies.—These attain the same size and are similar in shape to those on agar, but the violet fluorescence is not seen, nor is the pigment produced with any constancy. When it is produced, it is at first yellow, becoming later golden-brown. Rapid liquefaction takes place, the liquefaction being of the "plug" type, that is to say, the colonies do not spread, but sink rapidly downwards into the medium.

Gelatine Stab.—Liquefaction proceeds from above downwards, and is typically napiform or crateriform; growth takes place along the whole line of the stab, but it is more marked near the surface. Pigment is produced in the upper layers of old cultures.

Gelatine Shake.—Both surface and sub-surface colonies develop, liquefaction here also being from above downwards. From the fact that the lower colonies do not develop, the organism would appear to be aerobic. It is not a strict aerobe, however, for cultures in Buchner tubes grow well. Since also the bacillus grows in the poorly-oxygenated lower regions of a gelatine stab, it can better be described as a micro-aerophile and a facultative anaerobe.

Fish Gelatine.—Growth here resembles that on ordinary gelatine. Pigmentation of the medium was not observed.

Meat Bouillon.—The medium becomes turbid and a somewhat viscid white sediment is formed; there is no surface pellicle, and no flocculi are formed in the medium until the fifth week. Cultures about five weeks old show a golden-brown pigmentation which is more pronounced at the surface and does not extend to more than about one cm. below the surface of the broth.

Potato Medium.—Growth on potato-slopes in Roux tubes is moist, smooth, and glistening, as on agar slopes. A yellow ochraceous pigment is produced, which does not spread beyond the actual region of bacterial growth.

Litmus Milk.—The reactions of the organism in litmus milk are as follows:—

Acid: Produced in 18 to 20 days.

Gas: Negative.

Acid Curd: Negative.

Reduction: Litmus is slowly reduced and completely decolorized in 18 to 20 days.

Peptonization: Positive.

Solidified Ox Serum.—Growth abundant, with rapid liquefaction taking place along the line of culture, and with production of a dark brown colour.

Rabbit-blood-agar.—Growth is vigorous, with a yellow colour. Haemolysis is induced, and a zone of discoloration surrounds the line of culture.

Neutral-red-lactose-bile-salt-agar.—Growth is abundant, with production of a dark pigment.

Carbohydrate Media.—It is not advisable to keep sugar bouillons made up even when the broth is adjusted to neutrality or slight alkalinity. The medium frequently becomes acid on sterilization or with storage. Such acidity would favour hydrolysis of the contained sugars to other forms and so invalidate the results obtained. Accordingly, ordinary meat bouillon was tubed in 10 c.c. quantities to serve as a broth base, and one c.c. of a 10 per cent. sterile solution of the appropriate carbohydrate was added prior to inoculation. Durham fermentation tubes were inserted in the usual way to collect any gas that might be formed.

For each set of such inoculated sugar-media a bouillon tube without added sugar was inoculated as a control; this was to ensure that any

gas production in the other tubes did not result from fermentation of any unknown sugars possibly present in small amount in the meat extract used in the preparation of the bouillon.

Litmus paper was used in preference to an added indicator, since possible pigmentation in the medium might mask any colour change in the indicator; rough determinations of the hydrogen-ion concentration of the medium were made (*a*) from inoculated tubes after 14 days' growth and (*b*) from uninoculated control tubes. The results of one such series are set out below:—

	Saccharose.	Maltose.	Lactose.	Dextrose.	Adonite.	Xylose.	Arabinose.	Mannose.	Mannite.	Sorbite.	Galactose.	Levulose.	Raffinose.	Glycogen.	Inulin.	Sugar-free control.
Gas, - - -	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
pH of inoculated medium, -	6·5	8·0	7·0	8·0	7·0	8·0	8·0	8·0	7·8	8·5	6·8	6·8	8·0	7·0	8·0	7·2
pH of uninoculated control, - -	8·2	8·0	7·0	7·8	8·0	7·0	8·0	7·5	7·5	7·2	8·0	7·0	8·0	7·0	7·4	7·2

The above show the organism to be indifferent to carbohydrates; although growth is not inhibited, there is no gas production and no great change in the reaction of the medium. Older cultures in dextrose as well as in saccharose broth occasionally produce acid, but this is not a constant feature.

Peptone-water.—A suitable medium is 10 per cent. peptone in filtered sea-water, a modification of Dunham's solution. Growth in this medium is slow, turbidity not developing till the fifth or sixth day. There is no production of gas or of indol, but abundant formation of nitrite. Ammonia is sparingly formed. It is interesting to note that nitrates are abundantly formed as a degradation product from peptone, and ammonia only sparingly so.

Nitrates (0·25 per cent. nitrite-free KNO_3; 0·1 per cent. peptone in sea-water) are not reduced to nitrites; nitrites (0·25 per cent. KNO_2; 0·1 per cent. peptone in sea-water) are not decomposed. In the above work tests were also made from uninoculated tubes set as controls.

Conditions of Growth.

Foodstuffs.—Since this organism was originally isolated from sea-water, much of the cultural work involved the use of sea-water media. It is to be expected that it would thrive better in a nutrient medium

with a salt content approximating to that of the sea, but this bacillus was found to exhibit a wide range of accommodation to the necessary nutrient salts supplied. It grows equally well in a medium made up with sea-water or with tap-water and 0·5 per cent. NaCl.

On fish-extract media the growth is more luxuriant than on meat-extract.

Temperature.—The optimal temperature range is from 10°C. to 19°C. Above this, growth continues for three to five days, and is then arrested; the surface of the colony assumes a dry and punctulate appearance. After 10 days' incubation at 37°C., the organism dies off and is not recoverable from the culture. Incubated at 0°C., the culture shows no visible growth in the first 24 hours; multiplication does, however, take place, and after seven days a healthy culture is produced, comparable in amount to a three-day growth at 18°C. The original streak on an agar-slope is visible in such a culture as a punctulate line, subsequent growth being normal.

This shows that the organism does not live long above room temperature, and that low temperatures are only unfavourable in so far as they retard the rate of reproduction in the usual way. Here growth is not inhibited but simply arrested.

Hydrogen-ion Concentration.—It is known that a number of micro-organisms are specially sensitive to changes in the pH of the surrounding culture medium. For instance, the range tolerated by *Neisseria gonorrhœae* Bergey (*Gonococcus* Neisser) is from 7·8 to 8·4; other organisms are even more exacting, as for example *Hemophilus influenzae* Bergey (*Bacillus influenzae* Pfeiffer), for the cultivation of which the medium must have a pH of 7·8 to 8·0. It should, however, be borne in mind that the specific response of an organism to external conditions varies with the individual, and that although certain species such as the two enumerated above are intolerent of great variation in this respect, others may not be so sensitive. *Salmonella paratyphi* Bergey (*Bacillus paratyphosus* Brion and Kayser) serves to exemplify this, since, although for optimal growth the culture medium should have a pH of 6·2 to 7·2, the acid and the alkaline limits are 4·0 and 9·6 respectively.

In order to ascertain the effect of variation in hydrogen-ion concentration of the medium on the organism under consideration, a series of agar tubes was prepared with N/20 NaOH or N/20 HCl, having pH values approximately determined and ranging from 5·5 to 10·5. It was found impossible to use agar tubes of a less pH value than 5·5, because their acidity destroyed the solidifying properties of the agar.

Streak cultures on such a series of slopes show no appreciable difference either in the amount of growth or in its fluorescence.

However, the development of the brown pigment so characteristic of the bacillus depends on the pH of the medium. Between 7·5 and 8·0 the pigment is more marked; beyond this range, pigmentation does occur, but at a slower rate. Further work is being carried out on the conditions affecting pigment-production.

Oxygen.—The oxygen requirements of the organism have already been considered. As a free-living marine form it obviously is aerobic, but under laboratory conditions it can grow as a micro-aerophile or as a complete anaerobe.

Consideration of Affinities.

It has not been found possible to identify this bacillus with any known or previously described free-living heterotrophic chromogen.

(i) In certain of its morphological and cultural characteristics it resembles members of the Pasteurella group. In both cases we have small Gram-negative rods exhibiting only feeble powers of carbohydrate fermentation. But the Pasteurelleae (Trevisan) is a highly specialized group of organisms causing such diseases as fowl-cholera and swine-plague, and the optimal temperature for their growth is 37°C. There is no record of chromogenesis in the group. The bacillus under discussion cannot therefore be classified as a member of this family, since it cannot live many days at the temperature named.

(ii) Chromogenesis is characteristic of many free-living forms, notably Flavobacterium, Bergey, a common genus of water organisms exhibiting yellow or orange pigmentation on certain media. Chromogenesis, however, does not necessarily indicate phylogenetic relationship, and the bacillus under investigation differs in so many cultural and morphological characters from the known members of the genus Flavobacterium, Bergey, that it cannot be indentified with any species in that group.

(iii) It was brought to the writer's notice by Mr. A. C. Gardiner of the Alresford Fisheries Station that agar-cultures of this organism closely resembled *Bacillus salmonicida* Emmerich and Weibel, which causes the disease of furunculosis in salmon and trout, and which is pathogenic in a lesser degree to other cold-blooded animals. This disease is highly infectious, and in recent years there have been serious outbreaks in Irish,[3] Scottish,[4] and English[5] rivers; and although the disease and the causative organism have been known since 1894, there appear to be considerable dubiety and some amount of conflicting evidence as to the origin of the disease. The organism has been recorded from rivers

[3] Mettam, Ann. Rep. Fisheries, Ireland, Sci., Inv., 1914.

[4] Williamson, Fishery Board for Scotland, Salmon Fisheries, 1928, No. V.

[5] Masterman: Report upon the Epidemic of Salmonidae, Board of Agric. & Fisheries, 1912, (with an appendix by Arkwright).

during an epizootic, but it is not normally found free-living in river waters. There seems to be some evidence that fresh-run salmon may be infected before entry into the river[5]; this indicates the possible existence of this micro-organism in sea-water. The present writer therefore suggests that *B. salmonicida* may exist not only as a fish pathogen, but also as a free-living marine organism. Such a case would be comparable with *Bacillus tetani*, which may live as a harmless soil organism, and which becomes virulent only when it gains entry to tissues by way of broken skin and lives in a healing wound under anaerobic conditions.

Comparison of B. salmonicida and the described organism.— Morphologically and culturally these show great similarity, except that the former does not show violet fluorescence on agar—a constant feature in the latter. Both are Gram-negative, but the bipolar staining described by Marsh for *B. truttae* and by Arkwright and Williamson for *B. salmonicida* is not a distinctive feature of the form described in this paper.

The sugar reactions of the two bacilli are different. *B. salmonicida* is stated to ferment glucose and mannite; acid production appears to be a constant feature for all strains, with gas production in some cases. The free-living form under investigation has never been noted to produce gas from the carbohydrates used, and only in dextrose and saccharose is the formation of acid a marked feature. These are significant points of distinction, although there arises the possibility of its feeble fermentative powers being due to attenuation under conditions of laboratory culture.

Gelatine liquefaction in *B. salmonicida* is variously described as being from above downwards or from below upwards, with production of gas-funnels. In the organism considered here it is invariably infundibuliform or napiform. The differences here noted between the described organism and *B. salmonicida* are scarcely greater than those which subsist between the various strains of *B. salmonicida* itself, and the opinion is therefore expressed that they are two closely related forms. They are probably different "strains" or "biologic forms," one free-living in the sea, and the other a pathogen. It is a matter for experiment to determine whether the organism under consideration is potentially pathogen. At the request of Dr. Williamson, the writer has handed over a culture of this bacillus to enable its pathogenicity to be determined. *In the event of its being non-pathogenic, there would not be sufficient grounds for separating it as a species distinct from *B. salmonicida*, since

*Since the above was written, the organism has been found to be non-pathogenic to a frog after sub-cutaneous injection with 1/5 of an agar slope culture emulsified in sterile saline. I am indebted to Dr. Williamson for making this test and affording me this information.

even in the recognized forms of the latter, attention has been drawn to inconstancies in virulence, and the consequent probability that there are different varieties.[6]

It should be noted that this organism may either cause a general infection only, or produce in addition the so-called furuncles. Further, as Clayton[7] has recently demonstrated, it may exist in numbers in a perfectly healthy codling, so that the organism may be non-pathogenic under certain conditions. This lessens the distinction between the recognized form of *B. salmonicida* and the organism here under consideration.

Viability in Water.—There is apparently a marked contrast between these two organisms in their viability in water. With regard to *B. salmonicida*, the results of various workers do not agree, and the question of its viability in water calls for further experiment. Arkwright[5] finds that it does not live more than 19 hours in sea water, but that 25 per cent. sea-water and 75 per cent. tap-water had no lethal effect for 67 hours; Williamson (*loc. cit.*) states that it is recoverable after two days from unsterilized sea water and after three days from sterilized sea water.

The following tests were made to determine the viability in water of this bacillus which resembles *B. salmonicida*:—

Test I.—10 c.c. quantities of sterile solutions were prepared with tap water and evaporated sea salt, giving a range of salinity as under:—

0·0, 0·5, 1·0, 1·5, 2·0, 2·5, 3·0, 3·5 per cent.

The first of these would represent in salt concentration natural river water and the last sea-water.

Each tube was heavily infected with the bacillus, but no special precautions were taken to prepare emulsions containing approximately the same number of organisms, because the ultimate extent of bacterial growth in any culture is a function not of the initial numbers but of the total available foodstuffs.

From the above emulsions, loopfuls were plated on agar at intervals of two hours over a period of 24 hours. After five days' incubation at room temperature, all plates showed colonies of the bacillus.

Test II.—As above, but platings were made at 12-hour intervals over a period of five days. The results here again were in all cases positive.

It appears then that variation of salinity within the range occurring naturally is not an inhibiting factor. Other factors, then, such as

[6] Hulsow, Allgemeine Fisch. Zeitung, Nos. 10 and 18, 1913.

[7] Clayton, Report Dove Marine Laboratory, Northumberland, 1927.

competition with other micro-organisms may be unfavourable to the organism. Accordingly, the following viability tests were made:—

Test III.—Emulsions were prepared as in the preceding experiment, but for Tubes I to VII sewage-polluted water was used instead of sterile water, and inoculations were made at intervals of 24 hours. Tube VIII contained filtered unsterilized sea water taken off Greenock, where the bacterial content is very high. The results are as set out below:—

Salinity, per cent.		I 0·0	II 0·5	III 1·0	IV 1·5	V 2·0	VI 2·5	VII 3·0	VIII 3·5
Days.	1	+	0	+	+	+	+	+	+
	2	+	0	+	+	+	+	+	+
	3	0	0	0	+	+	+	+	+
	4	0	0	0	+	0	+	+	+
	5	0	0	0	0	0	+	0	+

+ = Growth on agar of this bacillus.
0 = No growth of this bacillus.

It will be seen from the above that, allowing for experimental error, in polluted waters the organism dies out more rapidly where the salinity is low. If then the same conditions hold in nature, this organism would not normally be able to live in river water, but could thrive in estuarine and marine habitats whatever the degree of competition with other micro-organisms. Further tests are being carried out along these lines.

This strain was isolated from a sea-bottom sample; other strains of the same organism isolated subsequently from Loch Striven and other areas were also obtained from sea-bottom samples taken above muddy layers. It is interesting to note in this connection the suggestion of Plehn (quoted from Williamson, *loc cit.*) that *B. salmonicida* may live for some time in mud.

In conclusion, the writer wishes to tender grateful acknowledgments to Professor D. Ellis, D.Sc., Ph.D., F.R.S.E., for his guidance and supervision of the work.

THE ROYAL TECHNICAL COLLEGE,
GLASGOW, C.1.

25/3/29.

I hereby certify that Miss Blodwen Lloyd M.Sc. has made satisfactory progress with her research work on the Survey of the Marine Bacteria of the Clyde area. She has made an interesting contribution to Science and the standard of her work appears to me to be worthy of the Doctorate of Philosophy for which she is ~~ap~~ a candidate.

David Ellis
Professor of Bacteriology.

Suggested Committee:

Professor Drummond (Chairman)
" Browning
" Ellis

THE NITROGEN CYCLE IN THE SEA.

In the sea, as on land, the majority of bacteria are metatrophic species, i.e. saprophytes which attack proteins and convert them into simpler compounds. There are also such prototrophic species as the Nitrogen-bacteria, the Sulphur-bacteria and the Iron-bacteria which have a much simpler metabolic cycle. The first of these groups, the Nitrogen-bacteria includes not only those prototrophic forms which synthesise inorganic nitrogen compounds from simpler ones, but also those saprophytes which under certain conditions decompose nitrates or nitrites.

Nitrogen-bacteria are classed as under:-

(i) Nitrogen-fixing forms. These are capable of 'fixing' nitrogen by synthesising nitrites or ammonium salts, as for example Pseudomonas radicicola found in symbiotic association with leguminous plants. Nitrogen-fixing organisms have been recorded from the sea, both free-living Azotobacter and species symbiotic with algae (23, 24), but there is little evidence to show that they play an important part in the formation of nitrogen compounds in the sea.

(ii) Nitrifying forms. These are able to oxidise (a) nitrites

to nitrates or (b) ammonium salts to nitrites, the two stages being effected by different organisms. Nitrate-builders and nitrite-builders may live together as loose symbionts, the nitrite-forming bacteria supplying the necessary raw material to the nitrate-builders, while these for their part would remove the nitrite as it is formed - a marked advantage to the nitrite-builders, since excess of the end-products of nitrite-building metabolism has a toxic effect on the organisms initiating the reaction. The existing theories of the food-cycle in the sea postulate the presence of such nitrifying species as a necessary source of nitrate in those sea areas removed from the influence of land drainage. Nitrite and nitrate bacteria have been isolated from inshore waters, but there is much uncertainty as to the actual part played by such species under natural conditions.

(iii) Denitrifying forms. These decompose (a) nitrates, reducing them to nitrites or less frequently to ammonia, or (b) nitrites with formation of nitrogen principally and small quantities of ammonia and nitrous oxide. Some few organisms are able to reduce nitrates completely, but in general denitrification, like nitrification is usually accomplished in two stages by different species. A number of denitrifying organisms have been recorded from seawater, but little is known as to their undoubted activities in the

sea.

Nitrogen-fixation.

In the soil the main natural method by which elemental nitrogen may again be returned to the metabolic cycle is by way of such nitrogen-fixing organisms as Clostridium and Azotobacter. In the sea, however, the continual drain upon the contained nitrates by marine plants is compensated by a renewal from the land in all those sea areas which are not far removed from neritic conditions. Such a renewal from land drainage might in itself be an adequate source of nitrate.

In the open oceanic regions, the only sources of nitrate are the atmosphere and the products of bacterial nitrification. Unless the former is sufficiently productive to support the phytoplankton of the open seas, then it would appear that nitrogen-fixing micro-organisms are responsible for nitrate-production. If these are inadequate for the nutrition of marine plants it would be necessary to assume that the microphytic plankton can utilise as a source of nitrogen the ammonium compounds produced by ordinary saprophytic bacterial action. From practival as well as theoretical considerations it would be of interest to ascertain whether such nitrogen-fixation occurs in the sea.

There does not appear to be much information as

to the existence of free-living nitrogen-fixing bacteria in the sea. According to Johnstone (23) both Clostridium and Azotobacter have been recorded from a variety of habitats such as the sea bottom, the surface of plankton and the sea water itself. In recent years marine workers have endeavoured to isolate such species from the open sea. Drew (10 - 13) used a glucose potassium hydrogen phosphate medium and inoculated with sea water off the Bahamas, but obtained negative results. Similarly Berkeley, working with water from Vancouver, found no evidence of nitrogen-fixation when he inoculated into a mannite potassium hydrogen phosphate medium.

Experimental.

Berkeley's nitrogen-fixing medium was adopted (see Appendix 1. Medium M); test tubes containing 10 cc were inoculated from the samples obtained at intervals for the quantitative work. An uninoculated control tube was set in each case, and both the control tubes and cultures were tested at intervals of one month for ammonia and nitrite (for test reagents see Appendix 2).

Results. In no case was there evidence of nitrogen-fixation. One set of cultures, made from surface, 10 fathom, 20 fathom and bottom samples, Loch Striven 18.5.28 gave a strong nitrate reaction, with none in the corresponding control tubes. Sub-cultures were made from these into similar media, and bacterial growth took place. The sub-cultures, however,

gave negative results for ammonia, nitrite and nitrate.

Nitrification.

The nitrifying bacteria on land restore to the soil nitrogen-compounds in a form suitable for absorption by the ordinary green plant; the genera Nitrosococcus and Nitrosomonas are nitrite-builders, and Nitrobacter is a nitrate-building form. Concerning the problem of nitrification in the sea Berkeley writes: ' If marine plants require their nitrogen in the form of nitrate and no agency exists in the sea for oxidising the ammonia produced by the breakdown of complex organic compounds, they must depend upon nitrates produced by bacterial agency on land and washed into the sea'. (4). He does not consider the atmosphere as a source of nitrate.

Thomsen (36) records nitrate and nitrite builders from the bottom of inshore waters, but did not find them in the open sea; Berkeley, employing a similar medium, had negative results also for the open sea. He suggests that marine nitrifying organisms might require the presence of organic matter, and that an inorganic nutrient medium (see Medium M, Appendix 1) is not favourable for growth. Lipman also found that no nitrifying organisms can be isolated from water taken from the open sea, although cultures made from calcareous sand showed nitrification (26). Harvey on the other hand found appreciable nitrate formation of the order of 7 mg. per cubic metre in an ammonium-nitrogen sea water medium, while controls

in which bacterial action were stopped by the addition of mercuric chloride showed no such nitrate formation.

Experimental.

Inoculations were made regularly from sea water into a medium similar to that employed by Berkeley and Lipman. See Medium M, Appendix 1; uninoculated controls were set and at intervals both the cultures and the controls were tested for increased nitrite and nitrate.

Results. Bacterial growth took place in all cases, but it was scant and the contained organisms soon died. There was no evidence of nitrification in any culture. This culture medium does not favour growth, so that the absence of nitrification here cannot be taken as conclusive evidence that this does not take place.

Denitrification.

Nitrogen-fixing and nitrifying organisms have a narrow range of accommodation and specialised requirements. Denitrifying organisms are of wider occurrence, and the power of nitrate and nitrite reduction is common to many ordinary saprophytic bacteria.

There is a lack of uniformity as to the use of the term denitrification. Some writers use the term in its widest sense to cover not only the reduction of nitrate to nitrite and ammonia, but also the reduction of nitrite to nitrous

oxide and nitrogen. Others restrict the use of the term to the latter stage, which is marked by evolution of gas. This results in confusion, and it would therefore be expedient to bring the terminology in line with that used for the nitrate building and nitrite building bacteria. Here the term 'nitrification' has a general import, and the two stages in the process characterised as nitrate building and nitrite building respectively. By analogy it is proposed here to use the word 'denitrification' in a general sense, and the terms nitrate reduction and nitrite reduction for the two stages in the process.

Denitrifying bacteria were observed in the sea as early as 1892 by Russel (31), who found aerobic nitrate reducing bacteria in bottom muds. Some years later Brandt, in formulating a theory to account for the paucity of phytoplankton in the tropics, postulated the existence of such denitrifying forms, and suggested that their increased activities in the warmer waters reduced the avaible nitrate , with consequent reduction of the planktonic flora.

Baur made the first exhaustive study of such forms (2). He isolated from aquarium mud two new nitrite reducing species. Gran (18) later described three other denitrifying forms found in littoral waters. Drew (12) isolated Pseudomonas calcis, a tropical denitrifying species. He observed that such denitrifying species occur most commonly where there is a high organic content, as in a mangrove swamp.

Drew used a medium containing calcium malate, sodium hydrogen phosphate and potassium nitrate. Berkeley found that with this medium he obtained abundant reduction of nitrate to nitrite, but no gas production.

Experimental. Drew's denitrification medium, peptone water, fish broth with 0.25% added potassium nitrate or nitrite were the media employed. The above were tubed in 10 cc quantities and a Durham fermentation tube inserted to collect any gas that might be given off. (For media formulae see Appendix 1.)

Inoculations were made into a series of the above media from the samples obtained from time to time for quantitative work on the bacterial content of sea water. Each culture was inoculated with 1 cc. of the sample and an uninoculated tube set as a control.

Tests were made at intervals for reduction of nitrate to nitrite; the Griess-Ilosvay method was employed. (For test reagents see Appendix 2). Different media were tried experimentally, and gave the following results:-

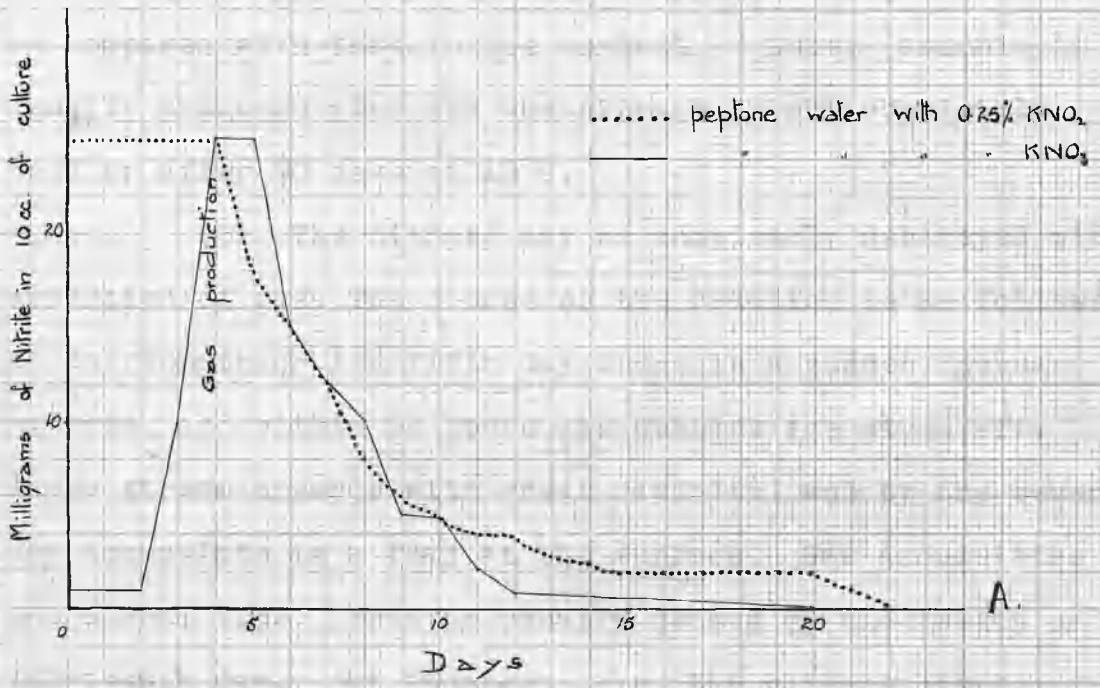
Nitrate peptone sea water. Medium H.

Bacterial growth invariably takes place. The nitrate may remain unchanged if there are no denitrifying bacteria present or if for any reason unknown conditions inhibit their activity. More frequently one of the following changes takes place:-

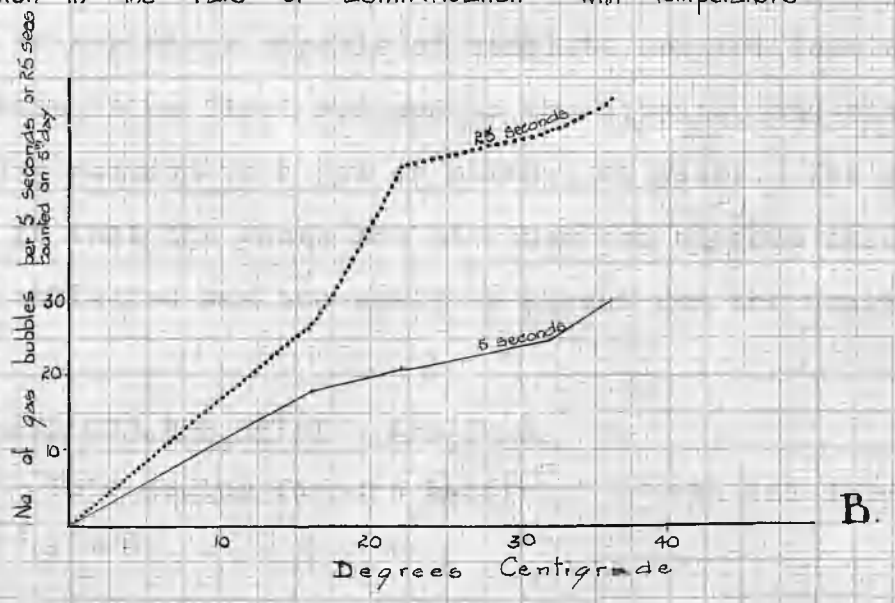
(i) The amount of ammonia may be greatly increased as compared with that in the control tube; this may or may not be

Fig. 10.

Denitrification by a mixed culture of marine micro-organisms at 18°C.



Variation in the rate of denitrification with temperature



accompanied by increased nitrates. In a mixed culture containing 0.025 gm. potassium nitrate, this is not completely used up after 90 days at 18° C.

(2) The amount of nitrite may be greatly increased as compared with that in the control tube; ammonia is usually produced also and the nitrate is not completely used up after 90 days at 18 C.

(3) The nitrate may be completely destroyed with evolution of gas. The course of the reaction is as follows:- At the fourth or the fifth day there is a sudden increase of nitrite, and within 24 hours gas bubbles are given off. These stream upwards with great rapidity, and by the seventh day accumulate as a foam at the surface. Gas accumulates in the Durham tube. Foaming usually ceased by the twelfth or thirteenth day. At fourteen days, the nitrate disappears and by twenty days, the nitrite also is destroyed. Fig. 10A.

In such a case we are dealing with an organism or mixture of organisms capable of complete denitrification, the nitrate being first reduced to the nitrite, and this in turn being reduced to a gas or mixture of gases. The probability is that the gases are nitrogen and nitrous oxide, but the gas collected was too small in amount for the qualitative test.

Nitrite peptone sea water. Medium I.

This medium favours bacterial growth and denitrification. Fig. 10 A.

Nitrate fish bouillon. Medium J.

and

Nitrite fish bouillon. Medium K.

In mixed culture rapid bacterial growth occurs, and a surface pellicle is formed. In the hitrate medium abundant reduction to the nitrite occurs, but there is no gas evolution. These media were adapted from the formulae of Baur. He obtained active gas production.

Medium M. Drew's denitrification medium.

In the foregoing media, the presence of peptone favours the development in mixed culture of most bacterial species. Drew's medium containing sodium hydrogen phosphate and calcium malate in seawater with added nitrate or nitrite is suitable for denitrifying forms but inhibits the development of other metatrophic bacterial saprophytes. Denitrification is readily obtained in this medium, but it does not proceed as rapidly as in a peptone-containing medium.

Conditions Affecting the Rate of Denitrification.

1. Temperature. A series of sub-cultures was made from an actively foaming nitrate peptone culture; these were incubated at different temperatures. On the 5th. day the number of gas-bubbles streaming upwards in 5 seconds were counted. The tubes were then replaced at incubation temperature for 30 minutes, and a second reading taken. This was repeated three further times so that an average of five observations could be obtained. The figures are given below, and the average results

shown in Fig. 10 B. A similar series was done for nitrite peptone.

	Nitrate.				Nitrite.			
	Inoculation Temperature.				Inoculation Temperature			
	16°	22°	32°	37° C	16°	22°	32°	37° C
1st. reading	20	36	31	40	7	7	9	12
2nd. "	15	15	21	31	4	11	12	13
3rd. "	15	24	23	29	7	9	10	10
4th. "	17	15	31	36	4	13	10	13
5th. "	12	16	19	25	5	8	12	9
Average no. of bubbles.	18	21	25	30	5	10	11	11

It will be seen that, within the range of temperature given, an increase of temperature favours increased rate of denitrification, and apparently this would continue until the thermal death point for the organism concerned would be reached. It may be noted the reaction is hastened past the optimal growth temperature for marine organisms.

2. Concentration of foodstuffs in the medium.

(a). Nitrate or nitrite. A series of cultures was set up in nitrate peptone media having varying concentrations of calcium nitrate, with the following results:-

% Concentration of KNO_3 :-	0.1	0.15	0.2	0.25	0.3	0.5	1.0	2.0	5.0
Degree of bacterial growth	+	+	+	+	++	++	+	-	-
No. of days before gas formed	8	8	8	7	5	4	-	-	-
No. of days before nitrate used	∞	∞	21	20	30	30	-	-	-

A similar series of culture in a nitrite peptone Medium gave the following results:-

% Concentration of KNO_2	0.1	0.15	0.2	0.25	0.3	0.5	1.0	2.0	5.0
Degree of bacterial growth	+	+	+	+	+	-	-	-	-
No. of days before gas formed	7	7	5	7	6	-	-	-	-
No. of days before nitrite used	20	21	20	20	20	-	-	-	-

From the nitrate series the following conclusions have been drawn: (i) bacteria are intolerant of a nitrate concentration over 2%, and do not grow; (ii) within the limits that permit growth, an increase in the nitrate supply hastens the rate of denitrification; (iii) where the available nitrate is small in amount, it is only reduced in part, and some residual nitrate is left after as long as 30 days.

In ~~the case of~~ the nitrite medium there is a similar intolerance of high salt concentration, but the rate of denitrification does not appear to vary with the nitrite content.

(b) Peptone. Routine cultures were usually employed at a peptone concentration of 1%. The following series of cultures was made in media of varying peptone content, with the following results:-

% Concentration of peptone:-	0.1	0.2	0.5	0.7	1.0	1.5	2.0	2.5	3.0
Bacterial growth	+	+	+	+	+	+	+	+	+
No. of days before gas produced	10	7	7	7	7	4	4	4	5
No. of days before nitrite used	∞	40	28	22	20	18	19	19	19

From the above it is concluded that the rate of denitrification varies as the peptone concentration up to 3%.

3. Presence of other organisms.

In the preceding section dealing with the identity of the organisms isolated, several species were noted as capable of nitrate or nitrite reduction in pure culture. It has been found that in all cases denitrification proceeds more rapidly in mixed culture than in pure cultures of any one organism. In these experiments, mixed sub-cultures of bacteria have been employed; these were chiefly Bacillus sp. (Type V), Bacillus sp. (Type XIII), Achromobacter sp. (Type I), and Achromobacter sp. (Type VII). If any one of these species is present with non-denitrifying forms, rapid denitrification

takes place if other conditions are favourable. Fig. 10 A gives a general curve for the rate of denitrification (i) from a nitrate culture and (ii) from a nitrite culture, as estimated by the amount of residual nitrite present at daily intervals. The method of estimating the nitrite present is given in Appendix 2.

SUMMARY.

1. A bacteriological water-sampler, similar to that described by Wilson, has been constructed, and samples have been taken at three stations in the Clyde area at monthly intervals over a period of ten months. Cultures have been made from these under standardised conditions, and the number of colonies grown in five days counted.

2. No regular seasonal variation in the number of bacteria was found in any place.

3. A marked decrease in the number of bacteria from surface to bottom was noted; in Loch Long and Greenock there was an increase also in the number of bacteria at the bottom.

4. Diurnal quantitative variation in a winter series of samples showed a maximum in the early night, with a decrease through the early hours of the next morning. Diurnal variation in a spring series of samples showed that the numbers increased through the night to a maximum at 3 a.m.

5. The common species were found to belong chiefly to the genera Flavobacterium and Achromobacter in Bergey's family of the Chromobacteriaceae.

6. The principal cultural and morphological characters of the common species are given; where possible these have been identified with, or classified near to, already known species.

7. One organism with distinctive chromogenesis on agar has been selected for special study.

8. No evidence has been found of nitrogen-fixing bacteria, nor of nitrite- and nitrate- building bacteria.

9. Denitrifying bacteria, both nitrate-reducing and nitrite-reducing forms were commonly found in seawater.

10. Some laboratory conditions affecting the rate of denitrification in cultures are considered.

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Appendix 1. Culture Media.

Medium A. Standard Agar.

Prepared according to the formula of Giltner in his Microbiology, p.40. New York, 3rd edition, 1926.

Medium B. Sea salt Agar.

Medium A modified as under:-

(a) NaCl is omitted and 1 litre of sterilised filtered sea water is substituted for tap water. Or:-

(b) NaCl is replaced by 30 grams evaporated sea salt. (Tidman's sea salt is a commercial preparation found to serve this purpose well).

Medium C. Fish Agar.

Heat 1 kilog. Codfish in 1 litre of sea water for 3 to 4 hours at 60 to 70° C. Strain through muslin, filter and make up to 1 litre with distilled water. This serves as standard fish extract. Procedure from this point is as for medium A.

(Modified from Baur, Wiss. Meeres. VI. 1901).

Medium D. Fish Gelatine.

Prepared as medium C, but substituting 15% gelatine for 1.5% agar.

(Modified from Baur, Wiss. Meeres. VI. 1901).

Medium E. Standard Gelatine.

Prepared according to the formula of Giltner in his

Microbiology, p.35. New York, 3rd edition, 1926.

Medium F. Conradi-Drigalski Agar.

Giltner, p.387.

Medium G. Peptone Water.

1.0% peptone in filtered sea water.

(a modification of Dunham's peptone water).

Medium H. Nitrate Peptone Seawater.

Peptone 1.0%, and potassium nitrate 0.25% in filtered sea water. Autoclave for 15 minutes at a pressure of 2 atmospheres.

Medium I. Nitrite Peptone Seawater.

Peptone 1.0%, and potassium nitrite 0.25% in filtered seawater. Autoclave for 15 minutes at a pressure of 2 atmospheres.

Medium J. Nitrate Fish Bouillon.

Peptone 1%, and potassium nitrate 0.25% are added to standard fish extract (see note under MediumC).

Medium K. Nitrite Fish Bouillon.

Peptone 1%, and potassium nitrite 0.25% are added to standard fish extract. (Adapted from Baur, loc.cit)

Medium L. Nitrification Medium.

Ammonium sulphate 0.02% is dissolved in filtered sea water, and basic magnesium carbonate is added in excess (Adapted from Lipman and Berkely).

Medium M. Nitrogen-fixation Medium.

Mannite 2 grams.

K_2HPO_4 0.5 grams.

Seawater 1 litre. (Berkeley's medium).

Medium N. Denitrification Medium.

KNO_3	0.5 gram.
Na_2HPO_4	0.25 "
Ca Malate	5.0 "
Seawater	1 litre. (Drew's medium).

Medium O. Lactose Bile-salt Agar.

Peptone	20 grams.
Sodium taurocholate	5.0 grams.
Agar	15 "
1% Neutral Red	2.5 cc.
10% Lactose	50 cc.
Water	1000 cc.

Procedure is as for standard agar, except thet the neutral red and the lactose are added after clearing with egg-albumen. (Taken from Ellis: Bacteriology for Chemical Students).

Appendix 2. Test Reagents.

1. Nitrite. Griess-Ilosvay Test.

Solution I. Sulphanilic acid.....0.5 g.
Glacial acetic acid.....30 cc.
Distilled water..... 120 cc.

Solution II. α-naphthylamine..... 0.2 g.
Glacial acetic acid.... 20 cc
These are heated, and then made up to 150 cc with 20% acetic acid.

The two solutions are mixed in equal quantities immediately before use.

For quantitative work, colorimetric comparisons are made with a standard of known nitrite content. To determine the nitrite content of a culture, 0.1 cc is withdrawn with a sterile pipette and one drop of the mixed reagent added. At the same time 0.1 cc is withdrawn from each of a series of standard solutions of known nitrite content and a drop of the reagent added to each.

These are carried out on a glass plate viewed against a white background, and the pink colour developed is matched against the appropriate standard.

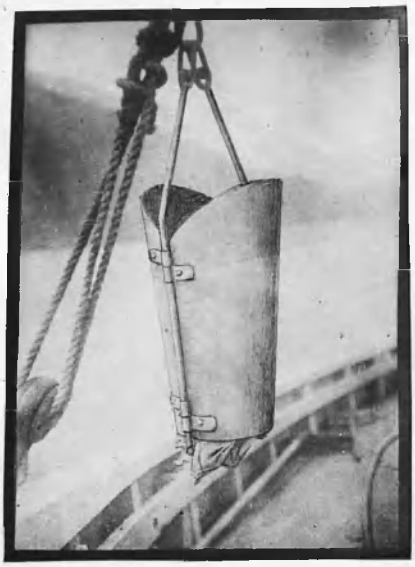
2. Ammonia. Nessler's Reagent.

3. Nitrate. (a) Diphenylamine..... 0.5 g.
conc. Sulphuric acid.. 100 cc
Water..... 20 cc.

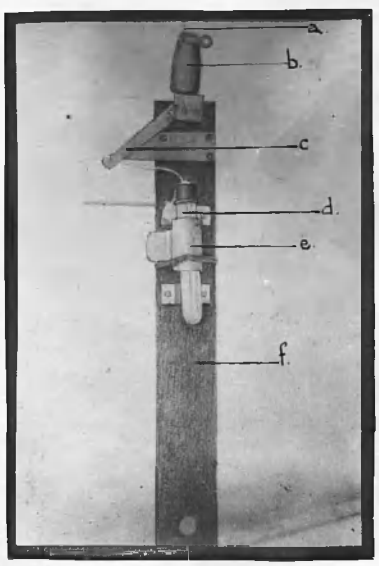
Add 2 cc concentrated sulphuric and 5 drops of the above reagent to 1 cc of culture. A blue colour is produced in the presence of nitrate. This test is valid in aqueous solution only if nitrites are absent.

Plate I

1. The "Nautilus"



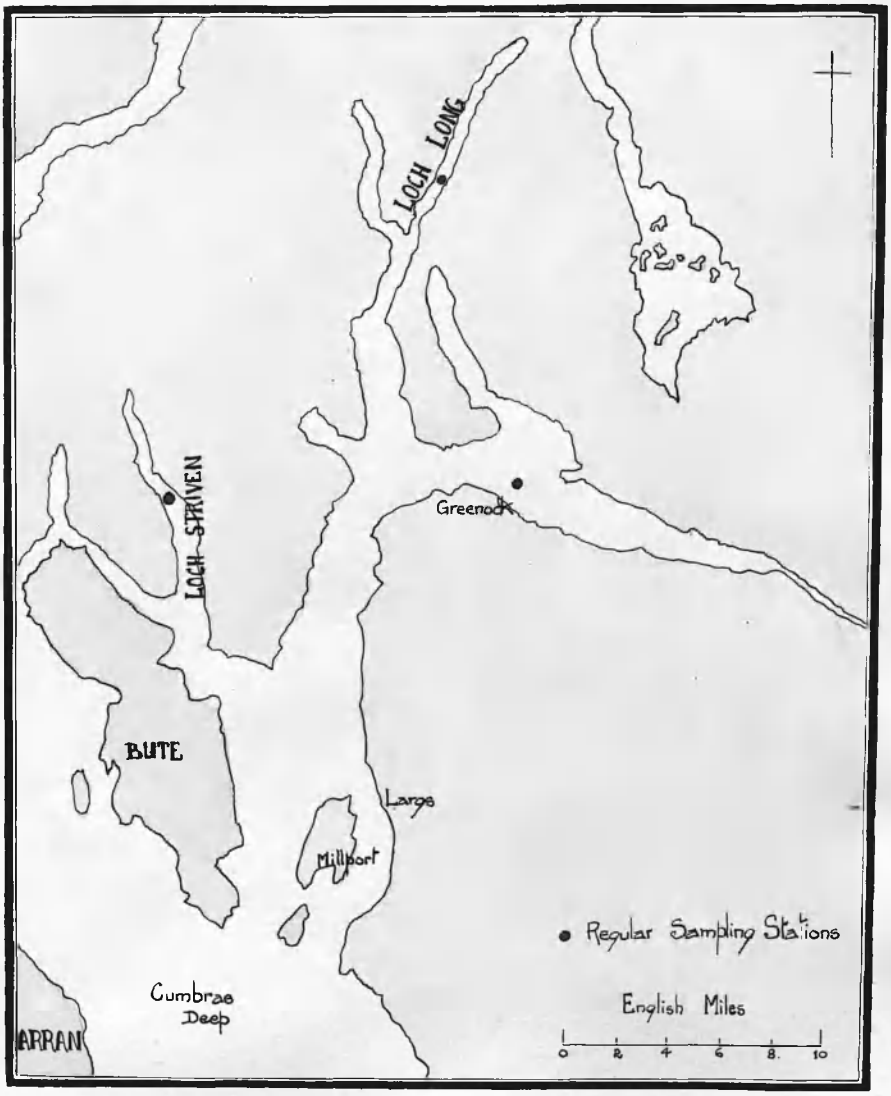
2. Robertson mud-sampler



3. Water-sampler

a. Lowering wire
b. Messenger
c. Breaker
d. Sterile sampling tube.
e. Clamp
f. Sinker.

Plate II



The Clyde Sea-Area

Plate III

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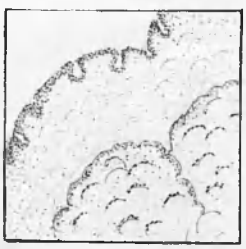
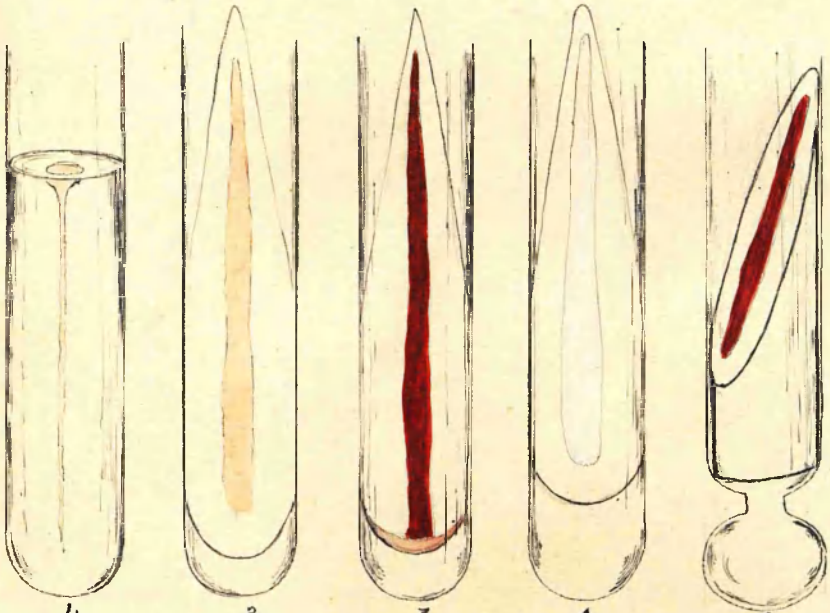
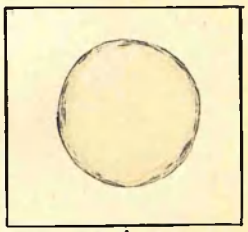


8

1. Gelatine-stab culture, five days at 22°C.
2. Agar-slope culture, five days at 22°C
3. Potato-culture, ten days at 18°C.
4. and 5. Nitrate-gelatine-stab cultures, showing in the lower part gas bubbles developed as a result of denitrification. ×2.
6. Agar colony, five days at 32°C. ×2.
7. " " " " " " ×6
8. Part of agar colony . . . ×60.
9. Cells from a 3-day agar slope. Unstained preparation. ×1000.

Plate IV

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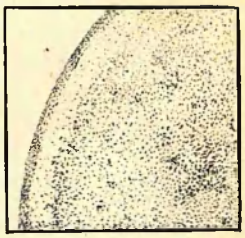
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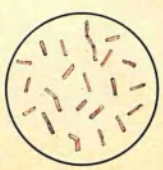
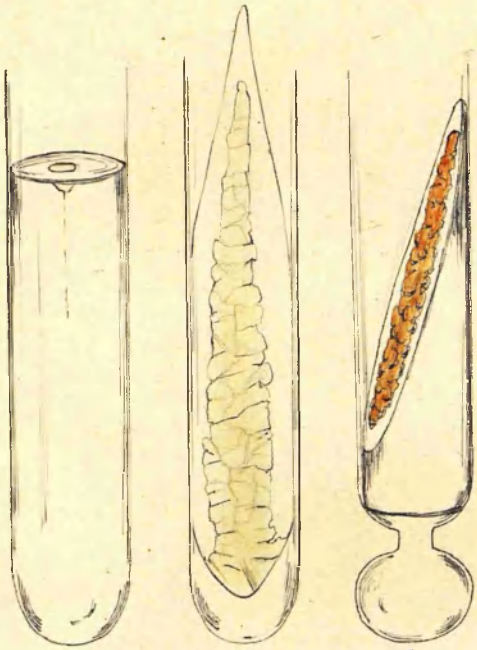

10.

1. Gelatine-stab culture, 7 days at 18°C, var. a.
2. Mannite-agar slope culture, var. b, 7 days at 18°C
3. " " " " var. b, " " "
4. " " " " var. c. " " "
5. Potato-culture, var. b, 5 days at 18°C
6. Surface colony, var. a, from a 7 day agar-plate incubated at 22°C. ×5.
7. Margin of an agar-colony. ×25
8. Surface colonies on agar, 7 days at 18°C. var. b. Nat. size
9. Surface colonies on agar, 7 days at 18°C. var. a. Nat. size.
10. Cells from a 24-hour broth culture. Stained acid fuchsin. ×1000.

Plate V



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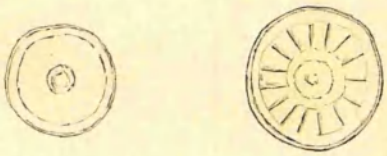

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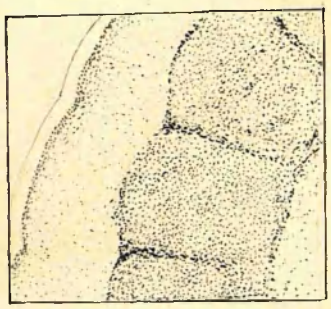

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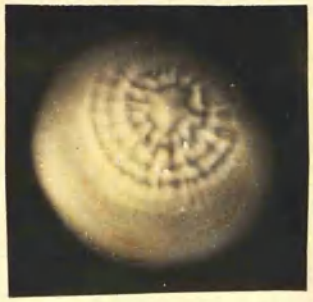

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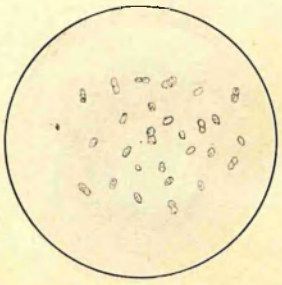

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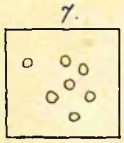
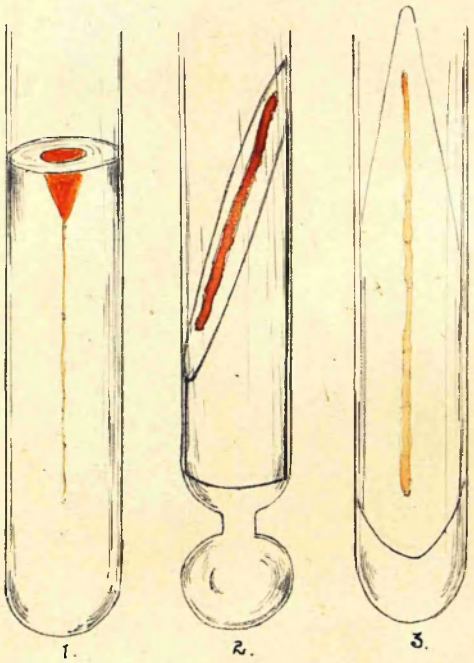
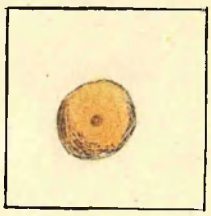


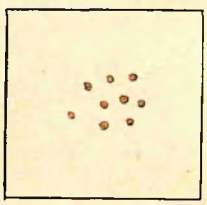
7.

1. Gelatine stab culture, 7 days at 18°C.
2. Agar-slope culture. 7 days at 18°C.
3. Potato-culture, twelve days at 20°C
4. Surface colony from agar plate, 5 days at 22°C. x4. Form with concentric markings
5. Surface colony from agar-plate, 5 days at 22°C. x4. Form with concentric and radial markings.
6. Margin of agar colony. x30.
7. Agar colonies. Nat. size
8. Cells from a 24-hour agar-slope. x1000
9. Single colony, asymmetrical. x10.

Plate VI







Flavobacterium sp.

1. Gelatine stab culture, 15 days at 22°C.
2. Glycerine Potato culture, 15 days at 18°C
3. Agar Slope, 6 days at 22°C.
4. Surface colony on agar, 6 days at 22°C. x5.
5. The same; natural size.
6. Cells from 24-hour agar slope. Stained fuchsin. x1000.

Plate VII

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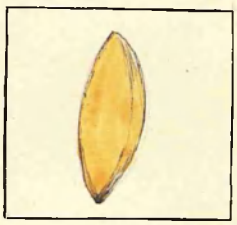
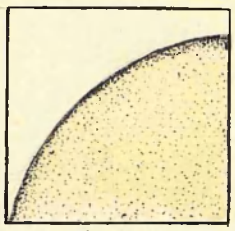
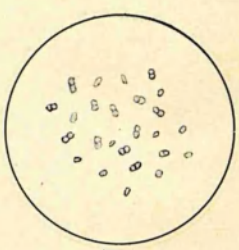


4.



5.

6.

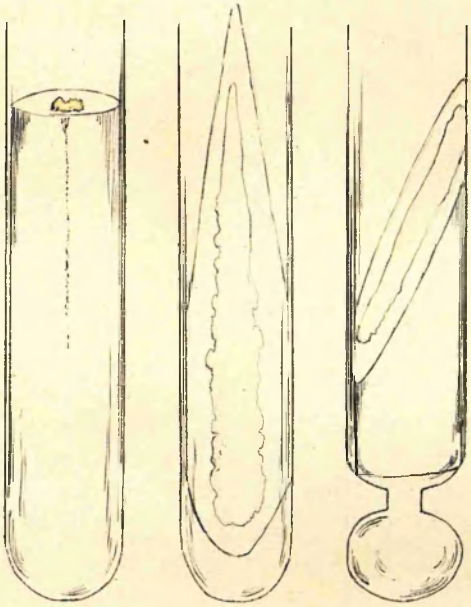


7.

Achromobacter sp.

1. Gelatine stab culture, 8 days at 18°C. x2.
2. Agar slope culture, 3 days at 22°C
3. Potato slope culture, 5 days at 18°C.
4. Sub-surface agar colony, 5 days at 18°C. x10.
5. Margin of the same. x25.
6. Agar colonies, 5 days at 18°C.
7. Cells from a 3-day agar culture. Stained with methylene blue. x1000

Plate VIII

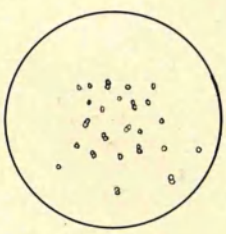


1. 2. 3.

4.

5.

6.

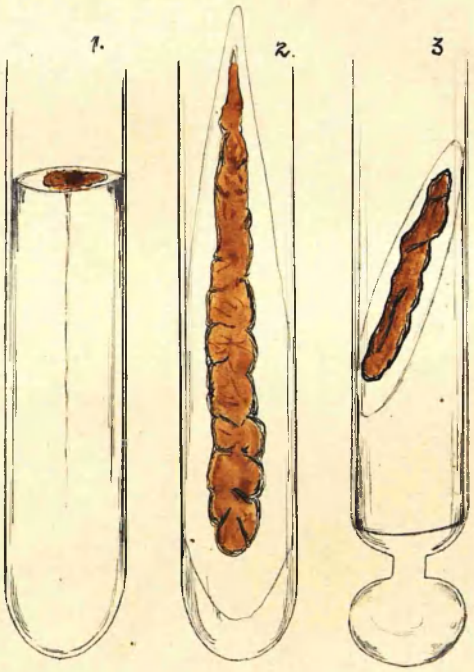


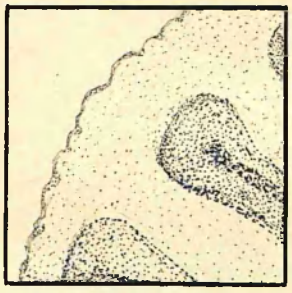
7.

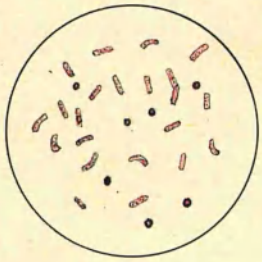
Achromobacter sp.

1. Gelatine stab culture, 5 days at 18°C
2. Agar slope culture, " " "
3. Potato slope culture " " "
4. and 5. Surface colonies on agar, 7 days at 18°C. x 2.
6. Margin of agar colony. x 30
7. Cells from agar culture. x 1000

Plate IX



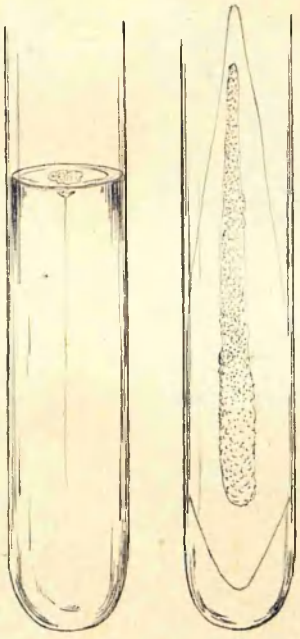




Bacillus sp.

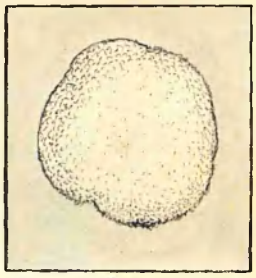
1. Gelatine stab culture, 5 days at 18°C.
2. Agar slope culture, 10 days at 18°C
3. Potato slope culture, " "
4. and 5. Surface colony on agar. ×2.
6. Margin of the same. ×20
7. Cells from 10-day agar culture, with spores. Stained carbol-fuchsin. ×1000.

Plate X.

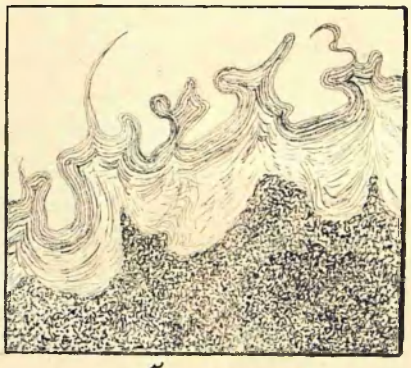


1. 2.

3.



4.



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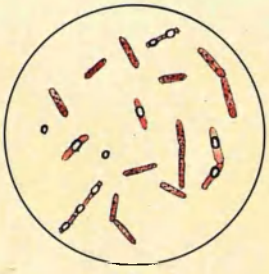


6.

7.

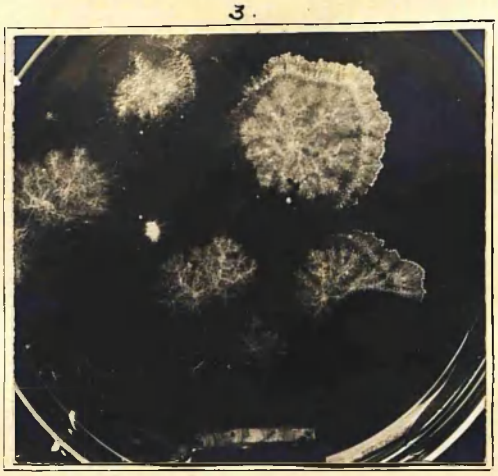
Bacillus sp.

1. Gelatine stab culture, 5 days at 18°C.
2. Agar slope culture " " "
3. and 4. Agar surface colonies. x5.
5. The same, x30.
6. Sporing cells from an old agar culture. Stained fuchsin. x1000.
7. Margin of agar colony. x30

Plate XI

1.

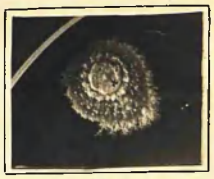
3.



4.

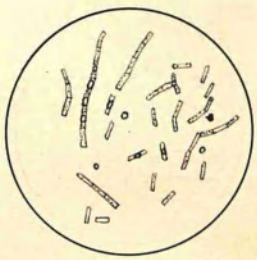
5.

2.



7.

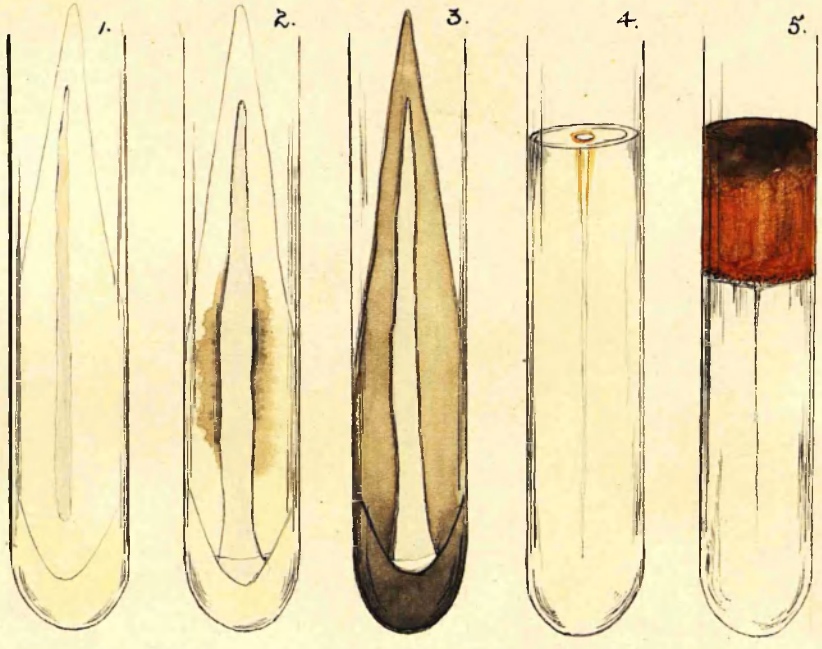
Bacillus sp.



6.

1. Gelatine-stab culture, 7 days at 18°C. X2.
2. Agar slope culture. Nat. size. . . . .
3.–5. Colonies of different types, all from the same original colony. Nat. size.
6. Cells with spores. ×1000.
7. Agar colony. Nat. size.

Plate XII



1. Agar-slope culture, five days at 22°C.
2. " " " fifteen " " "
3. " " " four weeks " "
4. Gelatine-stab " five days " "
5. Gelatine-shake " four weeks " "
6. Agar colony, surface, ten days at 18°C. x 10.
7. Cells from a 24-hour agar-slope, stained fuchsin. x 1000