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Heterotrophic Bacteria of North Sea Water

James Iain Walker Anderson

Marine bacteriology has received little attention in this country and no report, published during the past forty years, could be discovered where a British bacteriologist had investigated the bacterial flora of the North Sea. Since knowledge of this flora is virtually non-existent this investigation set out to develop methods for the study of the quantitative and qualitative nature of the aerobic heterotrophic bacteria in North Sea water.

Techniques for the isolation and further study of marine bacteria have been reviewed and methods developed and applied to the analysis of several water samples collected at different seasons from a station ten miles E.S.E. of Aberdeen. Seven media were compared for their suitability in isolating marine bacteria: organisms were isolated by plating 0.5 ml. aliquots of sea water on the surface of previously dried agar plates and incubating at 20°C for fourteen days. One medium, containing 0.25 per cent peptone, 0.25 per cent yeast extract, 0.01 per cent ferric phosphate and 75 per cent "aged" sea water, gave consistently higher counts than the other media used.

Approximately seven hundred isolates were studied in detail using a new scheme for their characterisation and qualitative comparisons made of the organisms detected in the various water samples by means of the different media. The composition of the medium had little effect on the qualitative nature of the flora isolated but there were striking differences in the bacterial populations of different water samples.

The genera, or groups, of micro-organisms found in North Sea water have been: Achromobacter, Bacillus, coryneform bacteria, Flavobacterium/Cytophaga, micrococci, Nocardia, Pseudomonas, Streptomyces, Vibrio, and yeasts.

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Heterotrophic bacteria of North Sea water.

by

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Thesis presented for the Degree of Doctor of Philosophy.

University of Glasgow,
July, 1962.

Acknowledgements.

I wish to acknowledge, with gratitude, the provision of a research grant by the University of Glasgow during a part of this work and, later, the award of a research studentship by the Department of Scientific and Industrial Research.

To Dr. John R. Norris, I express my appreciation and warmest thanks for the guidance and advice freely offered throughout the period of this investigation and for the help in the preparation and checking of the thesis.

Mr. F. Lonsdale has co-operated in supplying glass-ware and equipment when necessary, although this was often a strain on the routine of our Department. I owe him thanks for this and for help in preparing some of the photographs in the thesis.

Part of this work was carried out at Torry Research Station, Aberdeen, and I wish to express thanks to Dr. G.A. Reay, O.B.E., Ph.D., F.R.S.E., the Director, for the facilities granted, and to the skipper and crew of R.V. "Sir William Hardy" who were so helpful during the collection of water samples at sea. While working at Torry Research Station, I benefited much from the advice of Dr. J.M. Shewan and his staff, and for this I am very grateful. I wish to thank Dr. Shewan, particularly, for making available to me the galley-proof of his translation of Kriss' book on marine microbiology.

Finally, I wish to express my thanks to the technical staff of the Bacteriology Department, Anderson College, and to Mrs. Marshall, the secretary, for assistance during the past three years.

"Oh, what an endless work have I in hand,
To count the sea's abundant progeny!"

Edmund Spenser,
"The Faerie Queene".

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SECTION I.

GENERAL INTRODUCTION AND REVIEW OF LITERATURE.

1. GENERAL INTRODUCTION AND REVIEW OF LITERATURE.

1. 1. The Marine Environment.

Ocean waters cover 70·8 per cent of the world's surface (Sverdrup *et al.*, 1942). This represents a total area of 361 million square kilometres and, since the mean depth of ocean waters is 3,795 metres, a total volume of 1,370 million cubic kilometres.

Salinity is expressed as grams per kilogram of sea water and is represented by the symbol ‰. Generally the salinity of sea water is between 33‰ and 37‰. Where there is high rainfall, or dilution by rivers or melting ice the salinity may be considerably lower, while in isolated seas and where there is a high rate of evaporation, as in the Red Sea, salinity may be 40‰ or more. However, "regardless of the absolute concentration, the relative proportions of the different major constituents are virtually constant" (Sverdrup *et al.*, 1942). Figure 1, taken from Harvey (1957), and compiled from the data obtained by Dittmar who collected seventy-seven samples of water during the "Challenger" expedition in 1873-6, shows the relative proportions of the major constituents found in sea water.

At least sixty chemical elements have been detected in sea water and of these sodium chloride makes up 86 per cent of the total. An average sea water sample will have 5 mgm. per litre of organic carbon and less than 1 mgm. per litre of fixed nitrogen which includes organic nitrogen compounds, nitrate, nitrite and ammonia (ZoBell, 1959).

Figure 1.

Percentage composition of salts in ocean water.
(Harvey, 1957).

Na'	30•4%	Cl'	55•2%
Mg''	3•7	SO_4''	7•7
Ca''	1•16	Br'	0•19
K'	1•1	H_3BO_3	0•07
Sr'	0•04	(HCO_3 and CO_3'')	0•35

Minor constituents, 0•02 - 0•03%.

An investigation of the sea water off the coast of Bermuda reported by Krogh (1934a), showed a uniform content of nitrogen and organic carbon from the surface to the bottom at a depth of 5,400 metres. He found 0·244 gm. of nitrogen and 2·36 gm. of organic carbon per cubic metre. If this nitrogen is taken to be protein and the organic carbon to be carbohydrate then it is equivalent to 1.5 gm. protein and 5·9 gm. carbohydrate per cubic metre. This represents three hundred times the total organic dry substance of living organisms in the water at any given time. A comparison is drawn between good farmland, where the available nitrogen under one square metre to a depth of 25 cm. is approximately 400 gm., and North Sea water where the available nitrogen under one square metre to a depth of 100 metres is roughly 40 gm. On the same basis there are 210 gm. of phosphoric acid in 25 cm. soil per square metre, and only 15 gm. in 100 metres water per square metre.

The phenomenon of upwelling which results in water of lower density and temperature rising to the surface may occur anywhere in the sea but is most conspicuous off the western coasts of continents where the prevailing winds carry surface waters away from the land, and particularly in the Antarctic Ocean.

This has pronounced effects on the nutrient content of the water. For example, phosphorus, which is an essential element for all life, is incorporated into plant and animal cells and eventually sediments to the bottom and the upper water strata would be permanently depleted if it were not for the nutrient-rich water rising from the ocean depths.

The pH of sea water varies between 7·5 and 8·4. When the water is in equilibrium with the carbon dioxide of the atmosphere the pH is between 8·1 and 8·4. In regions where most of the oxygen has been consumed and where the carbon dioxide content is high the pH will reach its minimum value of 7·5.

The pressure in the sea is a function of depth and is nearly a linear function, pressure increasing by approximately 0·1 atmosphere per metre. Thus at a depth of 1 mile the pressure will be 1 ton per square inch (ZoBell, 1946).

Sea water can be divided vertically into three zones on a basis of light penetration (Sverdrup et al., 1942).

1). The euphotic zone which extends to 80 metres or more. This zone has an abundant supply of light and because of the photosynthetic processes taking place it is the site of primary productivity in the sea. The depth of this zone varies with latitude and with the amount of particulate matter in suspension. It is deepest of all in equatorial regions where there is intense direct sunlight.

2). The disphotic zone which extends from 80 to 200 metres is only dimly lighted and here there is little or no photosynthesis.

3). The aphotic zone which extends below about 200 metres and is in continual darkness. In this zone bacterial life abounds owing to the constant descent of organic matter from the euphotic zone.

As an indication of the variation which may occur with respect to light penetration into different water masses, photosynthesis has been detected to a depth of 18 metres in Woods Hole Harbour, 40 metres

in the Gulf of Maine, and 140 metres in the Sargasso Sea (Clarke, 1936).

An estimation of particulate matter in suspension showed 0.4 to 2·0 mgm. per litre in oceanic Atlantic water 100 miles beyond the continental shelf (Harvey, 1937).

Closely connected to the penetration of light into sea water is temperature, which ranges from -1·91°C., at which temperature sea water of salinity 35‰ will start to freeze, to 28 to 30°C., except for some localised areas of the littoral zone which may reach 38 to 40°C.

More than half of the surface waters of the world have a temperature between 15°C. and 30°C., but at depths of 200 to 1,000 metres this temperature falls to 5°C., while below 1,000 metres the temperature is between 5°C. and -1·5°C. Therefore 90 per cent of the marine environment is perpetually colder than 5°C. (ZoBell, 1946).

From the preceding brief outline it can be seen that the marine environment offers, with only a few localised exceptions, an unusual stability of physico-chemical conditions. As a result, marine bacteria are adapted to, and may be dependent on, the special conditions met with in the sea. This is particularly reflected in their salt and temperature requirements for growth (MacLeod and Onofrey, 1956; ZoBell and Conn, 1940); and many marine bacteria are adapted to grow under extremes of pressure which are lethal to terrestrial types (ZoBell and Johnston, 1949).

1.2. Review of Historical Literature.

Although one of the first accurately described bacterial species - *Spirochaeta plicatilis* - was isolated from sea water (Ehrenberg, 1838) it was not until the mid-eighteen-eighties that the first systematic studies of bacteria found in sea water were commenced. From that time until

the outbreak of World War One was a period of widespread exploration during which the ubiquity of bacteria throughout the marine environment was established. This period of exploration has been admirably reviewed by ZoBell (1946), and Benecke (1933).

Warming (1876) described many bacterial species he had come across in sea water off the coast of Denmark. He noted intensely pigmented spiral forms, rods and spherical forms, and recorded that many marine bacteria demonstrated a marked plasticity of form.

Certes (1884), while on the Talisman Expedition, collected one hundred samples of sea water and bottom deposits, some from depths as great as 5,000 metres. Aerobic heterotrophs were isolated from ninety-six samples. He isolated no anaerobes.

During the Plankton Expedition of the Humboldt Foundation in the Atlantic Ocean, Fischer (1894) studied the role of bacteria in the biology of the sea. He found an average of 1,085 bacteria per ml. in one hundred and seventy-five samples of water, the highest count being 39,400 bacteria per ml. Fischer used nutrient gelatin or fish gelatin prepared with sea water for cultivating his organisms. More than twice as many bacteria were isolated from the Baltic and North Sea as from similar depths in the open ocean. He reported maximum counts at 200 to 400 metres and thereafter bacterial numbers decreased with depth, until below a depth of 1,100 metres very few bacteria were found. Fischer suggested that the great variation in numbers of bacteria in his samples may reflect the fact that bacteria are not free-living but grow primarily on the dead bodies or excretions of marine plants and animals. He also noted a close association between high bacterial counts and

plankton organisms. The majority of Fischer's isolates grew only, or preferentially, in nutrient media prepared with sea water or with a solution of sodium chloride isotonic with sea water. Most of his organisms were Gram negative and motile, with a predominance of spirilla or vibrios and pleomorphic rods. Yeasts were regularly isolated, so too were lower fungi, particularly near land. Gram positive cocci and spore-formers were absent.

Forster (1892) demonstrated the occurrence of bacteria in the North Sea throughout the year. His isolates could multiply at 0°C.

Bacteria were found in all samples of water and bottom deposits collected by Russell (1891) in the Gulf of Naples, from depths as great as 1,100 metres and at distances up to 10 miles from land. The bottom slime contained 25,000 to 50,000 bacteria per ml. while water rarely contained more than 100 organisms per ml. The isolates Russell observed grew best at temperatures lower than 25°C. Very few strains tolerated temperatures as high as 37°C.

At Woods Hole, Russell (1892) examined samples collected at depths as great as 140 metres and up to 100 miles from land. He demonstrated the presence of 5 to 120 bacteria per ml., with little variation with depth. An average of 17,000 bacteria per ml. was found in bottom deposits. Most of his isolates were aerobes or microaerophills which liquified gelatin and digested casein. Many reduced nitrate. Pigment production and pleomorphism were common.

Frankland and Burgess (1897) demonstrated an average of 51 bacteria per ml. in sea water a few miles from the North Cape at a depth of 3 feet.

Between 8 and 140 bacteria per ml. of water were isolated by Minervini (1900) from the North Atlantic at depths between 5 and 10 metres. Vibrio species were the most common.

Schmidt-Neilsen (1901) encountered an average of 26 bacteria per ml. of surface water from the Oslo Fjord. He found that numbers increased from the surface down to 25 metres. A pigmented organism which he called a variety of Bacillus prodigiosus occurred regularly.

An analysis of surface water samples collected near Ross Island by Ekelöf (1907) during the Swedish South Polar Expedition revealed 4 to 5 bacteria per ml. Most of the bacteria isolated were curved or rod shaped.

While on the Scottish Antarctic Expedition, Pirie (1904) isolated bacteria in most water samples taken near the Orkney Islands. Most of his isolates were spirilla or rods. He found no nitrifying bacteria but denitrifiers were common.

Samples of sea water from the Atlantic Ocean between Europe and South America collected and examined by Otto and Neumon (1904) had an average of 60 bacteria per ml. in the open ocean 5 metres below the surface. Below 100 metres numbers decreased sharply with depth. They described the predominant organisms as vibrios and rod-shaped bacteria resembling Proteus vulgaris, Escherichia coli and Pseudomonas fluorescens.

Gräf (1909) investigated water samples from the Atlantic Ocean, and in surface water more than 120 miles from land he found an average of 42 bacteria per ml.

While sailing between Scotland, Iceland and Spitsbergen, Hesse (1914) collected surface water in a sterile sailcloth sampler.

Investigation of these samples revealed between 45 and 10,000 bacteria per ml., the largest bacterial populations occurring near land and where the warm Atlantic water meets the cold Arctic water. In this region the sudden change in temperature causes the death of many organisms thereby providing a plentiful food supply for bacteria. The majority of Hesse's isolates were motile rods or vibrios and were denitrifiers. They grew better at refrigeration temperatures than at 37°C., and room temperature was optimum for their growth. His isolates would grow in either sea water, or fresh water, nutrient media.

Issatschenko (1914) found nitrifiers, denitrifiers, sulphate-reducers, ammonifiers and other types of organisms in Arctic Seas to depths of 65 to 100 metres.

This brief sketch of the activities and findings of the early marine bacteriologists may be summarised as follows: bacteria could be isolated from sea water both near land and in the open ocean; great depths and accompanying pressures provided no barrier to the survival of bacterial species; sea water could contain up to a few hundred cultivatable bacteria per ml., though there was an indication that they were not free living, but attached to various particles suspended in the water; a diversity of physiological types could be demonstrated; morphologically the isolates were mainly motile rods or spirals, and a high proportion were pigmented.

1.5. The Isolation of Marine Bacteria.

1.5.1. Sampling Device.

Indispensable for the microbiological analyses of water masses is an efficient device for collecting water samples from

any depth. More than one hundred different samplers have been employed by microbiologists and many of these are described and criticised by ZoBell (1941a, 1946).

The most common faults exhibited by samplers used by early workers were: a) inability to obtain water from a given depth free from contamination by water from any other depth, b) failure to function under the great pressures encountered in the depths of the oceans, and c) bactericidal action of metals used in the construction of the sampler.

ZoBell (1941a) overcame the difficulties involved and described a simple and efficient sampler easily constructed in the laboratory. It consists of a metal frame incorporating a messenger-activated breaking mechanism which can be used with either glass bottles or collapsable rubber bulbs. The glass bottles can be used to a depth of 200 metres and the rubber bulbs to any depth in the sea.

A short piece of pressure resistant rubber tubing, closed with small-bore glass tubing which has been sealed at one end, is fitted to the neck of the container. The bottles are sterilised by autoclaving and sealed immediately so that upon cooling they remain partially evacuated. The rubber bulbs are similarly sterilized and collapsed by hand prior to sealing.

The container, in the metal frame, is attached to a standard hydrographic cable and lowered to the required depth. When the glass capillary tubing is broken the rubber tubing straightens out so that water is taken in from a point several inches away from the carrier and cable which are possible sources of contamination. The container always has some residual air which is expelled during the ascent of

the sampler as the pressure decreases. Because of this the intake of water from above the sampled area is prevented.

1.3.2. Effects of storing sea water samples.

Sea water isolated in a glass container and held at temperatures between 20°C. and 30°C. undergoes rapid changes in its bacterial population and chemical constitution (Whipple, 1901; Keys *et al.*, 1935; Waksman and Carey, 1935; ZoBell, 1936; ZoBell and Anderson, 1936; Lloyd, 1937; Harvey, 1941).

Keys *et al.*, (1935) demonstrated a rapid increase in bacterial numbers and a reduction of oxygen content of sea water on storage in a glass container. If the water was kept at 5°C. these processes were much slower. Waksman and Carey (1935) obtained similar results in their experiments and noted a direct parallel between bacterial multiplication, oxygen consumption and nitrogen liberation. They concluded that sea water contains sufficient organic matter in solution to support, under suitable conditions, an extensive bacterial population, and that the magnitude of the change in bacterial content, which can increase by as much as ten thousand times in a few days, reflects the content of organic material (ZoBell and Anderson, 1936). The bacterial flora undergoes qualitative changes as well as quantitative changes during storage. ZoBell and Feltham (1934) isolated twenty-six different types from a fresh sea water sample and only four different types after twenty-four hours' storage.

The ratio of water volume to area exposed to the walls of the container is directly proportional to the maximum numbers of bacteria which will develop in a stored water sample. Maximum numbers per

ml. will develop in a small container where a relatively large surface area is in contact with the walls (ZoBell and Anderson, 1936).

ZoBell (1936) found that in a water sample held at room temperature the maximum bacterial population was reached at three to five days, when numbers were estimated by plating water samples on a nutrient agar medium. Although bacterial numbers apparently continued to fall after five days, the oxygen consumption increased to a maximum at twenty days. This apparent anomaly was resolved when he demonstrated large numbers of periphytic bacteria firmly attached to the walls of the container. With similar evidence, i.e. falling bacterial numbers and increasing oxygen consumption, Waksman and Hotchkiss (1937) suggested that nannoplankton (plankton measuring less than 50µ) may increase in numbers at the expense of the bacteria present.

The foregoing results indicate that the organic matter in sea water which is roughly 5 mgm. per litre (Krogh, 1934b) is capable of sustaining a much larger number of bacteria than is actually found in open sea water. Keys et al., (1935) state that "the principal limitation to bacterial growth in pure sea water is lack of available organic carbon".

It has been conclusively demonstrated that a major factor involved in the rapid increase in bacterial numbers with the storage of a sea water sample is the provision of solid surfaces for the accumulation of organic matter and for the attachment of periphytes (ZoBell and Anderson, 1936; Stark et al., 1938; Heukelekian and Heller, 1940; Harvey, 1941; ZoBell, 1943; ZoBell and Grant, 1943).

Chemically cleaned glass slides accumulate measurable amounts of organic matter at their surface after being immersed for some hours in lake water (Stark et al., 1938). ZoBell (1943) could stain a thin film of organic material on the surface of glass slides which had been left in sea water for a few days. The same author added glass wool to sea water to give 2 to 20 cm.^{2} of glass surface per ml., and found that 2 to 27 per cent of the organic matter was adsorbed by the glass, the amount adsorbed being proportional to the surface area exposed. Harvey (1941) found that the adsorption of organic matter from dilute solution took place primarily at the glass-water-air interface.

It has been suggested recently by Kriss (1962) that not only is there a relative increase in concentration of organic substances in the vicinity of solid surfaces but there is also a qualitative change in the molecular form of proteins when they become adsorbed on a surface. Such modified proteins, he suggests, are more susceptible to enzymic attack. This occurs "throughout the entire water mass at points of contact of the suspended organic and inorganic particles with the water. The sinking particles, as they descend to the bottom of the sea or ocean, can form microzones resulting in the accumulation and transformation of the organic substances......".

ZoBell (1943) believes that most bacteria in the sea are associated with solid surfaces, e.g. plankton, and has demonstrated that many marine bacteria have a filament or stalk which is not apparent under normal laboratory conditions. Some species appear to secrete a cement while others produce a mucilagenous slime.

All of these mechanisms permit periphytes to attach themselves to surfaces, often so firmly that they cannot be dislodged by fast running water.

Investigations into the minimum concentration of organic material which will permit the growth of marine bacteria by ZoBell and Grant (1943) showed that as little as 0·1 mgm. per litre of glucose, succinate, malate, glycerol, starch, lactate, ethanol or asparagine was sufficient to initiate multiplication. Heukelekian and Heller (1940) observed that Escherichia coli did not grow in a medium containing glucose and peptone when their concentration was less than 2.5 ppm., unless glass beads were added to the culture flask. The growth promoting effect of the increased surface area due to the addition of glass beads was noticeable up to a nutrient concentration of 25 ppm., and when glucose and peptone were present at greater concentrations bacterial growth was similar in flasks with or without additional glass beads. When investigating the nutritional requirements of plankton bacteria Jannasch (1958) found that the limiting concentration for the growth of starved cells of Pseudomonas fluorescens was 0·01 mgm. per litre for ammonia-N and slightly higher for nitrate- and amino-N.

1.3.5. Growth requirements of marine bacteria.

Most isolates from sea water remote from terrestrial contamination have a specific requirement for sea water for growth (ZoBell, 1941b). Neither synthetic sea water nor other isotonic salt solutions were satisfactory substitutes. Although unable to identify the factor in sea water which is essential for the growth of his

isolates, ZoBell (1941b) remarks that "there seems to be something in sea water besides its mineral constituents which is essential for the growth of marine bacteria, because not even isotonic concentrations of sea salt were as good as natural sea water". However, after some weeks of laboratory cultivation most marine bacteria having an initial requirement for sea water for growth become adapted to grow in fresh water media (ZoBell and Michener, 1938).

These results are in direct conflict with those of MacLeod *et al.* (1953, 1954, 1956) who could detect no superiority in the growth promoting effects of natural sea water over artificial sea water when culturing marine bacteria. Moreover MacLeod and Onofrey (1956) produced a salt solution much simpler than the artificial sea water proposed by Lyman and Fleming (1940) which was as suitable as natural sea water for the growth of their isolates. At the same time MacLeod and Onofrey (1956) could not substantiate the findings of ZoBell and Michener (1938) since none of their isolates could be adapted to grow on fresh water media even after repeated subculture, but they did find that in five out of six cases rate and extent of growth was increased by using half-strength artificial sea water supplemented with iron.

Using marine bacteria requiring sea water for growth, MacLeod *et al.* (1956, 1957a, and 1957b) showed an absolute requirement for Na^+ and K^+ ions and variable requirements for Mg^{++}, Ca^{++} and Cl' ions. The required concentration of Na^+ has been shown to vary with the substrate supplied to the cells (MacLeod *et al.*, 1958). These authors suggested that the variation in the Na^+

requirement of cell suspensions with substrate could best be accounted for by assuming that the di- and tri- basic acids, but not the monobasic acids and hexose sugars, form complexes with Na^+ before crossing the cell membrane.

Tyler (1959) worked with one hundred marine pseudomonads which required sea water for growth upon initial isolation. Forty-eight of these failed to grow at any salt concentration other than that of sea water. All of these pseudomonads required NaCl for good growth, about half required KCl, and most required Mg^{++} salts.

Working with thirteen marine bacteria Ostroff and Henry (1939) found that a wide range of nitrogenous compounds could be utilised for growth. Amino acids resulted in the best growth and cyclic compounds were least satisfactory.

The oxidation and utilisation of twenty-one amino acids as the sole carbon and nitrogen source for a marine bacterium were investigated by Tomlinson and MacLeod (1957). Any one of sixteen of these amino acids could serve as a sole nitrogen source, thirteen were readily oxidised but only five could serve as the sole carbon source. MacLeod *et al.*, (1954) studied the nutritional requirements of thirty-three bacteria. Nineteen strains could grow in a medium containing any one of several organic compounds as a source of carbon and energy, ammonium sulphate as a source of nitrogen, and a mixture of inorganic ions. Seven strains grew feebly or not at all unless certain amino acids were supplied as a source of carbon and energy. Four strains required amino acid supplements in addition to a non-amino acid source of carbon and energy and five strains

grew only in the presence of one or more vitamins or other growth factors.

The nutrition and metabolism of marine bacteria have tended to be neglected, and most of the information about the detailed physiology of marine heterotrophs is to be found in the publications of MacLeod and his associates as described. Notwithstanding the necessity for much more comprehensive surveys of marine microbial populations along the lines initiated by MacLeod, it appears already that in marine bacteria we have a flora at once similar and dissimilar from the more widely known and understood soil flora.

The likeness is apparent when the range of biochemical activities is compared. Within the two groups, that is, soil and marine bacteria, is to be found an equally broad spectrum of enzymic activity which can be equated in general terms, although there do exist specific activities peculiar to one or the other. For instance, the hydrolysis of agar, a common activity among marine micro-organisms, is rare or absent among soil types.

A striking dissimilarity exists, however, with respect to the salt requirements of the two groups. Although native terrestrial bacteria can sometimes become adapted to survive or even to multiply successfully in the marine environment, and some indigenous marine forms can grow under terrestrial conditions, there does exist a group of marine bacteria having an obligate requirement for certain ions at concentrations found in natural sea water. The need for salts at specific concentration levels arises only in part from osmotic requirements and the detailed relationships between ions and

enzymic systems have still to be elucidated.

Effects of Pressure.

Certes (1884) found that marine bacteria survived a pressure of six hundred atmospheres of nitrogen and concluded that the hydrostatic pressure of sea water does not influence the vertical distribution of micro-organisms in the ocean.

Using more carefully controlled techniques ZoBell and Johnston (1949) subjected a wide range of terrestrial and marine bacteria to pressures up to six hundred atmospheres. At 30°C. the growth of most terrestrial bacteria was retarded by pressures of six hundred atmospheres and in no case was there perceptible multiplication at this pressure. In marked contrast, marine varieties isolated from the ocean depths, in particular "Bacillus submarinus" and "Bacillus thalassokoites", grew readily under six hundred atmospheres at both 30°C. and 40°C. "Indications are that hydrostatic pressure, within the range occurring in the deep sea, is a thermodynamic factor of co-ordinate importance with temperature in affecting the distribution and physiological activities of micro-organisms" (ZoBell and Oppenheimer, 1950). In general, lower temperatures accentuate the growth-retarding and lethal effects of pressure, while at high temperatures the effect of pressure is less pronounced (ZoBell and Johnston, 1949). ZoBell and Johnston were able to grow Pseudomonas xanthrochrus under four hundred to six hundred atmospheres at 40°C., a temperature which is lethal to this species at normal pressure.

The effect of pressure upon bacterial enzyme systems has been shown to be irregular. For instance the succinic dehydrogenase

system of *Escherichia coli* is inactivated by moderate hydrostatic pressures (Morita and ZoBell, 1956) while the activity of aspartase of the same species is accelerated up to six hundred and eighty atmospheres. In general starch hydrolysis, nitrate reduction and urea decomposition by certain bacteria, are retarded by pressures of the order that occur in the sea, while bacterial oxidation of ammonia to nitrite, and the liberation of ammonia from amino acids are accelerated by such pressures (ZoBell, 1955).

Hydrostatic pressure has a pronounced effect on cell morphology. This was demonstrated by ZoBell and Oppenheimer (1950) using *Serratia marinorubra* which at atmospheric pressure is a small rod and when subjected to six hundred atmospheres appears as filaments up to 200µ in length. The high pressure has apparently interfered with the cell dividing mechanism, since on return to atmospheric pressure the long filaments fragment into typical short rods after a few hours. Other species examined showed a loss of flagella at elevated pressures which was reversed and motility regained on return to normal pressure.

ZoBell and Johnston (1949) introduced the term "barophilic" to characterise organisms occurring in the ocean depths whose growth or metabolism is favoured by pressure. Such micro-organisms have since been isolated and described on many occasions (ZoBell and Oppenheimer, 1950; ZoBell, 1955; ZoBell and Morita, 1957).

Isolation Media.

The success of any cultural method for the quantitative assessment of a microbial population "depends largely upon the

capacity of the nutrient medium to satisfy the cultural requirements of the micro-organisms. The cultural requirements embrace not only energy and food requirements but also the proper hydrogen ion concentration, osmotic pressure, surface tension, oxidation-reduction potential, physical consistency, temperature and other environmental conditions" (ZoBell, 1946).

The comprehensive investigations reported by ZoBell (1941b) describe the effects of varying the chemical constitution of media on the isolation of marine bacteria. He demonstrated that peptone was superior for the isolation of marine bacteria when compared with algal and fish infusions, carbohydrates, organic acids and various nitrogenous compounds.

In order to obtain reproducible results ZoBell and Anderson (1936) state that it is necessary to use "aged" sea water for nutrient media, i.e., sea water which has been stored in non-metal containers for a few weeks. By this means the organic content of the water is stabilised and the slightly bacteriostatic effect of raw sea water is destroyed (ZoBell, 1946).

The medium recommended by ZoBell (1941b) as being best for giving "maximum and reproducible plate counts" is medium 2216. It has the following composition :-

"Aged" sea water	1,000·0 ml.
Bacto-peptone	5·0 gm.
Ferric phosphate	0·1 gm.
Bacto-agar	15·0 gm.
pH after sterilisation	7·5 to 7·6.

After many experiments the following medium was accepted as being best for consistent high counts at Woods Hole, Massachusetts (Reuszer, 1933):-

Peptone	1·0 gm.
Glucose	1·0 gm.
K_2HPO_4	0·05 gm.
Agar	15·0 gm.
Sea water	1000·0 ml.

ZoBell (1941b) found only 55 to 73 per cent as many colonies on this medium as on medium 2216 in a comparison of both Pacific and Atlantic waters. The addition of ferric phosphate to the Woods Hole medium resulted in counts almost as high as medium 2216. ZoBell (1941b) also found that maximum counts were obtained at pH 7·5 to 7·8 though they were comparable from pH 7·0 to 8·0.

Jones (1957) pointed out that yeast extract was "very stimulatory" to the growth of marine bacteria. The stimulatory effect of yeast extract on marine heterotrophs was confirmed by Carlucci and Pramer (1957) who obtained an increase in numbers of 25 per cent over ZoBell's medium 2216 when 0·01 per cent yeast extract was incorporated in the plating medium.

The addition of 10 ppm. of Tween 80 to culture media increased the counts obtained by Jones and Jannasch (1959) but produced irregular results when employed by Buck and Cleverdon (1961) who postulate that the disparity of findings between themselves and Jones and Jannasch (1959) "may be due to differences in susceptibility to Tween 80 of the predominant bacterial floras of the two areas at the time of sampling or to limits imposed by numbers of samples or to distinct differences in shaking procedure."

1.3.4. The enumeration of marine bacteria.

The enumeration of the bacteria in a sample of sea water presents many complex problems. There are two principal techniques used; the cultivation of organisms present and the counting of colonies produced, and direct counting using the microscope.

Cultural methods.

One of the most popular methods for cultivating marine bacteria entails mixing a molten agar nutrient medium at 42°C. with 1·0 ml. of sea water, or of a suitable dilution of sea water. A serious drawback of this method is the exposure of the water sample to 42°C. ZoBell and Conn (1940) showed, using gelatin plates, that many fewer colonies developed after a sample had been exposed to a plating temperature of 42°C. than when a plating temperature of 30°C. was used. The same authors found that 80 per cent of the bacteria in an average sea water sample were killed in 10 minutes at 40°C.

The alternative method of conducting plate counts is to inoculate the surface of the nutrient medium after it has been poured and cooled. Carlucci and Pramer (1957) report that this method gave 30 to 40 per cent fewer colonies than the pour-plate method attributing this to the growth of "micro-aerophilic or anaerobic bacteria below the surface of the agar in pour plates." In contrast, Buck and Cleverdon (1960) found that surface inoculated plates always gave higher counts than pour plates. Kriss (1962) believes that the best way of culturing marine bacteria is to spread 0·5 ml. of sea water on the surface of agar plates. Working with pure cultures Hentges and Fulton (1960) found similar numbers growing on spread- and on pour-

plates but they decided that spread plates were much easier to count.

Jones (1957) found that when sea water inocula were shaken with 10 ppm. of Tween 80 prior to plating the numbers of bacteria which developed on the agar plates were increased by an average of 3·4-fold.

Gunkel *et al.*, (1960) found that the volume of medium used in the pour plate method could substantially influence the development of both the number and kinds of bacterial colonies, and stressed that it was important to standardise the volume used.

The numbers and kinds of bacteria which will produce visible colonies on isolation plates are influenced by the temperature and period of incubation. ZoBell and Conn (1940) obtained maximum counts when their plates were incubated at 18 to 22°C. for one or two weeks. Only a very few colonies developed after incubation at 30 to 37°C., and although nearly all isolates could multiply at 0°C. there were no true psycrophiles. Figure 2, compiled by ZoBell and Conn, summarises their results.

Buck and Cleverdon (1961) found that incubation at 25°C. gave higher counts than incubation at 15°C. although the temperature of the water at sampling was nearer 10°C. The optimum temperature for incubation of sea water samples has been shown to vary with the composition of the isolation medium (Jones, 1960). His results indicate an "interrelationship between organisms, medium, temperature and time."

Marine bacteria have been cultivated on the surface of membrane filters through which has been passed a volume of sea water. Micro-organisms present in the water sample are held back by the filter

Figure 2.

Relative number of colonies appearing on nutrient agar incubated for different periods of time at different temperatures, the plate counts being expressed as percentages of the average plate count after 18 days at 18°C. (ZoBell and Conn, 1940).

Incubation time.	Incubation temperature.						
days.	4°C %	12°C %	18°C %	22°C %	25°C %	30°C %	37°C %
2	0	18	50	50	41	44	8
4	0	28	41	60	65	61	12
7	4	48	67	82	78	69	12
10	9	67	91	96	84	71	10
14	17	90	98	97	85	79	
18	26	97	100	98	82	65	
21	59	98	93	87	74	59	

which, when placed on a pad previously soaked in a fluid nutrient medium, will allow micro-colonies to develop. These can be counted with the aid of a microscope after only a few hours in a moist chamber (Oppenheimer, 1952). The same author observed a 50 per cent increase in counts on membrane filters when compared with conventional pour plate counts, but Kriss (1962) noted a marked irregularity of counts using this method. For instance, from the same water sample he passed 200 ml. through one filter and 75 ml. through another, and obtained fewer colonies on the membrane surface from the larger sample. Kriss (1962) expresses the opinion that the isolation of heterotrophs on complex media in Petri dishes gives a distorted picture of the marine microbial flora due to the selection of specific nutritional groups dependent on the cultural conditions employed. Nevertheless he believes that such cultural methods are at present irreplaceable.

Jannasch (1958) filtered sea water through membranes and counted colonies arising when the filter was placed on a pad soaked in nutrients and suggested from the distribution of his isolates on the filter surface that a main source of error when estimating bacterial numbers by plate counts may be clumping.

Direct Methods.

When it is not important to isolate organisms for further study and a sample is being investigated only with respect to the numbers present, direct methods of enumeration may be used. These entail examining a water sample microscopically and counting the bacterial cells visible. Obviously when dealing with water from

the open sea which may contain only a few hundred bacteria per ml., it is not feasible to make a direct count with any accuracy unless the organisms present can be concentrated into a smaller volume. A variety of methods for concentrating the microbial population of lake water have been described (Kusnetzov and Karzinkin, 1931; Snow and Fred, 1926; Collins and Kipling, 1957). None of these methods is suitable for the concentration of sea water since sea salts precipitate out and interfere with microscopic examination.

Cholodny (1929) passed lake water through a membrane filter having a pore size of 2μ, but stopped the process while a few ml. of water remained on top of the filter. This served to concentrate all the micro-organisms from a large volume of water into a few ml. He transferred measured quantities of this concentrate on to a glass slide and after staining with erythrosin he estimated from 100 to 1,000 times as many organisms by this method as by the plate count method. Cholodny appreciated that the drawback of this method was the inevitability of counting both living and dead organisms.

Jannasch (1955) and Ehrlich (1955) successfully applied this method, with modifications, to the enumeration of sea water bacteria and gave details of the technique used. The major deviation from the technique of Cholodny involved filtering all of the water through the filter, until it was dry, and examining the organisms directly on the surface of the membrane itself.

If glass slides are immersed in water and left for a period of time periphytic bacteria will attach themselves to the glass surface. If these slides are removed and stained it is

possible to count the number of organisms attached over a described area (Henrici, 1936; Hotchkiss and Waksman, 1936). Although these authors found only a limited positive correlation between plate counts and attachment counts, and ZoBell (1946) expressed doubts about its usefulness in the estimation of marine bacterial populations, this method has been widely employed by Kriss (1962) and Kriss and Mitskevich (1959, 1960) for qualitative and quantitative analyses of water masses and they have observed, attached to glass slides, many morphological types never encountered on isolation plates.

Cviic (1960) compared direct counts from the surface of membrane filters with plate counts, and noted more than a thousand fold superiority of counts by the direct method. He stressed, however, the importance of freeing the membranes from contaminating organisms which would give rise to an over-estimate of numbers. A similar relationship between plate counts and counts on the surface of membrane filters was reported by Wood (1950, 1955).

An evaluation of five different cultural methods, and two direct microscopic methods was carried out by Jannasch and Jones (1959). The cultural methods employed were; macrocolony counts on nutrient agar, silica gel, and membrane filters; microcolony counts on membrane filters; and the extinction dilution method. Direct microscopic counts were made of organisms on membrane filters and of micro-organisms transferred from membrane filters to glass slides. In all of the cultural methods a medium containing peptone and yeast extract was employed. The extinction dilution method

and the microcolony membrane filter method gave counts 25 to 35 times higher, respectively, than did any of the macrocolony methods. Direct microscopic counts on membrane filters were approximately 150 times higher than plate counts, and the numbers of microbes transferred from membrane filters to glass slides were roughly 2,000 times higher than plate counts. Figure 3, compiled by Jannasch and Jones (1959), gives a comparative summary of their results for all counting methods used.

It is interesting to note that Jannasch and Jones (1959) observed large numbers of spirilla-like organisms by direct microscopy, but isolated none of these types by cultural methods.

1.4. Bacteria in Sea Water.

1.4.1. Numbers and distribution.

Before an estimate of the extent of bacterial activity in any body of sea water can be attempted an indication of the numbers of bacteria and of their distribution throughout a water mass and of the factors influencing this distribution, is essential.

The first real attempt to investigate the effects of external influences on the distribution of bacteria in sea water was made by Lloyd (1930) working in the Clyde Sea area. Unfortunately she used media prepared with tap water throughout this investigation, but nevertheless she was able to obtain some interesting information on the distribution and variation of bacterial numbers in the area.

In summary, Lloyd's (1930) work indicated that surface water supported a greater bacterial population than water at

Figure 3.

Ratio of microbial counts, range of values, and per cent error of five different methods compared to plate counts computed from all data. (Jannasch and Jones, 1959).

	Plate method	Macro-colonies on MF *	Serial dilution method	Micro-colonies on MF	Direct counts on MF	Cholodny method
Mean	1	1·16	21·8	52·1	147	2100
Range	-	0·2 - 4·0	0·3 - 55	1·6 - 34	15 - 840	108 - 9700
Per cent error	-	17·1	19·2	12·7	8·5	20·7

* Membrane filters

lower levels, though there was a more marked fluctuation of numbers at the surface than at depths below 10 fathoms. In Loch Striven, an uncontaminated sea loch, there was an average of 80 bacteria per ml. at the ssurface but only 20 per ml. at 10 fathoms. There was an indication of diurnal variation at lower levels with a tendency for higher counts during the night. On the other hand little numerical variation with season was apparent. Lloyd postulated the division of marine bacteria into two groups; organisms living on and attached to particles, and free floating bacteria whose source of food is organic matter dissolved in sea water.

Waksman (1935) decided that the conditions responsible for the distribution of bacteria in sea water were :- 1) the supply of nutrients, which included the abundance, nature and extent of decomposition of organic matter, and the availability of essential elements, and 2) the environmental conditions such as aeration, temperature and salinity.

When attempting to correlate any variation in bacterial counts with obvious changes in environmental conditions, e.g. depth, season, distance from land, the results of Reuszer (1933) and ZoBell and Feltham (1934) should be considered. The findings of the latter two workers are particularly striking evidence of variation of numbers when water samples are collected at the same place at virtually the same time. The analysis of ten, 10 ml. samples collected at one place within a few seconds showed an average deviation of 85·5 per cent from the average plate count, and when only 1·0 ml. samples were collected and plated directly, the counts deviated by as much as 900 per cent from the mean.

Reusner (1953) found that the effect of land drainage on the numbers of bacteria in sea water did not extend beyond one mile from the shore. In shallow regions, where water was mixed from top to bottom, he found a considerably higher bacterial content, ranging from 20 to 200 organisms per ml., than in open sea water where 1 ml. was often free from cultivatable heterotrophs.

ZoBell and Feltham (1934) failed to detect any significant diurnal variation in numbers, or an increase in numbers from the surface down to 25 metres. From the open sea maximum plate counts are obtained in the zone from the surface down to 50 metres, while below 100 metres only a very few bacteria are present (ZoBell and Feltham, 1934; Wood, 1953).

In a study of Atlantic coastal water off the coast of Wales, Hudleston (1955) noted a suggestion of seasonal variation in microbial counts, but no significant variation of numbers with depth from the surface down to 8 fathoms.

Jannasch and Jones (1959) found that the largest counts of bacteria occurred in surface waters, decreasing down to 75 metres, with an increase in abundance at 200 metres. A marked decrease in bacterial numbers was observed just below the thermocline as reflected by macrocolony counting methods but not by direct microscopic methods.

In the Adriatic Sea, Cviic (1960) studied the vertical distribution of bacteria and found that the maximum population of bacterial heterotrophs appeared in the euphotic zone, and that numbers diminished with depth. The highest microbial populations were apparent in spring and summer.

The relationship between bacteria and plankton in sea water has been investigated by numerous workers (Renner, 1935; Waksman et al., 1937; Gylio, 1955; Devèze, 1955; Jannasch, 1958b; Oppenheimer and Vance, 1960; Kriss, 1962). The occurrence and distribution of plankton certainly influences the bacterial populations of the seas and oceans but the exact mechanism of this interrelationship is by no means clear.

Waksman et al., (1937) found that dead marine diatom plankton underwent rapid oxidation and decomposition when added to fresh sea water and this was accompanied by bacterial multiplication. However, living diatoms added to sea water were rather resistant to bacterial attack.

The resistance of living planktonic organisms to bacterial attack has been further demonstrated by Devèze (1955), who noted a lag of three weeks between a plankton outburst and an increase in bacterial numbers, and by Oppenheimer and Vance (1960) who showed by microscopic examination that diatoms, flagellates, ciliates, and blue-green algae did not support an attached bacterial population, and apparently had some protective mechanism against bacterial attack.

1.4.2. Characterisation of marine bacteria.

Most marine bacteriologists have been content to estimate the size of bacterial populations, and to assess the relative proportions of the morphological forms isolated. In only a few publications can information be found about the physiology and classification of bacteria isolated from sea water. There are, however, numerous publications giving detailed information on the physiology and classification of the bacterial flora of marine

fish (Wood, 1950; Shewan *et al.*, 1954; Tarr, 1954; Liston, 1955; Georgala, 1957; De Silva, 1960).

Figure 4, compiled from the data of several investigators, gives an impression of the proportion of morphological types found in sea water, fresh water, soil, and on the surface of cod fish.

It appears from this table that the microbial flora of sea water is composed mainly of rods, most of which are Gram negative. A notable exception to this is the flora of Eastern Australian waters as reported by Wood (1953), who describes a much higher proportion of Gram positive rods and cocci. The microbial flora of fresh water and of cod fish is equatable with the general trend to be found in sea water. Soil, however, would appear to present an entirely different picture with a predominance of Gram positive types.

Descriptions of genera and species encountered in sea water are given by ZoBell and Upham (1944); Wood (1952 and 1955); Bunt (1955); Brisou (1955); and Kriss (1962).

About 80 per cent of the organisms catalogued by ZoBell and Upham (1944) were Gram negative rods, predominating in the order, *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Achromobacter* and "*Bacterium*". Under suitable conditions 70 per cent of their isolates were chromogenic.

As a general rule marine bacteria tend to be proteolytic rather than saccharolytic (ZoBell, 1942). The same author reported the ability of marine bacteria to attack a wide range of complex organic compounds such as starch, cellulose, agar, chitin and lignin.

Figure 4.

The aerobic bacterial flora of different environments. (as %).

Authors	Total G+ %	Total G- %	G+ Rods %	G- Rods %	Total Rods %	Total Cocci %	Vibrio Spirilla %	Actino-Mycetes %	Other %
ZoBell and Feltham (1934) Sea water (Pacific)	>15	>80	10	80	93	5	Few	Few	Few
ZoBell (1942) Sea water (Pacific)	*	Most	*	*	63	5	32	*	*
ZoBell (1946) Sea water (Pacific)	5	95	2	95	97	5	0	0	0
Wood (1953) Sea water (Australia)	55	61	16	81	77	19	0	<1	<1
Hudleston (1955) Sea water (East Atlantic)	4	96	0	96	96	4	0	0	0
Kriss (1962) Sea water (Indian Ocean)	*	*	*	*	89	10	0	Few	Few
Taylor (1942) Fresh water lake	5	95	4	95	99	1	0	0	0
Topping (1937) Soil	75	27	54	27	81	0	0	19	0
Georgala (1958) North Sea Cod	10	89	0	84	95	1	5	0	Few

* Information not available

Sea water collected from off the eastern coast of Australia has been analysed with respect to bacterial types by Wood (1953), who found that *Mycoplana* was by far the largest group comprising about 60 per cent of his total isolates. *Corynebacterium* and *Staphylococcus* were commonly isolated, and *Pseudomonas* occurred only rarely. Wood's predominant group - *Mycoplana* - was first isolated from soil (Gray and Thornton, 1928) and until Wood's report had never been encountered in sea water. It seems most likely that this group would be classified by other workers as belonging to another genus, and possibly would be referred to as *Pseudomonas* species. This would account for the apparent rarity of pseudomonads in Australian waters.

Spore-formers have been isolated only infrequently from sea water, although Newton (1924) found them regularly. The unusually high proportion of spore-formers isolated by Newton is probably due to the fact that all of her water samples were collected near sources of terrestrial contamination.

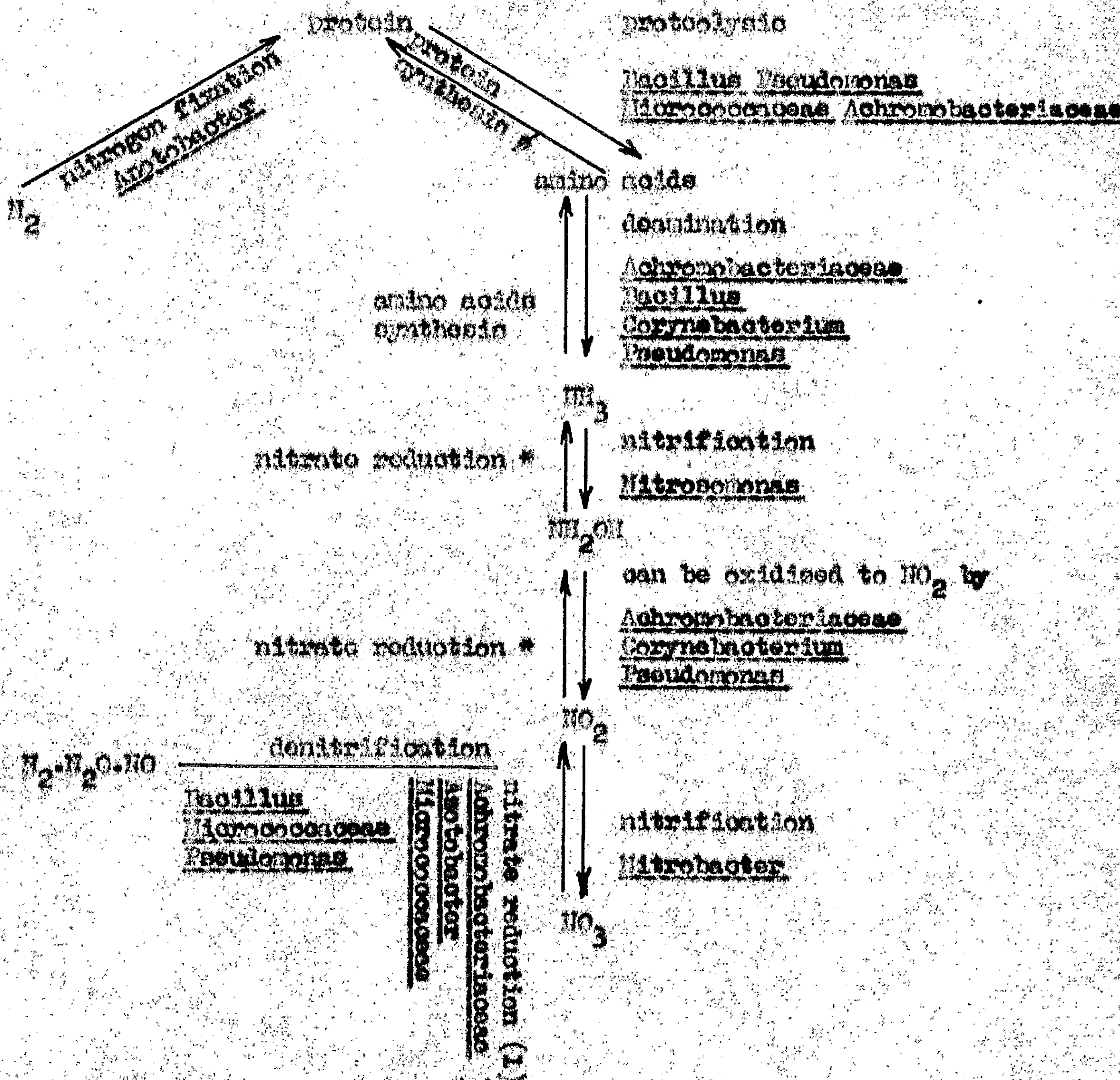
1.4.5. *Cycle of life in the oceans.*

The importance of bacteria in the cycle of life in the sea, and in particular their role in the nitrogen cycle, has been extensively studied by Waksman and his co-workers (Waksman, Carey and Reuszer, 1933; Waksman and Carey, 1935; Waksman, 1934; Waksman and Renn, 1936). The problem of the biological cycling of nitrogen has been investigated by Botan *et al.*, (1960), who determined the numbers and kinds of micro-organisms concerned in the aerobic decomposition of nitrogenous matter in a fresh water lake. Figure 5, taken from Botan *et al.*, (1960), summarises the conclusions drawn from the results of their

Figure 5.

Nitrogen Utilization Scheme

(slightly adapted from Dotan *et al.*, 1960)



* Same organisms as reaction (1).

≠ All organisms studied.

1. Organisms listed are those which have been isolated and appear to play a positive role in this study. 2. Organisms are listed in two ways: (1) family names if more than one genus of a family is involved, (2) generic names are used if only one genus of a family is involved in a specific reaction.

investigation.

Although figure 5 is based upon results obtained from a fresh water lake it is probable that a similar system functions in the balance of nitrogen in sea water.

1.5. Russian Advances in Marine Microbiology.

The achievements of Russian workers in marine microbiology and their significance have tended to be neglected by Western investigators. This is probably due, in the main, to the volume of obscure publications in the Russian language. However the treatise on Marine Microbiology by Kriss (1959) has been translated by Shewan (Kriss, 1962) and gives an excellent account of the advances made in this field by Russian workers mainly during the last fifteen years. A digested review reporting the advances of the Russian School has been compiled by Sieburth (1960), - "Soviet Aquatic Bacteriology: A Review of the Past Decade".

Russian investigations have been concentrated on quantitative aspects of microbial life in the oceans, there being numerous reports on the biomass (an estimation of the total living weight of bacteria in a water body) and distribution of morphological types, and on the cycling of elements brought about by diverse bacterial metabolic processes. Kriss (1962) writes, "It is not possible to estimate in a quantitative manner the dynamically grandiose processes of metamorphosis of organic matter and its formation from mineral substances both on dry land and in the world ocean without taking account of the mass of living micro-organisms and of their work,

both creative and destructive, in establishing the cycles of biogenic elements."

Using plate- and membrane-filter- cultural methods and the glass slide technique developed by Cholodny (1929), the distribution of bacterial biomass and its productivity have been determined throughout the world's water basins. Detailed charts of the horizontal and vertical variation of microbial biomass have been compiled and these utilised to "map" currents and characterise water masses. - "Micro-organisms, due to their selective relationship to various forms of organic substances can be used as reliable indicators of the qualitative differences in the organic contents of the sea water and, consequently, they are more suitable for the detailed identification of the water masses than the present-day hydrochemical methods." (Kriss, 1962).

1.6. Summary.

From this review of the literature the following points are especially pertinent to the work in hand.

1). Sea water samples when isolated in glass containers undergo rapid and striking changes with respect to their microbial flora. Ideally, samples should be plated immediately on board ship, but since this was not possible during the present investigation the bottles were covered in ice and transported to the laboratory as quickly as possible. In no case did more than six hours elapse between collection and plating of samples.

2). Such is the extent of the variation in bacterial numbers in water samples collected seriatim from the same place that any attempt to estimate the effects of depth or locality, etc., on the bacterial population must involve a comprehensive programme of sampling over a period of months or even years. Desirable as such a programme would be it was quite outwith the scope of the present work. For this reason no attempt was made to estimate absolute values of bacterial biomass in North Sea water, or to attach any significance whatsoever to variation in numbers among samples. One locality and depth were chosen at the outset and within the limits of navigational accuracy all collections were taken from this position.

3). There are numerous reports demonstrating that slight variation in the chemical composition of isolation media can have pronounced effects on the quantitative assessment of a marine microbial flora. However, no report could be discovered where the influence of the isolation medium on the qualitative nature of the flora was considered; unless of course where a selective medium had been used deliberately to single out a particular physiological type.

4). Bacteriological information on the North Sea is available from only two sources: a) from the work at Torry Research Station, Aberdeen, where Dr. Shewan and his associates have investigated the flora of marine fish, and b) from the results of early workers, e.g. those who sampled waters of the Norwegian fjords (Schmidt-Nielson, 1901; Föyn and Gran, 1928).

The information accumulated from these sources is of doubtful value in an appreciation of the numbers and types of

bacteria which may be expected to occur in the water of the open North Sea. Although there is undoubtedly some correlation between the surface flora of fish and the native microbial population of the water itself, the surface of fish is such a selective environment that it can hardly be expected to reflect accurately the nature of a free-living population of marine bacteria; and because of recent developments in sampling techniques and cultural methods only limited significance can be attached to the results of Schmidt-Nielsen, and Föyn and Gran.

Against this background, the aims of the present study have been to evaluate: 1) the relative merits of various nutrient media in the quantitative assessment of the heterotrophic bacterial population of North Sea water, and 2) the influence of these media on the qualitative nature of the flora, using a new scheme of identification developed during the course of the investigation.

SECTION 2.

THE ENUMERATION OF HETEROTROPHIC BACTERIA IN NORTH SEA WATER.

2. THE ENUMERATION OF HETEROTROPHIC BACTERIA IN NORTH SEA WATER.

2.1. Introduction.

Before undertaking the main body of work it seemed necessary to carry out some preliminary investigations which would enable a rigid scheme of work to be formulated. This was done during the winter months of 1959-60. A brief summary of this undertaking and of the significance of the results obtained with reference to the later work, is presented here.

Samples of water were collected from several stations between ten and thirty miles off the coast of Aberdeen, from depths of ten, thirty and fifty metres, and plated on ZoBell's medium 2216 (ZoBell, 1941) and the sea water agar* standardly used at Torry Research Station, as described by Georgala (1957). Two methods of plating were employed: 1) 1 ml. samples of sea water were spread over the surface of previously dried nutrient agar plates and 2) agar cooled to 42°C. was mixed with 1 ml. samples of sea water in Petri dishes. Duplicate plates were inoculated throughout, and colonies were counted after ten days at 20°C.

* Lab-Lemco Meat Extract 5·0 gm., Difco Peptone 10·0 gm., Difco Agar 15·0 gm., and "Aged" Sea water 1,000 ml.

The conclusions drawn from this preliminary investigation can be summarised as follows: 1) with the nutrient media used, North Sea water yielded from ten to one hundred colonies per ml., there being no apparent difference with depth or locality; 2) although only duplicate plates were available for counting, ZoBell's medium 2216 seemed to give higher counts than the Torry sea water agar; 3) there was no apparent difference in counts from surface inoculated- or pour-plates, but the former were more easily counted and certainly more suitable for picking off separate colonies for sub-culturing.

On a basis of these findings it was decided: 1) to collect all samples from a station ten miles E.S.E. of Aberdeen, at a depth of ten metres since this site showed no gross difference in counts from any other sampled. It was sufficiently far from shore to minimise the possibility of terrestrial contamination but near enough to the laboratory to allow plating of samples a few hours after collection; 2) to surface-inoculate plates, mainly because the counting and isolation of individual colonies would be facilitated, but also because results of Shewan and Hayes (pers. com.) indicated that exposure to a plating temperature of 42°C. has a deleterious effect on specific physiological types, particularly on the genus Flavobacterium.

2.2. Materials and Methods.

2.2.1. Collection of water samples.

The sampling area throughout this investigation (Figure 6) was a station ten miles E.S.E. of Aberdeen (Lat. 57°6'15"N, Long. 1°44'15"W). The sea bottom at this locality is at a depth of 120 metres and is of a sandy nature. The salinity of the sea water at the sampling area is 34·7‰. The water temperature varied only slightly throughout this investigation - from a minimum of 8°C. during the October, 1961 sampling trips to a maximum of 11°C. at the June, 1960 sampling trip. (This information was kindly supplied by Dr. B.B. Rae and Mr. R.E. Craig of the Department of Agriculture and Fisheries for Scotland, Aberdeen). No figures were available for the organic matter content of the water at this area.

Water was collected by means of a J-Z water-sampling device and a glass container as described by ZoBell (1941a).

Sea water samples were obtained on the following dates: Samples A and B, 25th May, 1960; samples C and D, 14th December, 1960; samplesE, 2nd October, 1961; samples F and G, 3rd October, 1961; samples H and J, 5th October, 1961. On each trip two samples of roughly 100 ml. each, were collected at a depth of 10 metres within a few minutes of each other. Immediately upon the withdrawal of the sample the broken capillary tube was sealed and the bottle buried in crushed ice, where it remained until the plating procedure was commenced three to six hours later.

2.2.2. Preparation of isolation media.

"Aged" sea water was filtered through Green's No. 904

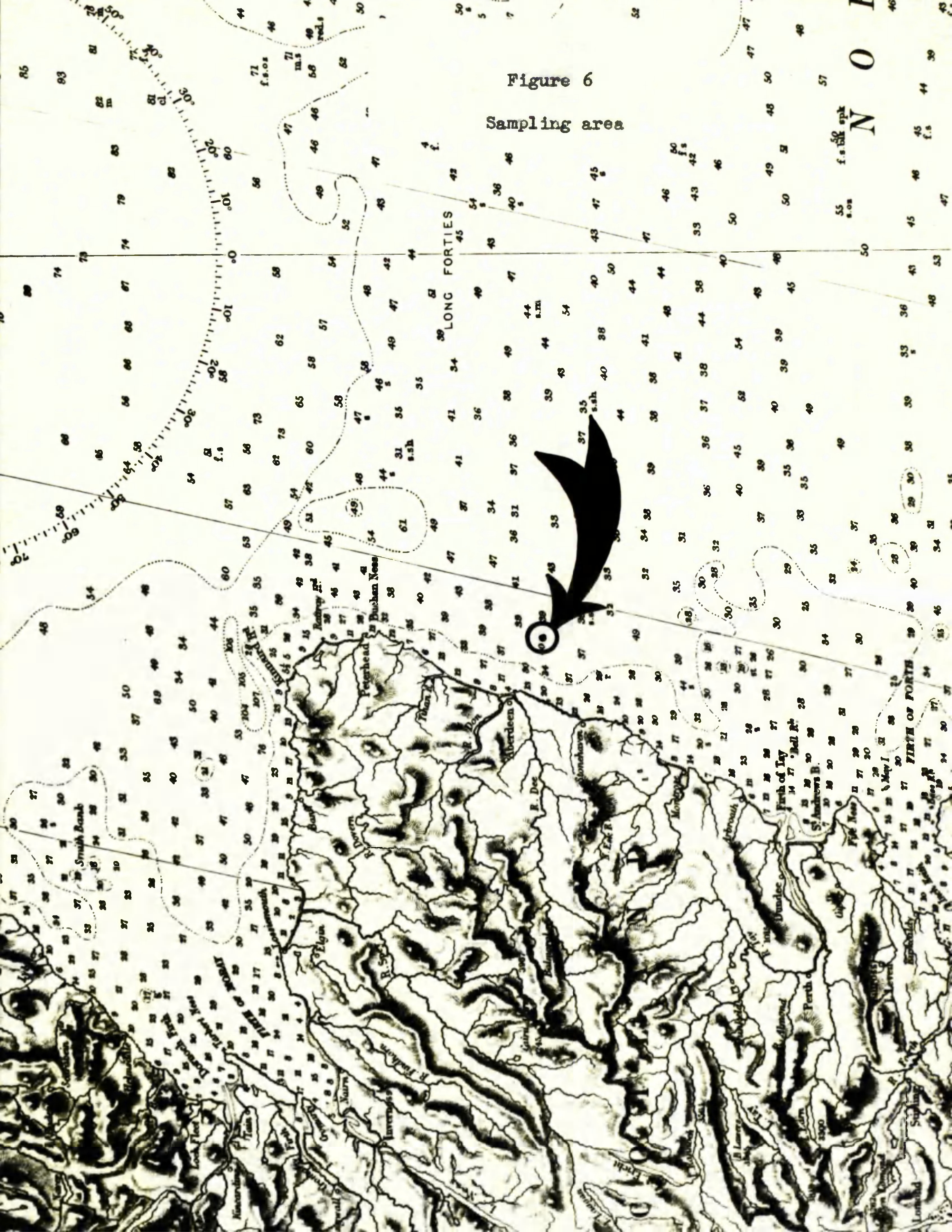


Figure 6

Sampling area

filter-paper to remove any particulate matter, and then diluted (v./v.) with distilled water in the proportion, three parts sea water to one part distilled water. The required quantity of agar was added and dissolved by steaming, whereupon all of the nutrient constituents were added and dissolved by thorough shaking.

15 ml. amounts of the medium were then dispensed into 25 ml. screw-cap containers using an automatic 15 ml. ejector pipette. In this form media were sterilised by steaming for 20 to 30 minutes on three consecutive days.

Throughout this investigation "aged" sea water was used in the preparation of media. This was sea water which had been stored for at least three months in polythene containers at room temperature. It was found that sea water which had been stored for less than three months, when incorporated in media, resulted in unpredictable pH changes during sterilization. This was not the case with sea water "aged" for more than three months, when the pH of the media used was 7·4 to 7·6 after sterilization.

2.2.3. Composition of isolation media.

ZoBell's medium 2216 (ZoBell, 1941) was used as the standard medium throughout the investigation. It was slightly modified from the original, in that 75 per cent "aged" sea water was used. This medium will be referred to as medium 1.

Composition of medium 1:- 5·0 gm. Difco peptone, 0·1 gm. ferric phosphate, 15 gm. Difco agar, 750 ml. "aged" sea water, 250 ml. distilled water. pH after sterilization 7·4 to 7·6.

Medium 2 was very similar to medium 1, the only alteration in composition being the reduction of peptone content to 2·5 gm. per litre.

Medium 3 had the same constituents as medium 2 with the addition of 1 gm. of glucose per litre.

The work of ZoBell (1941) indicates that media of similar composition to media 2 and 3 could be expected to give plate counts comparable with medium 1. They were included in this series to investigate possible qualitative differences in the flora isolated, since Holding (pers. com.) had suggested that with soil bacteria, some Gram positive species were inhibited by peptone concentrations above 0·25 per cent in the isolation medium. It seemed possible that organisms active in the dissimilation of glucose might be encouraged by the inclusion of this sugar in medium 3.

Medium 4. Here, peptone and ferric phosphate were omitted completely and replaced by yeast extract and sodium glycero-phosphate. It had the following composition: 2·5 gm. Difco yeast extract, 2 gm. sodium glycero-phosphate, 15 gm. Difco agar, 750 ml., "aged" sea water, 250 ml. distilled water. pH after sterilization 7·4 to 7·6.

Medium 4 was designed with a view to estimating the value of yeast extract as a replacement for the peptone and iron deemed necessary by ZoBell (1941) for the isolation of heterotrophic bacteria from sea water. It did not seem desirable to omit phosphate from this medium. Experiments in the preparation of medium 4 showed that inorganic phosphates resulted in heavy precipitation when added to sea water while sodium glycero-phosphate caused little precipitation.

These four media were used for the analyses of the first two water samples of the series, in June 1960. For subsequent samples the following media were also used.

Medium 5 was basically the same as medium 2 with the addition of 2·5 gm. of yeast extract per litre.

Medium 6 was the medium described by Georgala (1957), made up with 75 per cent sea water.

Medium 7 was basically the same as medium 4 with the addition of 2·5 gm. peptone per litre.

Medium 6 cannot be related to any of the other six media. This medium was included in the investigation in an attempt to estimate the value, in the microbial analysis of sea water, of using a medium developed with a view to isolating the microbial flora of marine fish.

The composition of all these media used is presented in figure 7.

2.2.4. Preparation of plates.

A few days before the collection of a water sample nutrient agar plates were poured - each Petri dish receiving exactly 15 ml. of medium. The poured plates were then stored at 3-5°C. until a few hours before the water samples were due to arrive in the laboratory when they were removed and placed in an incubator at 50°C. for two to three hours with the lid of each plate slightly removed. This process served to dry the plates sufficiently to allow 1·0 ml. of sea water to be absorbed within half an hour of being spread over the surface.

Figure 7.

The composition of media used for the isolation of heterotrophic bacteria from North Sea Water.

Medium Constituents	Medium Composition in Grams per litre of 75% sea water						
	1	2	3	4	5	6	7
Peptone	5•0	2•5	2•5	-	2•5	10•0	2•5
Yeast Extract	-	-	-	2•5	2•5	-	2•5
Meat Extract	-	-	-	-	-	5	-
Glucose	-	-	1•0	-	-	-	-
Fe Po_4	0•1	0•1	0•1	-	0•1	-	-
Sodium glycero-phosphate	-	-	-	2•0	-	-	2•0
Agar	15	15	15	15	15	15	15

2.2.5. Inoculation of sea water samples.

The first two samples of the series were plated on media 1, 2, 3, and 4, and thereafter all seven isolation media were compared.

The following procedure was adopted.

1) A uv sterilized inoculation room was used.

2) Throughout all of the plating procedure one control plate, representing each of the media being compared, was exposed to the atmosphere of the inoculation room as a check against contamination.

3) Bacteriological analyses were conducted concurrently on two separate water samples collected at the same time, from as near to the same position as possible.

4) Before inoculation each water sample was shaken by hand for five minutes.

5) Ten plates of each medium were inoculated from each water sample.

6) All of the plates to be inoculated with one sample were suitably labelled and thoroughly mixed so that there was no routine order of inoculation. This was done to randomize the effect of any change in the bacterial flora of the water which might take place during the period of plating.

7) From the first two water samples, 1·0 ml. aliquots of sea water were spread over the surface of the agar, and for all subsequent samples, 0·5 ml. aliquots were used. Spreading was accomplished by carefully rotating the plate until all the surface was wet.

8) After the inoculation was completed all plates, including the control plates, were incubated at 20°C. for fourteen days.

Figure 8.

The average microbial counts per 1·0 ml., and the standard deviations, of two samples of North Sea Water, when plated on four different media.

Date of Collection	Sample		Medium 1	Medium 2	Medium 3	Medium 4
25/5/60.	A	$\bar{x}$	111·1	185·4	175·8	132·9
		s	28·95	48·66	25·28	55·24
	B	$\bar{x}$	125·1	198·2	165·1	185·8
		s	15·57	57·44	51·74	57·60

$\bar{x}$ = mean value of plate count from ten replicate plates.

s = standard deviation.

2.2.6. Counting of Colonies.

The control plates produced an average of less than one contaminant per plate throughout the series. When it is considered that each control plate was opened for the same time as the isolation plates in the drying incubator, and roughly seventy times longer in the inoculation room, then aerial contamination could have no significant effect on the quantitative analyses of the water samples.

Colonies on plates were counted by naked-eye against an illuminated ground-glass screen.

Occasionally it was not possible to estimate the number of bacterial colonies on an isolation plate because of the presence of spreading or swarming species.

2.3. Results.

Groups of ten replicate plates of media 1, 2, 3 and 4 were surface inoculated with 1·0 ml. aliquots from water sample A and from water sample B. The average numbers of bacterial colonies which developed after fourteen days incubation at 20°C., and the standard deviation of each group of ten replicate plates from their mean value, are presented in figure 8. (A full list of plate count values is presented in Appendix 1.)

From these mean values and standard deviations, the significance of the differences of the plate counts obtained using these four media was calculated by the following formula:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\frac{s_1}{N_1} + \frac{s_2}{N_2}}$$

where, $\bar{x}_1$ and $\bar{x}_2$ are the mean values of two sets of plate counts from one water sample, s_1 and s_2 are the corresponding standard deviations and N_1 and N_2 represent the number of replicate plate counts from which the mean values are calculated.

In entering the statistical distribution tables of t, to obtain the values of P (probability), the degrees of freedom (DF) were calculated from the expression, $DF = N_1 + N_2 - 2$.

Plate counts were accepted as having a real difference in value when P = <0·05, i.e. a 5 per cent level of significance was used.

An example is presented to demonstrate the calculation involved. - With sample A are the counts obtained from medium 2 significantly higher than the counts obtained from medium 1?

$$t = \frac{185 \cdot 4 - 111 \cdot 1}{\frac{48 \cdot 66}{10} + \frac{28 \cdot 95}{10}}$$

$$= 9 \cdot 55$$

$$DF = 10 + 10 - 2 \quad = 18$$

$$\text{then} \quad P = < 0 \cdot 001.$$

Therefore the larger count on medium 2 cannot be ascribed to chance.

Statistical analysis, using the figures given in figure 8 demonstrated that media 2, 3 and 4 provided significantly higher counts than medium 1. The striking difference in value of medium 4 between samples A and B is inexplicable. However,

medium 4 was in both cases superior to medium 1.

The fact that media 2, 3 and 4 produced higher bacterial counts than medium 1 (ZoBell's medium 2216) which has been used so widely by marine bacteriologists for the isolation of bacteria from sea water, seemed to warrant a fuller inquiry into the use of various media for the enumeration of marine heterotrophs. Since the results of Jones (1957) and Carlucci and Pramer (1957) had indicated that yeast extract was stimulatory to marine bacteria, and the analyses of samples A and B had shown that the yeast-extract-containing medium 4 gave higher counts than ZoBell's medium 2216, it was decided to incorporate two more yeast-extract-containing media in the survey - media 5 and 7.

With samples A and B there were often around two hundred colonies per plate and it seemed desirable, for the sake of accuracy, to reduce this number for subsequent samples. Since dilution of the sea water would have introduced another variable factor it was decided to reduce the volume of sea water spread over the surface of each plate to 0·5 ml. As can be seen from the full list of plate counts presented in the appendix, on no occasion were there more than eighty-six colonies per plate from samples C to J, and the accurate counting of bacterial colonies was greatly facilitated.

The results obtained when all seven media under investigation were compared using seven different water samples (C to J), are presented in figure 9.

Figure 9.

The average microbial counts per 0•5 ml., and the standard deviations, of seven samples of North Sea water when plated on seven different media.

Date of Collection	Sample		Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6	Medium 7
14/12/60	C	$\bar{x}$	20•2	19•5	12•7	25•2	35•9	9•6	54•9
		s	8•50	4•72	14•20	8•98	11•30	4•57	1•47
	D	$\bar{x}$	56•6	31•5	31•2	37•4	40•8	24•4	37•2
		s	15•80	7•92	7•89	8•59	7•66	6•28	7•87
2/10/61	E	$\bar{x}$	19•4	13•8	14•5	16•6	29•3	6•1	32•7
		s	6•58	5•07	2•56	2•61	4•60	1•97	5•22
3/10/61	F	$\bar{x}$	21•8	25•5	39•8	19•8	55•0	16•5	48•3
		s	7•24	13•80	10•58	4•42	6•18	5•09	7•64
	G	$\bar{x}$	23•3	27•4	19•6	26•9	49•1	5•8	47•7
		s	11•34	15•80	7•56	5•99	6•9	4•07	12•1
5/10/61	H	$\bar{x}$	60•0	40•6	68•0	60•2	75•6	59•3	60•0
		s	9•65	12•51	11•76	10•75	6•12	7•95	12•7
	J	$\bar{x}$	15•0	12•5	9•9	8•4	20•1	5•6	15•6
		s	5•25	3•8	4•46	0•94	4•05	2•8	2•74

$\bar{x}$ = mean value of plate count from ten replicate plates

s = standard deviation

Statistical analysis of the figures given in figure 9 indicate that the highest bacterial counts were consistently obtained from media 5 and 7, while by far the lowest counts were obtained from medium 6. The relative merits of the other media varied from sample to sample, and there do not exist such clear cut relationships as are found between media 5, 7 and 6. However, media 1 and 4 often produced similar plate counts, somewhat lower than those of media 5 and 7, while media 2 and 3 usually gave lower counts than 1 and 4. Media 2 and 3 were able to support the growth of many more bacteria from the sea water than medium 6.

The relative merits of all media used, with respect to the production of maximum bacterial counts from water samples C to J are indicated in figure 10.

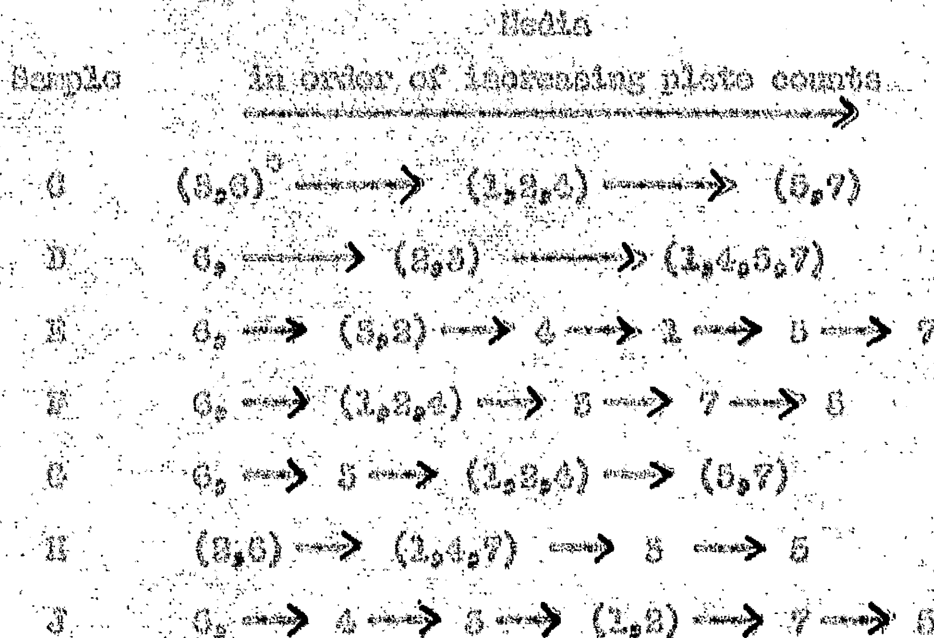
If the bacterial counts obtained on medium 1 are taken as a standard, equivalent to one hundred, and all other plate counts are expressed as a percentage of this arbitrary standard, the magnitude of the differences in plate counts among the seven media can be more easily assessed (figure 11).

2.4. Discussion.

The advantage of incorporating yeast extract in media for the enumeration of bacteria in North Sea water has been demonstrated. When compared with ZoBell's medium 2216 the colonial counts showed, with medium 5 an average increase of 161 per cent (range, 114 to 251 per cent) and with medium 7 an average increase of 149 per cent (range, 100 to 218 per cent) (figure 11). These results are

Figure 10.

The relationships of all media with respect to bacterial plate counts, as indicated by seven different water samples.



* Figures enclosed in brackets indicate that there was no statistically significant difference in bacterial counts on the corresponding media.

→ Indicates statistically significant difference in count.

Figure 11.

The influence of the composition of nutrient media on plate counts of bacteria from nine different samples of sea water (all plate counts being expressed as % of medium 1).

Media	WATER SAMPLES A	B	C	D	E	F	G	H	J	Average	Range
1	100	100	100	100	100	100	100	100	100	100	-
2	105	142	95	84	72	118	93	67	95	103·8	67-165
3	156	150	65	84	68	185	70	115	76	104·8	65-185
4	119	148	125	100	89	91	96	100	64	103·6	64-148
5	-	-	180	114	153	251	175	122	154	161·3	114-251
6	-	-	50	65	52	76	20	64	28	47·9	20-76
7	-	-	175	103	168	218	172	100	108	149·1	100-218

of a similar order to those reported by Carlucci and Pramer (1957) who obtained a 125·5 per cent increase in counts when ZoBell's medium 2216 was supplemented with 0·01 per cent yeast extract. These authors made this assessment from one water sample and eight replicate plates.

Why yeast extract should have such a stimulatory effect is uncertain, though it seems possible that when cultivated in the laboratory some marine bacteria may have a requirement for growth factors in excess of the concentration found in natural sea water. MacLeod and his associates (1954) have found some marine bacteria which had to be supplied with vitamins before they could grow, and it seems possible that the inclusion of yeast extract could permit the isolation of nutritionally fastidious organisms which would be unable to grow without a vitamin supplement. It is reasonable to assume that the beneficial effect is due to organic compounds in yeast extract and not to trace elements, since MacLeod and Onofrey (1956) noticed no stimulation of growth when yeast extract ash was added to culture media.

During this survey 0·25 per cent yeast extract was incorporated in the isolation media to ensure an excess of any growth factors present. This level of yeast extract is twenty-five times greater than that employed by Carlucci and Pramer (1957), and since these authors report from only one sample it is difficult to say whether this increase has been justified. However, the virtue of adding yeast extract to media for the isolation of heterotrophic bacteria from sea water has been conclusively demonstrated when the

results of Jones (1957) and Carlucci and Pramer (1957), and the present results are viewed collectively, though the optimum concentration has yet to be decided.

Medium 7 differs from medium 5 only in that sodium glycero-phosphate replaces ferric phosphate. ZoBell (1941b) found up to an 80 per cent increase in plate counts when traces of iron, as phosphate or citrate, were added to media whose only source of organic nitrogen was peptone. Since medium 5 produced, on an average over seven samples, a plate count increase over medium 7 of only 11 per cent when both are compared to medium 1 (figure 11) and statistical analyses showed that with just three samples out of seven were the counts on medium 5 significantly higher than on medium 7 (figure 10) it would appear that to a large degree yeast extract can supersede the requirement for an iron supplement. Further less direct evidence for this suggestion, can be obtained from a comparison of media 2 and 4, which on average over nine water samples produced comparable plate counts (figures 8, 9 and 10). In this case yeast extract has satisfactorily replaced peptone plus an iron supplement.

Reuszer (1933) incorporated glucose into his isolation media and ZoBell (1941b) found that the addition of glucose produced increased plate counts only when the concentration of peptone was low. In this investigation a comparison of media 2 and 3 shows that the addition of glucose had little or no effect on total plate counts (figures 8 and 9).

The desirability of including peptone in isolation media is borne out by comparison of media 4 and 7, though the concentration of peptone does not appear to be as important (compare media 1 and 2, figure 11) as indicated by ZoBell (1941b) who obtained plate counts 15 per cent lower with 0·25 per cent peptone than with 0·5 per cent peptone.

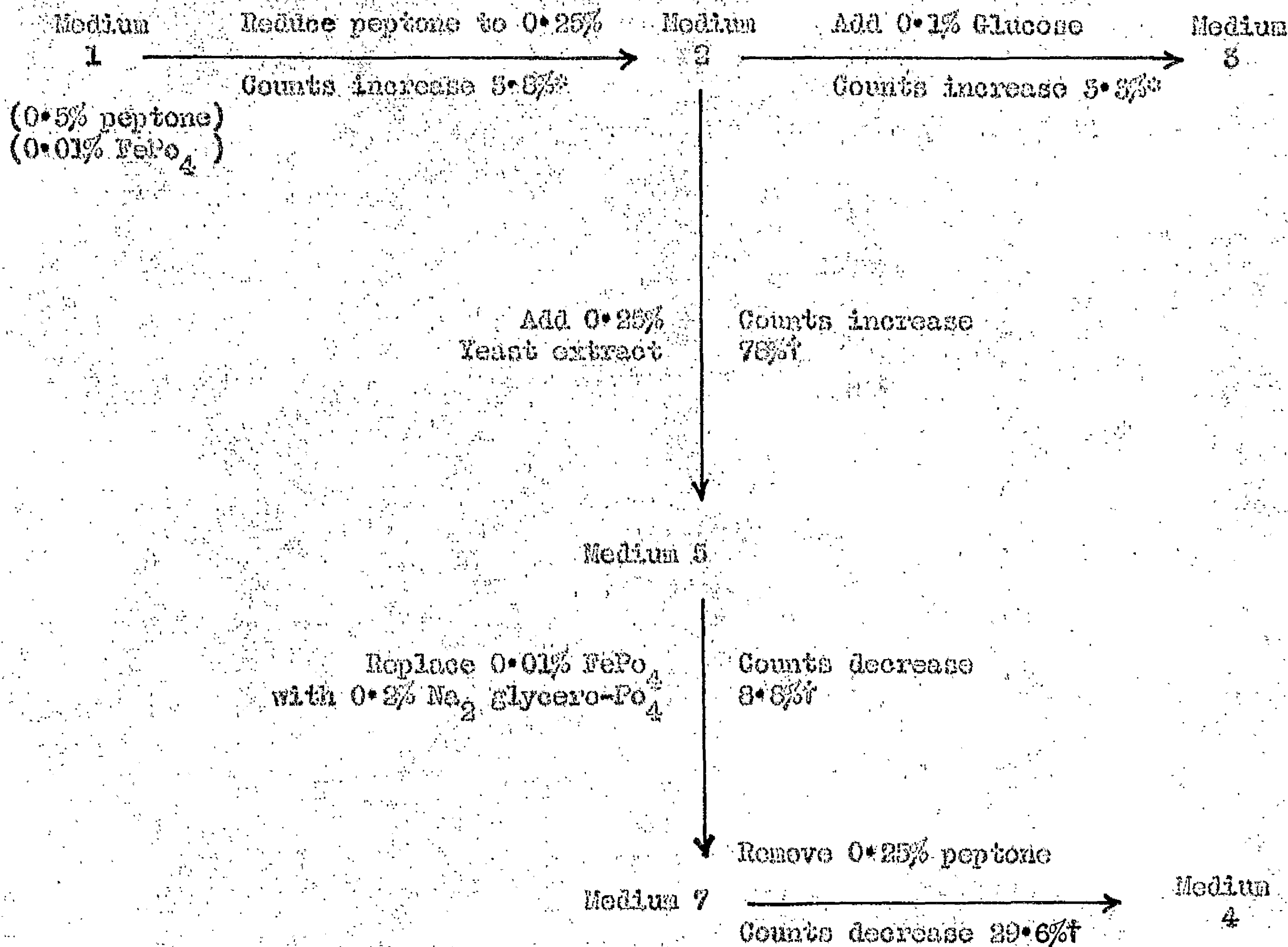
Medium 6, which was designed at Torry Research Station to isolate bacteria from the surface of fish, fared very badly throughout this series. This may reflect a basic difference in the nutritional requirements of bacteria from fish, and from the sea water itself. It would, however, be of interest to compare the merits of media 5 and 6 for the isolation of bacteria from the surface of marine fish.

The relationships existing among the media used during this investigation, (with the exception of medium 6 which is not directly comparable) and the effect on plate counts, of changing the composition, is presented in figure 12. The percentage values given in this figure are different from those presented in figure 11, where media were compared only with reference to medium 1. An examination of figure 12 will show the extent of the influence on plate counts of altering medium composition with respect to a single constituent. For example, the addition of 0·25 per cent yeast extract to medium 2 results in a plate count increase of 78 per cent.

It was discovered after the formulation of the media used during this investigation that media 5 and 7 were very similar in composition to that found to be best for the isolation of bacteria from soil, by Topping (1937). Her medium contained 0·25 per

Figure 12.

The effect on plate counts of changing medium composition.



† = these figures have been calculated from the sum of the average plate counts for each medium from water samples C to J. (see fig. 9).

* = Calculated as above from samples A to J (see figs. 8 and 9).

cent yeast extract and 0·25 per cent peptone, in tap water. This would suggest a broad similarity between the nutritional requirements of heterotrophic bacteria in the water of the North Sea and in the soil of the East of Scotland and Saxony, from which Topping isolated her cultures.

A notable fact which emerges from this survey is the inconsistency of relative merit among the media employed, from sample to sample (figure 10). The assessment of the value of any medium, for the isolation of bacteria from sea water, should involve the analyses of many difference water samples, using, if possible, ten replicate plates for each medium.

2.5. <u>Conclusions.</u>

1) The observation of Jones (1957) that yeast extract is stimulatory to marine bacteria, has been confirmed for aerobic heterotrophs from North Sea water.

2) A medium containing 0·25 per cent yeast extract, 0·25 per cent peptone and 0·01 per cent ferric phosphate, in 75 per cent sea water was best for producing maximum and reproducible plate counts.

3) A medium intended for the isolation of bacteria from fish, was inferior when compared with media designed to isolate bacteria from the sea water.

4) The relativelmerits of different isolation media varied widely from one water sample to another.

SECTION 5.

THE CHARACTERISATION OF HETEROTROPHIC BACTERIA ISOLATED FROM NORTH SEA WATER.

3. THE CHARACTERISATION OF HETEROTROPHIC BACTERIA ISOLATED FROM NORTH SEA WATER.

3.1. Introduction.

An estimate of bacterial numbers occurring in sea water is of little value unless the physiology of the bacterial population is understood. Until such information has been accumulated, figures indicating the fluctuation of microbial numbers with environmental variation are but bare facts, the significance of which can only be apparent when they can be correlated with specific anabolic or katabolic activities. There is no shortage of information about the extent of the bacterial population of sea water. However, the marine biologist is very far from reaching a comprehensive understanding of the biological processes with which marine bacteria are involved. The recent researches of the Russian workers described by Kriss (1962) have been directed towards large scale inquiries into the characterisation of microbial activities throughout the world's oceans. ZoBell (1946), Wood (1953, 1958) and Brisou (1955), on the other hand, describe isolated marine bacteria in some detail directing most of their attention towards identifying or classifying their isolates. The scheme of work to be described in this section was designed primarily with a view to providing information about the physiology of the bacteria isolated from North Sea water. Nevertheless, it was possible, as will be demonstrated when the results are considered, to designate most of the isolates to conventional genera.

Over and above the estimation of some of the activities of bacteria from North Sea water the design of experiments described in section 2 has enabled physiological types isolated on different media, and from different water samples, to be compared.

3.2. Materials and Methods.

3.2.1. Introductory comments.

A basic concept, accepted from the outset of this work, has been that the standardisation of procedure was of primary importance. By this is meant, that all isolates were treated in the same fashion and all biochemical tests (with a few deliberate exceptions which will be described) were carried out using the same basal growth medium supplemented with specific nutrients. In order that these requirements might be fulfilled it was necessary to evolve a scheme by which all isolates could be characterised with maximum convenience. This latter point, "maximum convenience", is of great importance when a hundred or more isolates are being handled. Not only had the overall scheme to be compiled, but suitable media for the various biochemical tests had to be designed, or adapted from other sources.

The idea for such a scheme came basically from the determinative scheme in use at Torry Research Station (Shewan *et al.*, 1960a,b), and although different in many respects, it retains some of the determinative tests which enabled Shewan and his associates to group bacteria, isolated from marine fish, into different genera. Rejected from the scheme of Shewan *et al.*, have been some tests designed to group bacteria into genera while failing to add to

our knowledge of what activities these bacteria might have in their natural environment. For example, antibiotic sensitivity was omitted, as was sensitivity to 2:4-diamino-6:7-di-isopropylpteridine (0/129) (Shewan *et al.*, 1954).

It must be stressed at this stage that the rejection of tests which might help to classify marine bacteria, ought not to be taken to imply that classification is considered unimportant in its own right in a study of marine bacteria, but that these more "artificial" tests (e.g. sensitivity to antibiotics) lacked priority in a scheme which has part of its value in simplicity.

Since the scheme used during this investigation is new, and the reasons behind its formulation in this manner are important when the results are considered, it will be described in detail in this section. The scheme is presented in tabular form in figure 15.

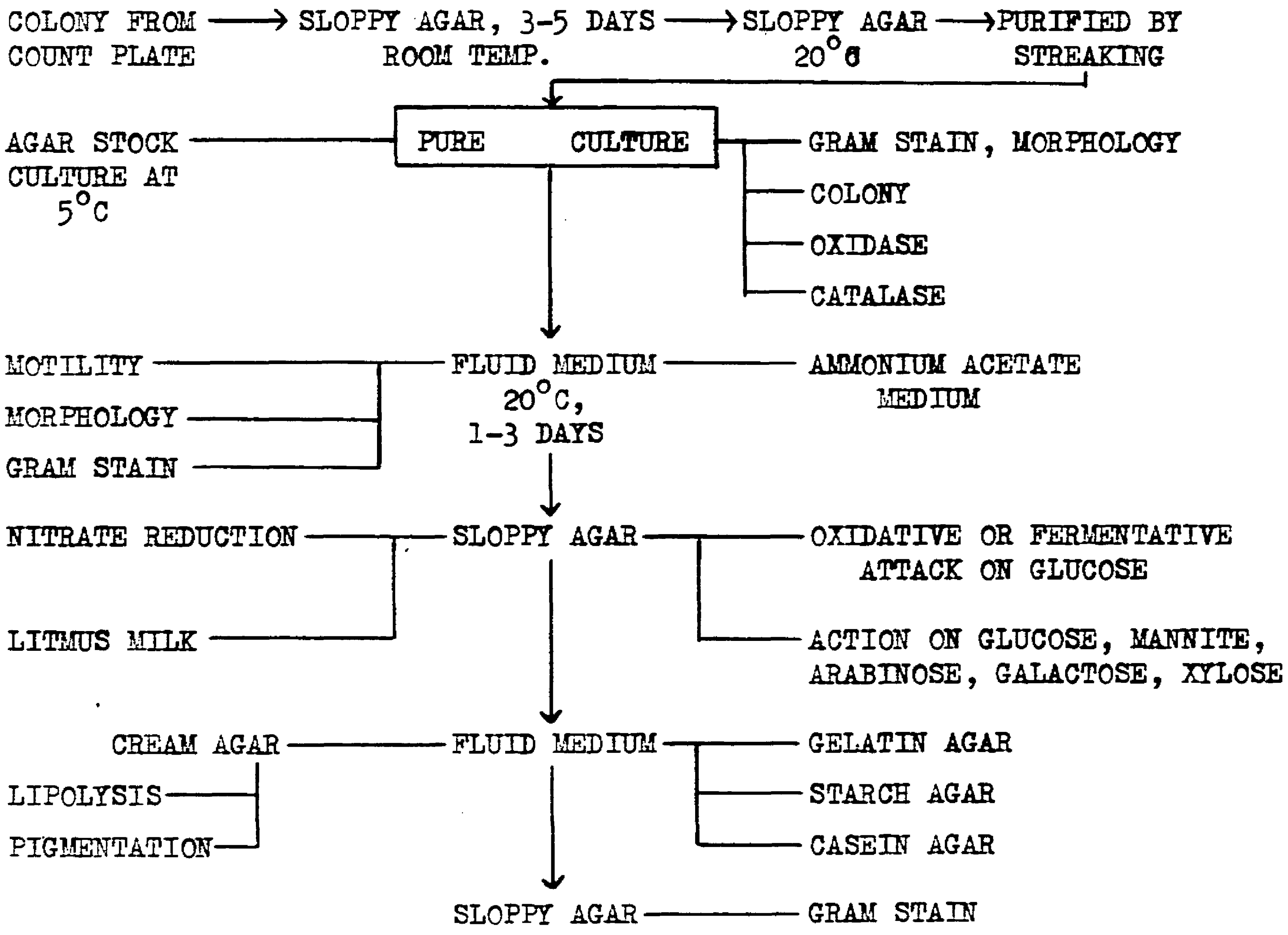
5.2.2. The scheme employed for the characterisation of aerobic heterotrophic bacteria from North Sea water.

1) The selection of colonies from count plates and the inoculation of a sloppy agar medium.

If any significance is to be attached to a variation in numbers of physiological types from one sample to another, or from one medium to another, then it is important that isolates for further study are selected completely at random. Liston (1955) proposed to describe population variation of the heterotrophic bacterial flora of skate and lemon sole caught in the North Sea, but he admitted

Figure 13

SCHEME FOR THE CHARACTERISATION OF HETEROTROPHIC BACTERIA FROM NORTH SEA WATER



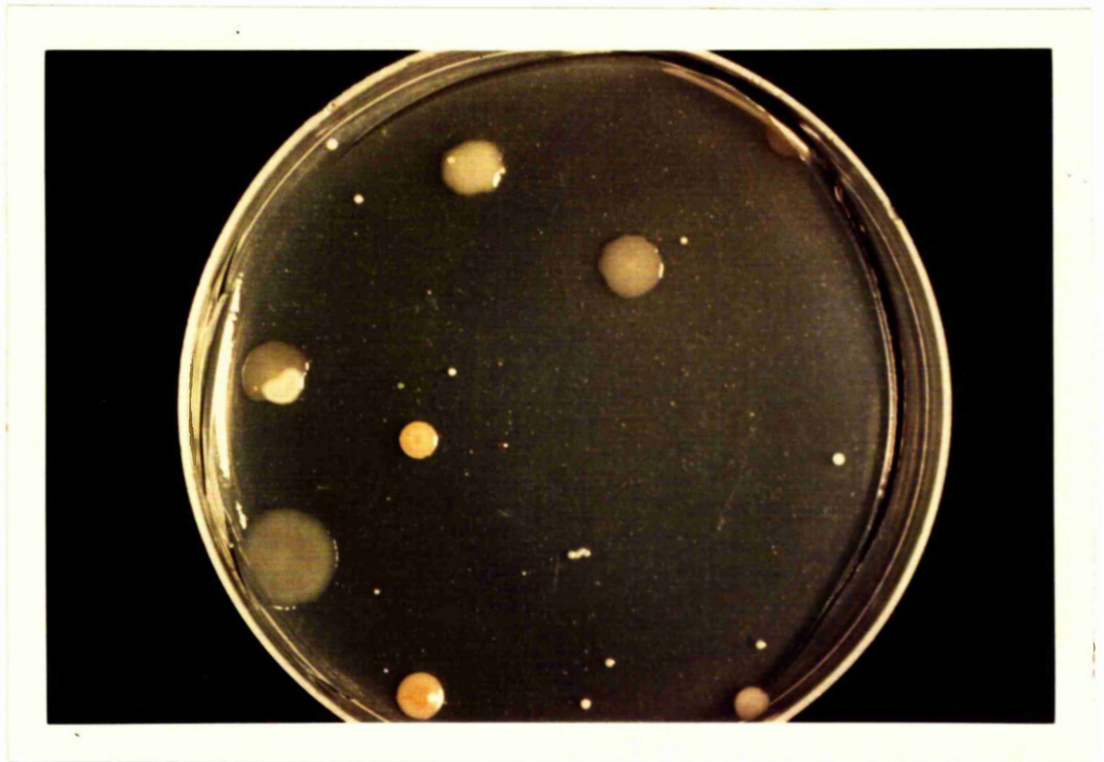
that when selecting colonies from his count plates he chose those having different appearances. Such a procedure can hardly be called random selection. It is appreciated, however, that when examining a count plate, brightly coloured colonies are eye-catching and it is difficult to reject them. To overcome this difficulty, one or two plates from each group of media were chosen because the colonies were well distributed over the surface, and then every colony was picked off from these plates for further study. Figure 14 shows a typical appearance of a count plate from which colonies were picked. With sample A, isolates from media 1 to 4 were investigated, while with samples C and G, isolates from media 1 to 6 were investigated. Between fifty and fifty-five colonies were picked from each medium. Where to pick all of the colonies from one or two plates would mean many more than fifty isolates, a plate was arbitrarily divided into sections and every colony within a section was picked off.

At the beginning of this investigation it became apparent that very many marine bacteria grew only slightly on ZoBell's medium 2216 (ZoBell, 1941b), after repeated sub-culture, and from sample A, twenty four per cent of the original isolates grown after the first sub-culture, were lost after several transfers through this medium.

On a few occasions when a mould contaminated a culture plate there was a marked stimulation of growth of the marine bacteria in its vicinity. This prompted the supplementing of ZoBell's medium 2216 with growth factors, including yeast extract. To a basal medium of 0·25 per cent peptone, 1·5 per cent agar and 0·01 per cent ferric phosphate, in 75 per cent "aged" sea water,

Figure 14.

Typical count-plate from which single colonies were chosen for detailed study.



yeast extract was added at various concentrations between 0·01 and 5·0 per cent, and agar plates of these various media were inoculated with a variety of marine bacteria, all of which were then stimulated to produce larger and more intensely pigmented colonies. The optimum level for growth stimulation, as measured by colonial size, was between 0·25 and 0·5 per cent yeast extract.

From these results a medium containing 0·25 per cent Difco peptone, 0·25 per cent Difco yeast extract and 0·01 per cent ferric phosphate, in 75 per cent "aged" sea water, and of pH 7·5, was chosen as the basal medium to be used throughout the scheme. It will be referred to as BM. It is coincidental that BM and isolation medium 5 have the same composition.

BM was usually prepared in 5 or 10 litre amounts, dispensed in 500 ml. screw-cap bottles and sterilised at 15 lbs. per square inch for 15 minutes. Before use, this medium was filtered through Whatman's No. 1 filter-paper, any further constituents added and the pH adjusted when necessary. When only agar was added to BM, sterilization was always at 15 lbs. per square inch for 15 minutes, but when any other nutritional constituent was added, the media were sterilised by steaming for 20 to 30 minutes on three consecutive days.

With water sample A, colonies selected for future study were sub-cultured on to agar slopes of ZoBell's medium 2216, and as just described many strains were lost after a few sub-cultures. With samples C and G, colonies were picked into BM plus 0·25 per cent agar in $4 \times \frac{1}{2}''$ test-tubes. In all cases, incubation at this stage was at

room temperature for three to five days, during which time all cultures were transported from Aberdeen to Glasgow.

This use of sloppy agar for the primary culturing of isolates from count plates was recommended by Miss Vera Collins who had found this technique stimulatory to the growth of fresh water bacteria. The use of yeast extract and the passage through a sloppy agar medium, reduced the loss of cultures from twenty-four per cent from sample A to twelve per cent from samples C and G.

It should be pointed out that this percentage loss was calculated from the number of strains which grew after the first sub-culture from the count plates. Approximately eight hundred and fifty colonies were picked off from the count plates throughout the investigation, but only seven hundred and eighty-three of these were viable in the first culture medium.

Among the isolates lost during sub-culture were a few _Chromobacterium_ strains identified by the intensive purple pigment produced. So although a few of these bacteria were isolated they do not feature in the final analyses of results.

2) Transfer of culture into second tube of sloppy agar.

This step was merely to ensure that all cultures were growing well, before the purification procedure was commenced. Cultures were incubated at 20°C. and examined daily until visible growth appeared.

3,4) Purification.

When visible growth was apparent in the BM sloppy agar (2), cultures were streaked to obtain isolated colonies on BM plus 1·5 per cent Difco agar. This procedure was continued until

a pure culture was obtained.

5) Stock culture.

A single colony was picked from each plate having a pure culture and inoculated on to the surface of a BM agar slope in a 6 x 5/8" test-tube. After incubation at 20°C., until good growth was apparent, this culture was stored at 5°C. Stock cultures were transferred every three months.

6) Fluid culture.

Before any further tests were carried out on the plate culture, a single colony was picked into BM and incubated at 20°C. for one to three days.

7) Gram stain.

Single colonies from the purification plates were picked and stained by Gram's method (Kopeloff and Cohen, 1928). From microscopic examination of this stain, the Gram reaction of the culture and the cell morphology were recorded.

8) Colonial appearance.

Individual colonies were examined under a plate microscope and characteristics recorded.

9) Oxidase test.

The method used for this test was an adaption of that described by Kovacs (1956). A strip of Whatman's No. 1 filter-paper was impregnated with a 1 per cent solution of tetramethylparaphenylenediami dihydrochloride and then laid on the surface of colonies on the purification plate. After a few seconds the paper was removed and dependent on the nature of the colonies they either remained adhered to the agar surface or they were removed with the

moist filter-paper. In both cases a positive oxidase reaction was reflected by the colonies turning dark purple in 5 to 15 seconds.

10) Catalase test.

This test was carried out by allowing a few drops of a 10 per cent solution of 10 vol. hydrogen peroxide to fall on the surface of colonies on the purification plate. Uninoculated plates were used for control purposes.

11) Growth in ammonium acetate medium.

Since all of the cultures isolated during this investigation were grown on media containing complex sources of nitrogen and growth factors, it was of interest to determine what proportion were able to grow in a much simpler medium. The medium designed for this purpose supplied nitrogen in only two forms - as nitrate and as ammonium acetate. It had the following composition: glucose, 5·0 gm.; sodium citrate, 1·0 gm.; sodium succinate, 1·0 gm.; sodium gluconate, 1·0 gm.; ammonium acetate, 1·0 gm.; potassium nitrate, 1·0 gm.; "aged" sea water, 750 ml.; distilled water, 250 ml. The solids were dissolved in the 75 per cent sea water and the medium was filtered through Whatman's No. 1 filter-paper. The pH of the medium was adjusted to 7·5 and it was dispensed in 5 ml. amounts in $4 \times \frac{1}{2}$" test-tubes. Sterilization was by steaming for 20 to 30 minutes on three consecutive days.

As soon as visible growth appeared in the BM fluid culture (6) the ammonium acetate medium was inoculated from it, using a straight platinum wire. These cultures were incubated at 20°C., for up to two weeks and the appearance of turbidity was taken

to indicate the ability of an organism to utilise ammonium acetate or potassium nitrate as a source of nitrogen.

On a few occasions, when only a very slight turbidity developed after prolonged incubation, a loopful of this culture was transferred to a fresh tube of ammonium acetate medium and once again incubated for up to two weeks. If a slight turbidity appeared once again, the organism in question was recorded as having the ability to grow in this medium.

12) Motility and cell morphology.

From the BM culture (6), after incubation for one to three days at 20°C., each culture was examined under phase contrast for motility and cell morphology. It is important, when dealing with marine bacteria, to examine the cell morphology in the living unstained state since many strains become very distorted by osmotic pressures when subjected to the normal microbiological staining procedures.

13) Gram stain.

The reaction of each pure culture to Gram's staining method was carried out here from a fluid medium (6), as a check against the previous Gram stain of the culture grown on solid medium.

14) Sloppy agar culture.

After the fluid medium culture (6) had been used for steps 11, 12, and 13, a BM sloppy agar culture was grown, in preparation for the next series of tests.

15) Oxidative or fermentative attack on glucose.

Hugh and Leifson (1953) described a medium and method for differentiating the process of acid production by many bacteria from carbohydrates, into the oxidative method,

which requires oxygen, and the fermentative method, which can be carried out anaerobically. This test was designed to enable this distinction to be made by a simple routine easily applied to the screening of large numbers of cultures. It involves inoculating duplicate tubes of a semi-solid medium, one tube of which is left open to the atmosphere, while air is excluded from the other by covering the surface with a layer of liquid paraffin. Since the publication of Hugh and Leifson's (1953) method other workers have described different media which they suggest may be more sensitive for the detection of acid produced (Board and Holding, 1960; Véron et Chatelain, 1960).

It was of interest in this investigation to differentiate those organisms producing acid from glucose, into the oxidative or fermentative groups. Before a decision was made as to what medium would be adopted for this purpose, experiments were carried out to compare the merits of the media of Hugh and Leifson, and Board and Holding, and an adaption of the basal medium already described (1), with respect to acid production by a variety of marine bacteria. The final composition of the three media compared, is presented below.

a) The medium of Hugh and Leifson was kindly supplied by Difco Laboratories. It was dissolved in 75 per cent "aged" sea water by steaming, and 0·5 per cent glucose was added. At this stage the medium was filtered, under pressure, through Green's No. 904 filter-paper and a layer of powdered cellulose. It had the following final composition: peptone, 0·2 per cent; sodium chloride, 0·5 per cent; dipotassium hydrogen phosphate, 0·03 per cent; agar, 0·3 per cent; bromothymol blue, 0·003 per cent; glucose, 0·5 per

cent; 75 per cent "aged" sea water.

b) The medium of Board and Holding was slightly adapted from the original and had the following composition: ammonium dihydrogen phosphate, 0·05 per cent; dipotassium hydrogen phosphate, 0·05 per cent; Difco yeast extract, 0·05 per cent; glucose, 0·5 per cent; agar, 0·5 per cent; bromothymol blue, 0·002 per cent; 75 per cent "aged" sea water. After dissolving the constituents by steaming, this medium was filtered as (a) above.

c) The medium developed during this investigation, for the differentiation of oxidative and fermentative acid production from glucose, and compared with (a) and (b) above, consisted of BM plus 0·5 per cent glucose, 0·25 per cent Difco agar and 0·002 per cent bromothymol blue.

The pH of all three media just described, was adjusted to 7·1. The media were dispensed in roughly 10 ml. amounts in 6 x 5/8" test-tubes, and sterilised by steaming.

Duplicate tubes of each medium were stab-inoculated, using twenty-four different marine bacteria, and one tube of each pair was covered with a layer of sterile liquid paraffin. Incubation was at 20°C., and cultures were examined daily.

Analysis of the results obtained from this experiment, showed that all three media were comparable detectors of acid production if the organism was able to grow. However, both Hugh and Leifson's medium and Board and Holding's medium were unable to support the growth of many marine bacteria. These media were designed, primarily, to support the growth of non-pigmented

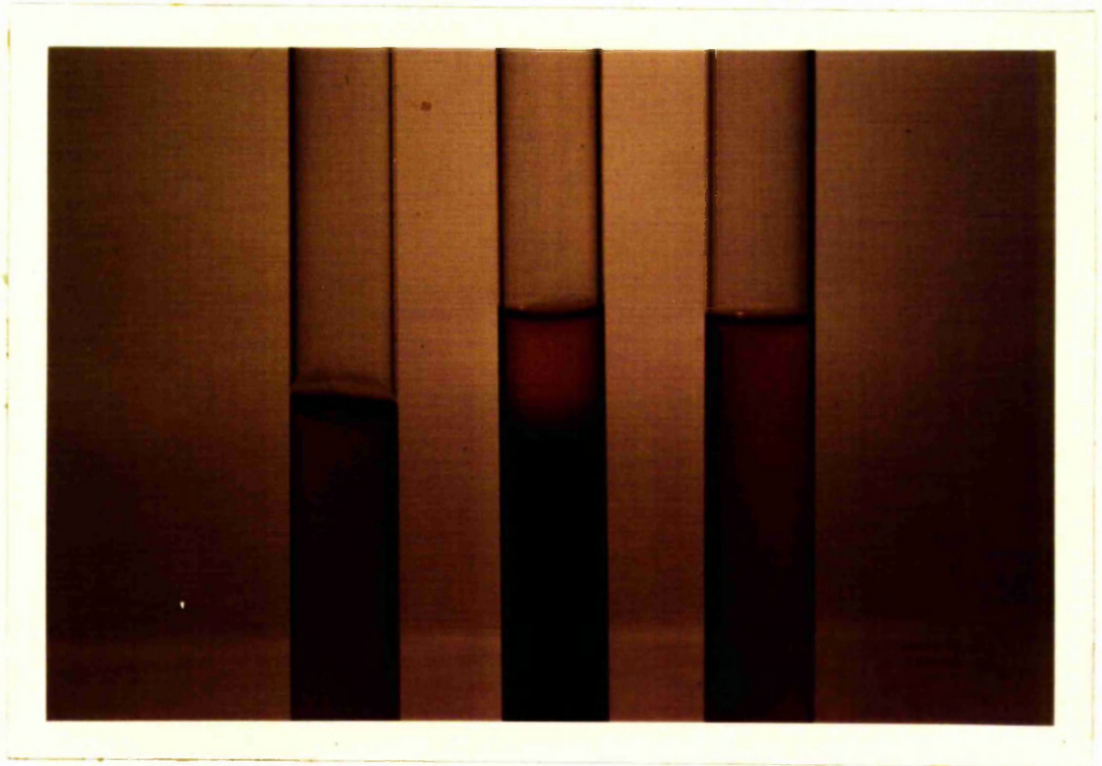
Gram negative organisms and it is probably significant that most of the strains failing to grow on these media were Gram positive, or Gram negative and pigmented. Figure 15 shows a typical result of this series of tests. The tubes on the left, containing the medium of Board and Holding, show no growth; the centre tubes contain the medium of Hugh and Leifson, and have slight growth and acid production, while the right-hand tubes, containing the medium developed during this work, have good growth and acid production is apparent throughout the medium. Somewhat surprisingly, a few cultures were unable to grow in this last medium. Since the only difference in composition between this medium and the standard medium on which all cultures were maintained, was an addition of glucose and bromothymol blue, one of these constituents had to be the inhibiting factor. Phenol red, at a concentration of 0·0015 per cent, was substituted in this medium for the bromothymol blue with the result that all isolates were able to grow.

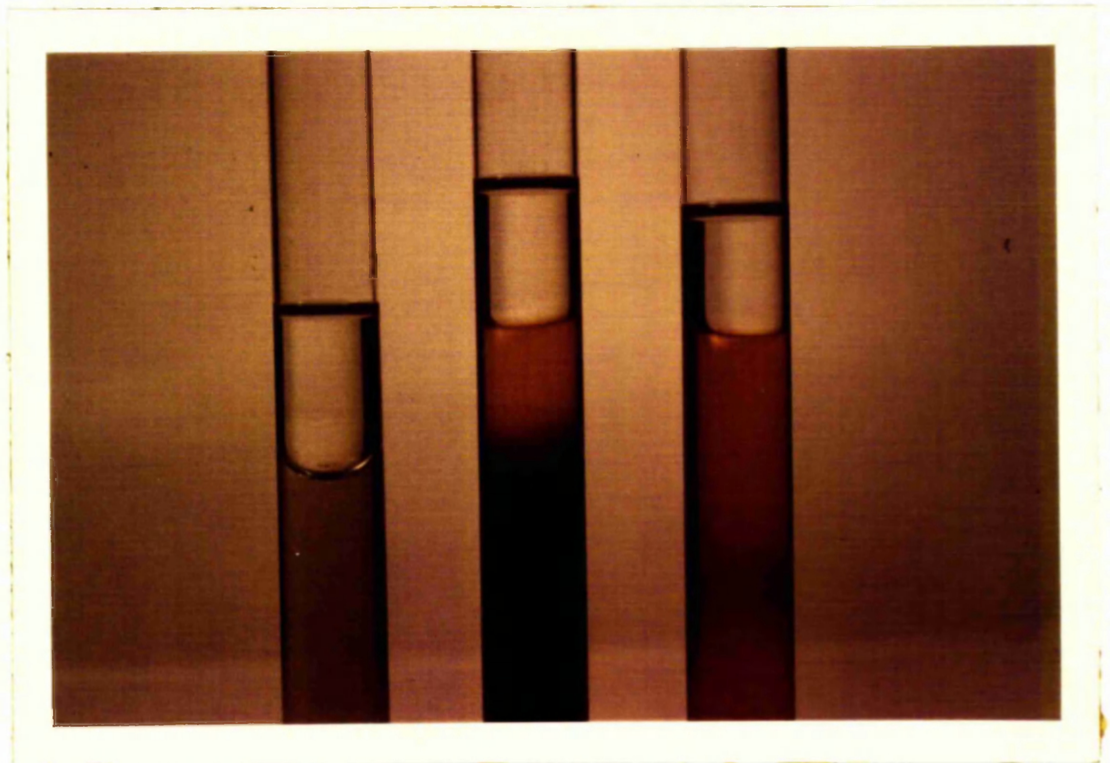
On a basis of the information gained from this preliminary investigation, a medium containing BM plus 0·5 per cent glucose, 0·25 per cent agar, 0·0015 per cent phenol red, and of pH 7·5, was adopted for the differentiation of oxidative and fermentative attack on glucose, throughout this survey.

It is realised that a medium containing 0·25 per cent yeast extract and 0·25 per cent peptone, will have a higher buffering capacity than the media of Hugh and Leifson and Board and Holding, and as such may not be as critical an indicator of acid production. However, it was judged to be advantageous to use one standard

Figure 15.

Comparison of three different media for the detection of oxidative and fermentative acid production (for full details see p. 62).





medium in which all isolates could grow well rather than to attempt to detect traces of acid produced by a few strains.

Although the tests described above were carried out in 6 x 5/8" test-tubes, during the actual survey, as an economy on medium and space, 5 ml. amounts were dispensed into 4 x ½" test-tubes.

Just before use, the tubes containing the medium were steamed to remove any dissolved oxygen and after cooling, were stab-inoculated, in duplicate, from a sloppy agar culture (14). After inoculation, one tube of each pair was covered with at least one inch of sterile liquid paraffin. All cultures were incubated at room temperature and examined daily until acid production was apparent or until they had been incubated for fourteen days, whereupon, if no acidity was obvious, the culture was recorded as not producing acid from glucose. Daily examination was necessary because many strains studied produced only transient acidity in the medium. Nevertheless, as stressed by Hugh and Leifson (1953), the incorporation of a small quantity of agar into sugar media, localises the acid production and therefore minimises the likelihood of acid being neutralised by other medium components.

If the pH of the medium became acid in only the open tube, the culture was designated as having an oxidative attack on glucose, while, if both the open and the sealed tubes became acid, the culture was designated as having a fermentative attack on glucose.

10) Action on a variety of sugars.

In the preparation of the media for these tests, mannite, maltose, arabinose, galactose and xylose, respectively, were

substituted for the glucose in the medium adopted for the differentiation of oxidative and fermentative attack on glucose (15). The medium was prepared in bulk with only the sugar omitted, sterilized at 15 lbs. per square inch and stored in 500 ml. screw-cap bottles. Before use, sugars were added to give a concentration of 0·5 per cent, the medium dispensed in 5 ml. amounts in $4 \times \frac{1}{2}$" test-tubes and sterilized by steaming.

Cultures were incubated aerobically in all of these tests and examined daily for the reasons already described (15). For these tests a note was kept of all strains producing transient acid in the different sugars.

Glucose and galactose were chosen to represent the monohexose sugars; glucose because of its key role in metabolic processes and galactose because it is the major constituent of agar which occurs extensively in marine algae. Mannite was included since it is a constituent of the brown algae, and xylose and arabinose to investigate the ability of marine bacteria to produce acid from pentose sugars. Maltose was included as a representative of the disaccharides but gave little extra information and should probably be omitted from further studies and as such has been omitted from figure 15.

17) Nitrate reduction.

To carry out this test 0·5 per cent Analar potassium nitrate was added to DM, the pH adjusted to 8·4, and the medium dispensed in 5·0 ml. amounts in $4 \times \frac{1}{2}$" test-tubes containing Durham tubes. Cultures were incubated in this medium at room temperature, for ten to fourteen days, before testing for nitrate reduction.

The production of nitrite was determined by adding, to each culture tube, a few drops of each of the following reagents (Manual of Microbiological Methods, 1957): 1) 8 gm. sulphanilic acid in 1 litre of 5N. acetic acid; 2) 6 ml. dimethyl-alpha-naphthylamine in 1 litre of 5N. acetic acid. Uninoculated controls of the medium used, gave a faint background pink colour after the addition of these two reagents. This was probably due to traces of nitrite in the Analar potassium nitrate. The production of nitrite by strains tested, was easily distinguished from this faint positive reaction given by the control tubes, by the deep red or pink colour which developed.

On a very few occasions, the addition of the reagents to the culture tube resulted in no colouration whatsoever, developing, indicating that all traces of nitrite had been removed. Since the addition of a little zinc dust to such tubes showed that nitrate was also absent, it was clear that the nitrite in the original medium had been reduced. It was thus possible to use this medium as a dual purpose test, i.e., if an intense red colour was produced nitrate had been reduced only to nitrite; if a faint pink colour, equivalent to the colour of an uninoculated control tube, developed, nitrate had not been reduced; while, if no colour at all developed, nitrate had been reduced past nitrite.

The ability of an organism to reduce nitrate to gaseous end-products, was detected by the inclusion of a Durham tube in each tube of medium.

18) Action in litmus milk.

This medium consisted of a 10 per cent solution of

skimmed milk powder in distilled water, with litmus indicator added. It was tubed in 5 ml. amounts in $4 \times \frac{1}{2}$" test-tubes, and sterilised by steaming. Only marine bacteria capable of growth in the absence of sea water would grow in this medium and as such it was of little general value. However, it was retained in this scheme because it enabled a group of micrococci to be distinguished. This will be discussed further in the appropriate section.

19) Fluid culture.

After tests 15, 16, 17 and 18 had been carried out each isolate was inoculated from a BM sloppy agar culture (14) into BM fluid culture for use in the next series of tests.

20) Gelatin hydrolysis.

The medium used for this test was adapted from Frazier (1926) and consisted of BM plus 1·5 per cent agar and 0·4 per cent gelatin. The pH was 7·5 and the medium was dispensed in 15 ml. amounts in screw-cap containers.

Cultures were spot-inoculated on the surface of this medium in agar plates, from a fluid culture (19), and after incubation at room temperature for three to five days, plates were flooded with a solution of mercuric chloride in hydrochloric acid ($HgCl_2$, 15 gm.; concentrated HCl, 20 ml.; distilled water, 100 ml.) (Frazier, 1926). This solution precipitates unchanged gelatin and leaves a clear zone where gelatin has been hydrolysed.

21) Starch hydrolysis.

This medium was prepared, and cultures inoculated, in exactly the same fashion as has been described for gelatin plates

(20), with the exception that 0·5 per cent soluble starch replaced the gelatin.

After incubation at room temperature for three to five days, plates were flooded with a one in ten dilution of Gram's iodine solution.

22) Casein hydrolysis.

The incorporation of casein into a sea water containing medium presented some technical difficulties. A 10 per cent skimmed milk solution was used as a source of casein and if this was added to a fluid sea water medium, the casein was precipitated. To overcome this, the preparation of this medium was divided into two steps: 1) a 10 per cent skimmed milk solution was prepared in distilled water and sterilized by steaming: 2) 1·5 per cent agar was added to BM and after dispensing in 15 ml. amounts in screw-cap containers, this was sterilized at 15 lbs. per square inch for 15 minutes. When the medium was required for use, 1·5 ml. amounts of the skimmed milk were added to Petri dishes and the BM agar melted and cooled to 42°C. When these two components were mixed in the Petri dishes, the agar quickly solidified and a smooth suspension of casein was obtained.

Cultures were inoculated as described for gelatin hydrolysis (20), incubated at room temperature and examined at intervals for up to fourteen days. Casein hydrolysis was apparent by the clearing of the medium around the bacterial growth.

23) Lipolysis.

The source of fat for this test was pure dairy cream, sterilized at 15 lbs. per square inch for 15 minutes.

BM agar was prepared and sterilized in 15 ml. amounts as described for the casein medium (23). Before use, 1·5 ml. of sterile cream was pipetted into 15 ml. of the molten agar and thoroughly mixed before pouring.

Cultures were spot-inoculated on this medium and incubated at room temperature in diffuse daylight for fourteen days.

After incubation, the bacterial growth was scraped from the surface of the agar, using a platinum loop, (this bacterial material was used in the next step) and the agar surface flooded with a saturated solution of copper sulphate in water, (Willis, 1960), and allowed to stand for 20 minutes when the excess solution was poured off. Copper sulphate reacts with fatty acids to form insoluble copper soaps which are bright blue coloured. The appearance of this intense blue stain under the bacterial growth was taken to indicate lipolysis. This reaction could be accelerated and made more intense by pouring hot copper sulphate on to the plates (Hobbs, pers. com.).

24) Pigmentation.

There have been numerous reports of the occurrence of "pigmented" marine bacteria. It became apparent during this investigation that there was a continuous spectrum of colour of bacterial colonies, from white or colourless to deep colours like golden yellow or chocolate brown. Where the line is drawn, and a culture designated pigmented or non-pigmented, is a personal and arbitrary decision.

In an effort to standardise results, British Standard Colour Charts were used for comparison with, and accurate

description of, the colour of bacterial growth after fourteen days incubation in diffuse daylight on the cream agar described (29). The colour charts used were British Standard 3810: 1949 (Fifth impression 1959, incorporating Amendments Nos. 1, 2 and 3) and British Standard 2660: 1955. The former charts were most commonly used since they gave a name to each colour, whereas the latter charts only had reference numbers. However, a few bacterial pigments could not be closely matched to the named colours and in these cases the corresponding reference numbers from the other charts were recorded. Representatives of all colours corresponding to any bacterial pigment encountered are presented in Appendix 2.

Colours have been divided into four groups: 1) all pale pigments and colourless; 2) more intensely coloured than group 1; 3) yellows and orange; 4) a few unusual colours rarely encountered, e.g., Eau-de-nil, Poppy.

The division of bacterial pigments in this fashion is no less arbitrary than the attachment of the epithet - "pigmented" or "non-pigmented" - by other workers. However, the colour chart does provide a standard reference. Organisms in group 1, have been considered to be equivalent to what most workers would assess as non-pigmented, while organisms in groups 2, 3 and 4 have been considered pigmented. No significance has been attached to the different colours within each group except in group 4 (to which only a very few isolates were designated), where such strikingly different colours are present.

The method used for the comparison of the colour of bacterial colonies with the charts, consisted of scraping off

growth from the cream agar as described (23) and placing it on a strip of Whatman's No. 1 filter-paper. The bacterial growth was then matched to a standard colour in daylight.

25, 26) Final check for purity.

After the inoculation of media for tests 20 to 23, a DM sloppy agar culture was inoculated from the fluid culture (19) and incubated at room temperature until growth appeared, when each culture was stained by Gram's method and examined microscopically. If the cell morphology was the same as previously recorded (7, 12, 15) and there was no apparent contamination, the results of all tests were finally recorded.

When dealing with large numbers of cultures in the manner described, it is likely that a few will be contaminated during the process. It was not possible to check each culture for purity in every medium and it was felt that the method devised for checking the purity at the last stage, was satisfactory. If the culture appeared to be impure it was repurified (4) and the characterisation process repeated.

3.2.3. The recording of results.

Since about eight hundred isolates were examined during this survey and as many as forty characters could be investigated for any one strain, it was essential that a suitable method for recording results should be developed. The use of a punch card system was found to be admirable for this purpose.

Punch Cards No. P.2. were obtained from the Copeland Chatterson Co. Ltd., Stroud, Glos., and each of the characters

Figure 16.

Punch card showing the coding system employed.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
1 7 4 2 1 7 4 2 1 7 4 2 1 7 4 2 1
A B C D

Water sample A
Water sample C
Water sample G
Medium 6
Medium 5
Medium 4
Medium 3
Medium 2
Medium 1
Pigment group 4
Pigment group 3
Pigment group 2
Pigment group 1
Peritrichous flagella
Polar flagella
Morphology indeterminate
Spore
Yeast
Filamentous
Chinese letters
Vibrio/spiral
Coccoid
Cocco-bacillus
Rod
Motile
Gram negative
Gram positive

Catalase
Oxidase
Growth in ammonium acetate
Fat (cream) hydrolysed
Casein (milk) hydrolysed
Starch hydrolysed
Gelatin hydrolysed
Nitrite reduced
Nitrate reduced
Litmus milk clotted
Litmus milk peptonised
Litmus milk alkaline
Litmus milk acid
Alkalinity xylose
Acid xylose
Alkalinity galactose
Acid galactose
Alkalinity arabinose
Acid arabinose
Alkalinity maltose
Acid maltose
Alkalinity mannite
Acid mannite
Alkalinity glucose
Acid glucose
Gas from glucose
No acid from glucose
Attack on glucose fermentative
Attack on glucose oxidative

PARAMOUNT REGD. TRADE MARK 53/C.C. 92778 C

Figure 17.

Punch card after all the information gained for one strain has been recorded.

Gram + Stout rods in Chinese letters No. 208
dividing into segments. Medium 1
Non-motile Sample C
Dissimilation of glucose - aerobic
Glucose, acid; Mannite, acid; Maltose, acid;
Arabinose, alkaline; Galactose, alkaline; Xylose, alkaline.
Litmus milk - no change.
Gelatin + Starch +
Milk - Fat -
Growth in Ammonium Acetate Medium +
Nitrate reduced
Oxidase - Catalase +
Pigment - Jasmine yellow
Colonies - Small, smooth surface, semi-opaque, entire.

PARAMOUNT REGD. TRADE MARK 53/C.C. 92778 C

investigated was assigned a number. Over and above the properties of each isolate, the water sample and medium from which each culture was isolated, were similarly recorded. An example of the cards and the coding system employed, is presented as figure 16. The two shorter sides of the card were used to record the number of the isolate and eventually the generic group to which it had been ascribed. This information does not feature in figure 16.

A typical card, after all the available information had been recorded and the corresponding holes punched, is presented as figure 17.

The use of the punch card system has greatly facilitated the analyses of results obtained. It offers a quick method for the assessment of any correlative characters and enable isolates, with any number of characters in common, to be selected with ease. The system has also been invaluable in the estimation of the distribution of characters among different media and water samples.

The use of punch cards as an aid to classifying yeasts, has recently been described by Shifrine and Miller (1961).

5.2.4. Flagella staining.

The staining of flagella was not carried out as a routine method during this survey, though selected motile cultures, as representatives of morphological and physiological groups, were so examined.

Cultures were inoculated on to BM plus 1·5 per cent agar slopes, or on to the same medium prepared with an artificial sea

water solution (MacLeod et al., 1954) as recommended by Girard and Cleverdon (1961), and incubated at 20°C. until visible growth appeared. Smears were prepared and stained as described by Leifson (1960).

5.5. Results and Discussion.

5.5.1. Introductory Comments.

Organisms isolated on count plates of samples A, C and G were selected for further study. As already described, it was important that this selection was completely at random, and to this end every colony was chosen from one or two plates to give approximately fifty isolates from each medium (5.2.2.). A total of seven hundred and eighty-three strains were grown in the primary sub-culture medium, and of these, six hundred and sixty-seven survived and were fully characterised according to the scheme described (figure 15). The extent of the losses of bacterial cultures varied from one sample to another. With sample A, one hundred and forty-seven strains were tested representing seventy-five per cent of the organisms grown after the first sub-culture. The corresponding figures for samples C and G, respectively, were three hundred strains, representing ninety-one per cent, and two hundred and forty-five strains, representing eighty-five per cent. This reduction in losses of cultures when samples C and G are compared with sample A has been ascribed to the use of a sloppy agar medium for the primary culturing of the isolates, and to the incorporation of yeast extract in all media used to maintain the cultures, from samples C and G (5.2.2.).

The reason for the death of cultures was not apparent, though the strains which were commonly lost had an extremely irregular cell morphology which suggested that some metabolic process, or processes, were unable to function efficiently under the growth conditions supplied.

The results obtained from this survey have been used to indicate the effect of medium composition on the pattern of biochemical activities of the micro-organisms isolated from each water sample, and also the extent of variation of characters and physiological activities encountered from different water samples. Isolates have been assigned, where possible, to genera, and the overall activities of these genera have been assessed and compared with a view to demonstrating their distinctive features.

3.3.2. Presentation of results.

The part of the investigation concerned with the characterisation of isolates involved the detailed study of six hundred and sixty-seven organisms. Each strain was subjected to twenty tests, the results of which provided forty characters for systematic purposes. The full collection of characters therefore contained approximately twenty-seven thousand units of information and it was necessary to reduce this mass of data to a form which would be both fully informative and readily assessable.

The use of punch cards, as described in section 3.2.3., provided a convenient means of storing and correlating information, and experience with this system suggests that it could find wide application for studies of this kind. For presentation in this

thesis, the results obtained by analysis of the punch cards have been summarised in the form of histograms (figures 18, 19, 20, 21, 37) and dendrograms (figures 24, 25, 27, 29, 38). Only in the case of those organisms which could not be assigned to a systematic group and with which characters could not usefully be correlated, has it been considered necessary, or desirable, to present the detailed reactions of each strain. Reference to the histograms and dendrograms will show that they yield all the information about the characteristics of the organisms which can be considered as important - and indeed, that they give much which is undoubtedly trivial.

3.3.3. The influence of the isolation medium on the assessment of microbial activities in sea water.

The effect of the composition of the isolation medium on the numbers of bacteria isolated from North Sea water has been demonstrated (2.5.), and with this information available it was of interest to assess the extent to which the medium influenced the qualitative nature of the bacterial flora isolated.

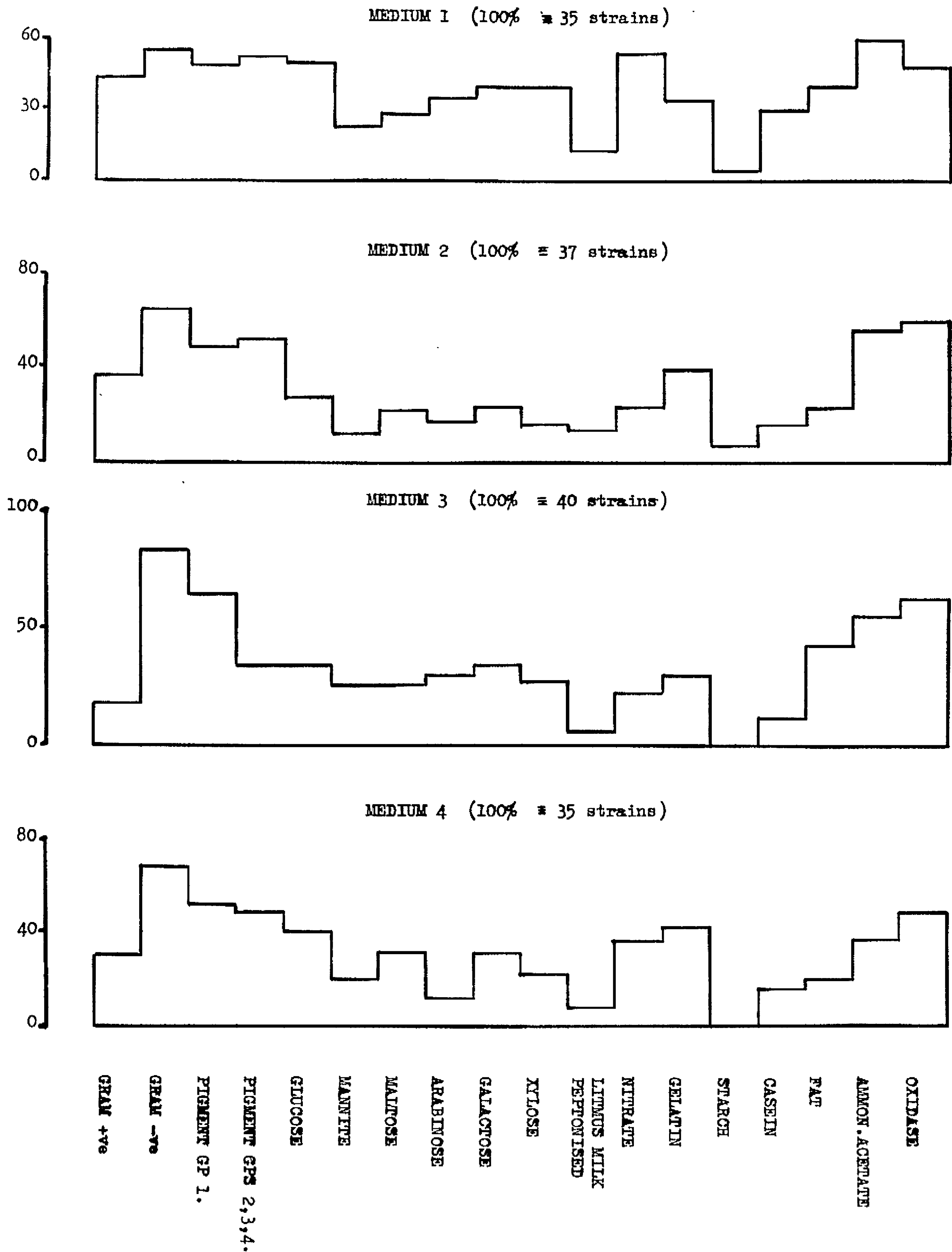
Water sample A.

Organisms from this sample were isolated on media 1 to 4 (2.2.3.) and characterised using the scheme already described. The results obtained have been analysed and presented as histograms (figure 18), a comparison of which indicates the essentially similar pattern of activities of organisms isolated on these four different media.

There was a higher incidence of Gram positivity, acid production from sugars and nitrate reduction among medium 1

Figure 18

Total activities of all organisms isolated from sample A on four different media



isolates than among isolates from media 2, 3 or 4. Roughly forty per cent of all isolates from media 1 and 5 were lipolytic, whereas only twenty per cent of isolates from media 2 and 4 were lipolytic. The yeast-extract-containing medium 4 had approximately twenty per cent fewer isolates able to grow on an inorganic nitrogen medium than the other media which had no yeast extract. Rather surprisingly, medium 2, which contained glucose, produced the smallest percentage of isolates with the ability to form acid from sugars.

Water sample C.

For this sample, organisms were picked from count plates of media 1 to 6 (2.2.3.) for further investigation and a comparison of the activities of the isolates from these media (figure 19) shows that medium composition had almost no effect on the overall pattern obtained; one exception was the higher incidence of lipolysis among organisms from media 3 and 5.

Water sample G.

As with samples A and C, the incidence of activities of isolates from water sample G, varied little from one isolation medium to another (figure 20). Organisms from medium 1 had a proportionately higher saccharolytic activity when compared with the other media.

It would appear from the results presented above that the composition of the isolation media used had little real effect on the overall nature of the bacterial population isolated. In other words, no matter which of these media had been used to isolate bacteria from North Sea water, a similar pattern of microbial activities would have been apparent. As has been shown previously (2.3),

Figure 19

Total activities of all organisms isolated
from sample C on six different media

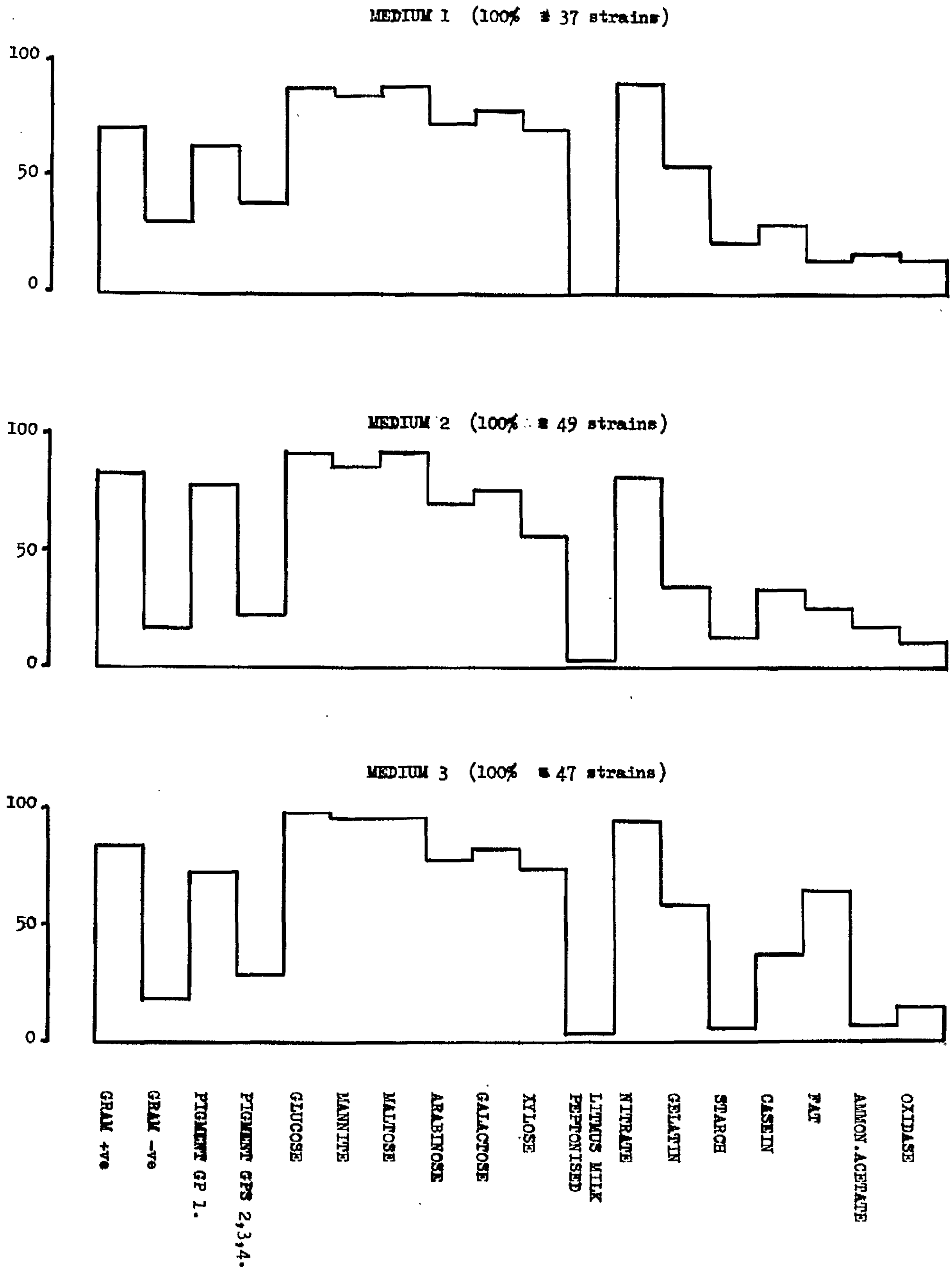
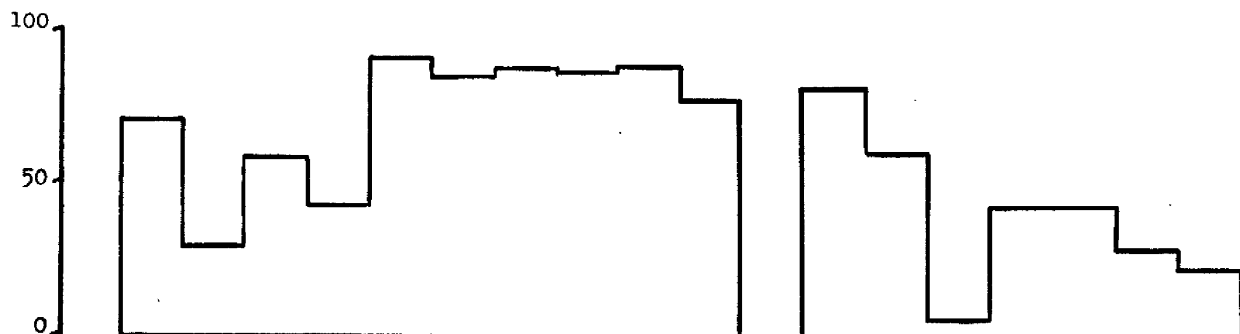
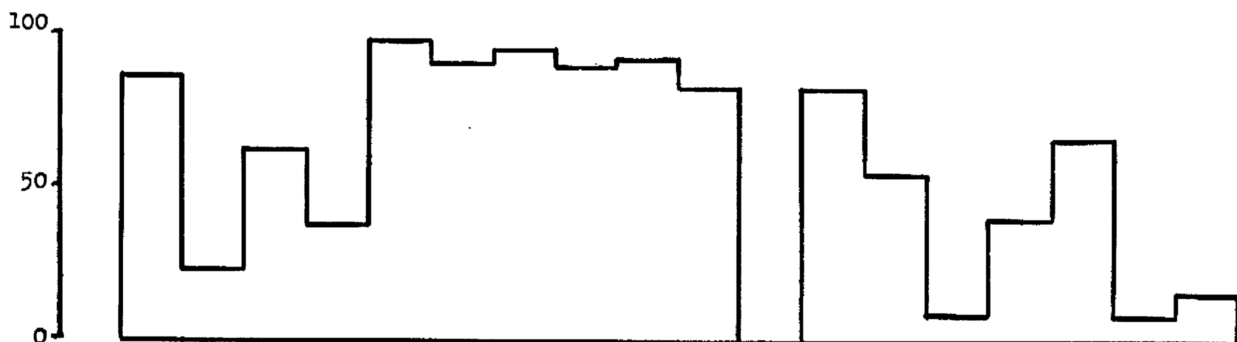


Figure 19 (continued)

MEDIUM 4 (100% = 43 strains)



MEDIUM 5 (100% = 46 strains)



MEDIUM 6 (100% = 52 strains)

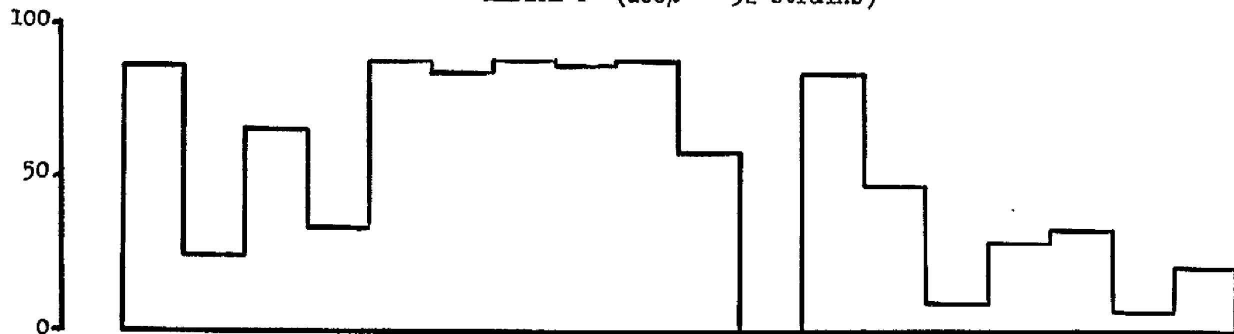


Figure 20

Total activities of all organisms isolated from Sample G on six different media

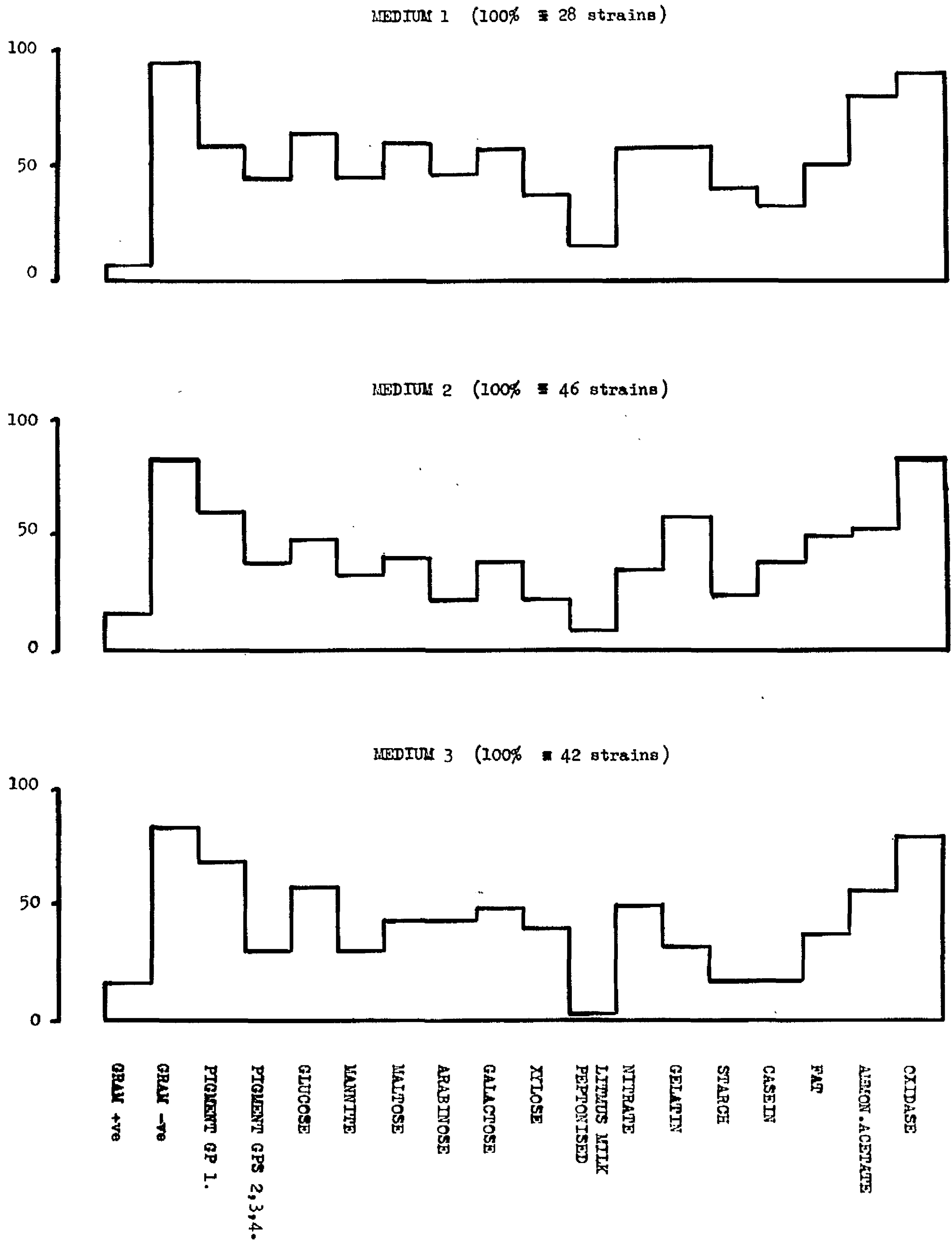
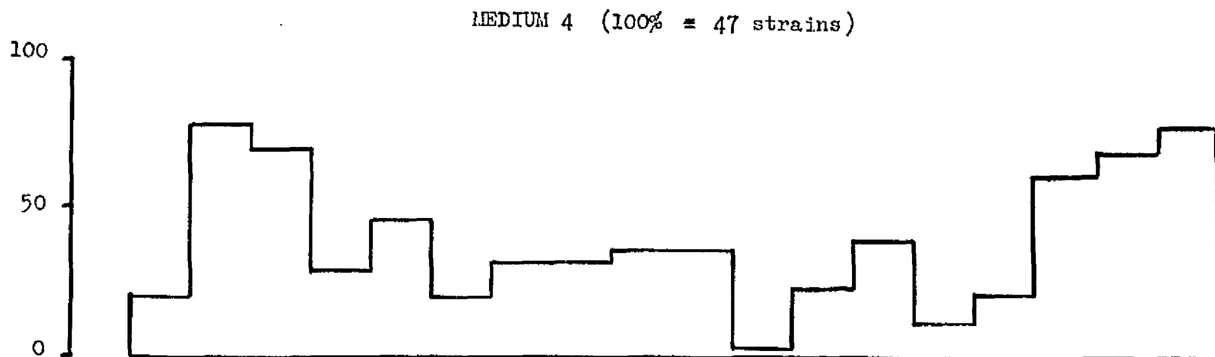
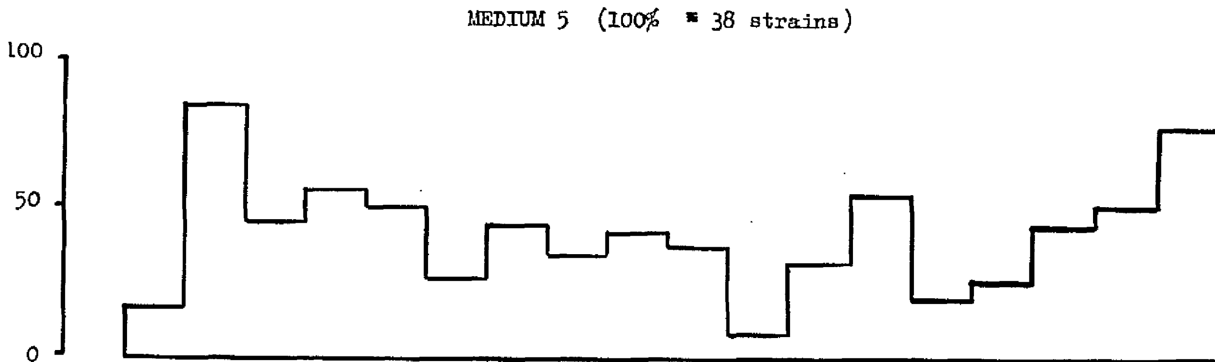
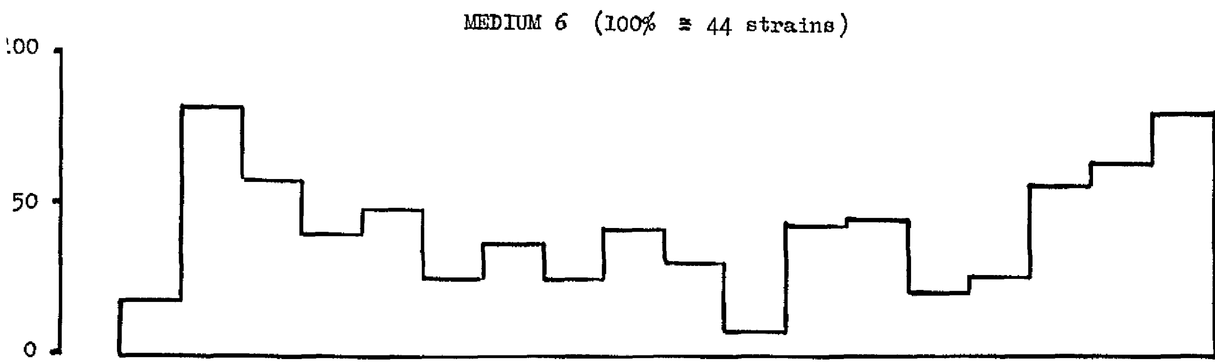


Figure 20 (continued)







GRAM +ve
GRAM -ve
PIGMENT GP 1.
PIGMENT GPS 2,3,4.
GLUCOSE
MANNITE
MALTOSE
ARABINOSE
GALACTOSE
XYLOSE
LITMUS MILK PEPTONISED
NITRATE
GELATIN
STARCH
CASEIN
FAT
AMMON.ACETATE
OXIDASE

however, the numbers of organisms isolated varied considerably from one medium to another, so although the choice of medium would not greatly influence an assessment of the qualitative nature of the physiological processes which could potentially occur in sea water, it would be of great importance for an accurate quantitative assessment.

For an investigation whose purpose was to estimate the extent of microbial activities in sea water, and where obviously the numbers of micro-organisms involved is important, it would be essential to employ a medium such as medium 5 which was known to give the highest possible counts. On the other hand, if the investigation were not concerned with the levels of microbial activities, but only with the possible occurrence of particular metabolic processes and the relative importance of these, the choice of isolation medium would be of little significance.

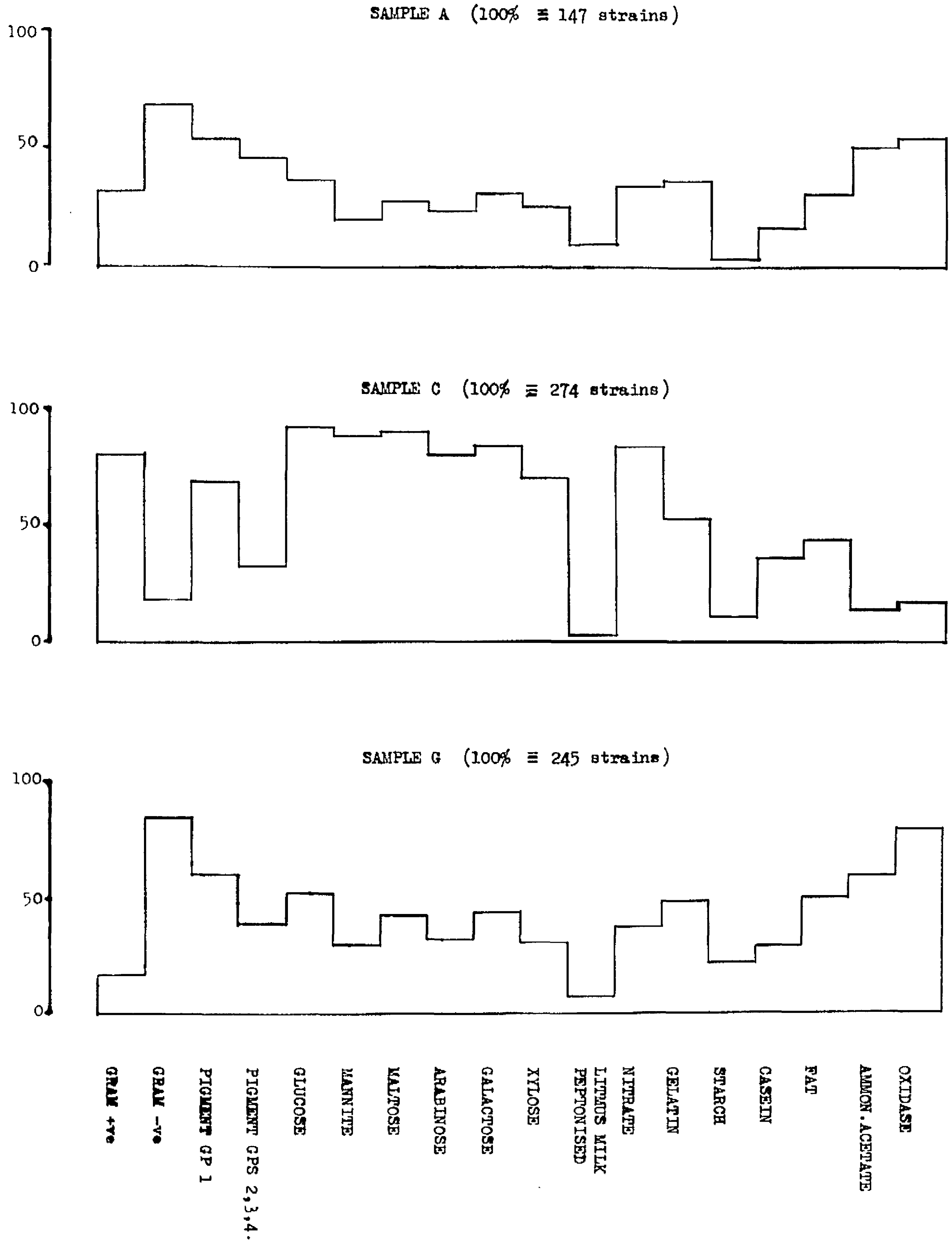
3.5.4. The variation of microbial activities in different water samples.

Since the influence of isolation medium composition was shown to have only a minor effect on the qualitative assessment of the marine bacterial flora, it seemed justifiable to group together the isolates from all media within each water sample and to compare the overall properties possessed by their respective bacterial populations.

Figure 21 indicates the distribution of properties among all isolates from water samples A, C and G, and demonstrates the extent to which different water samples may vary with respect to the potential physiological activities of their microbial population.

Figure 21

The distribution of characters of all isolates
from three different water samples



The incidence of Gram positive species was much higher in sample C (eighty-one per cent of all isolates), than in the other two samples (thirty-two per cent in sample A and only seventeen per cent in sample G). This basic difference in bacterial types in the three samples, as reflected by the Gram reaction alone, is borne out by a detailed comparison of the histograms in figure 21. Sample C presents a markedly different picture of microbial activities in sea water from that seen with samples A and G. For instance, the majority of isolates from sample C were saccharolytic and could reduce nitrate, while saccharolytic activity and nitrate reducing capacity in samples A and G, were confined to about fifty per cent of the isolates. On the other hand, between fifty and sixty per cent of the strains from samples A and G could grow in an inorganic nitrogen medium, while only fourteen per cent of the strains from sample C could grow in such a medium.

Samples A and G have the same general pattern of activities, though in sample G proportionately more isolates were active in producing acid from sugers and in hydrolysing gelatin, starch, casein and fat.

The qualitative assessment of islates in the manner described above, demonstrates strikingly the differences which may exist in the microbial flora of samples of sea water collected at various seasons, and emphasises the need - not always recognised by workers in this field - for detailed study of large numbers of strains isolated from repeated samples, before valid conclusions regarding the nature of the marine microflora, and the effects of changing

conditions on it, may be reached.

5.5.5. The characteristics of the predominant groups or genera, encountered in three samples of sea water.

The results obtained from the use of the scheme described have enabled most isolates to be assigned to one of six major groups or genera. The predominant groups encountered in North Sea water during this investigation were

micrococci	31 per cent;
Achromobacter	22 per cent;
coryneform organisms	12 per cent;
Pseudomonas	10 per cent;
Flavobacterium/Cytophaga group	7·5 per cent;
Vibrio	5·5 per cent
miscellaneous organisms	12 per cent.

The definition of these groups is not always obvious from studies of the literature and it has been necessary to make decisions regarding the criteria to be applied in assigning strains to particular groups. Such decisions have always been based upon the most recent or most soundly documented views expressed by authorities on the groups concerned and these are quoted in the appropriate sections. Where it has proved impossible to reconcile the views of prominent workers in a particular field, as with the micrococci for instance, a more arbitrary approach has been made. The characters used for the definition of these groups are summarised in figure 22.

As will be apparent from the discussion of the various groups, it has proved possible to sub-divide certain of them

into major sections and the numbers of strains involved render it reasonably certain that such sub-divisions represent definite taxa. There exists a temptation to attach considerable significance to such sub-divisions and give them generic or even specific status, drawing comparisons with similar treatment accorded to related organisms from other environments. This temptation has been resisted for two main reasons:

Firstly, the pattern of tests employed in this survey was designed to give information about the general physiology of the isolates and hence, as was considered of fundamental importance, about the biological potential of the sea itself. This means that certain tests which are primarily of the diagnostic nature (such as resistance to antibiotics, for instance) and which have found application in the systematic study of individual groups, have been omitted and it is not always possible to draw direct comparisons with the taxa proposed by other workers;

Secondly, consideration of the literature on these particular groups - which are not on the whole extensively studied - can only lead to the conclusion that the time is not yet ripe for their detailed classification. In the opinion of the present author much of the confusion existing in these groups at the present time stems from premature attempts at classification on a basis of insufficient information. Whilst it would certainly be possible to apply generic and even, in some cases, specific epithets to some of the divisions created in the present work, the extent of knowledge of these marine organisms is such as to render such designations

Figure 22.

A summary of the characters used to define the six major groups of isolates.

	Gram Reaction	Cell Morphology	Motility	Acid from Glucose	Pigmentation	Oxidase	Catalase
Pseudomonas	-	small rods	+ (polar)	oxidative or -	*	+	*
Vibrio	-	small rods	+ (polar)	fermentative (anaerogenic)	*	+	*
Flavobacterium	-	small very slender rods	± (peritrichous)	*	Group 3	*	*
Cytophaga	-	small very slender rods	swarming motility	*	*	*	*
Achromobacter	-	large cocco-bacilli	-	oxidative or -	*	*	*
Coryneforms	+	cocco-bacilli and larger rods without spores	*	*	*	*	+
Micrococci	+	cocci in irregular clusters	*	*	*	*	+

* Not used as a definitive character.

valueless and indeed harmful; since a name once applied tends to be perpetuated in the literature long after it has become meaningless as a result of later developments.

MICROCOCCI.

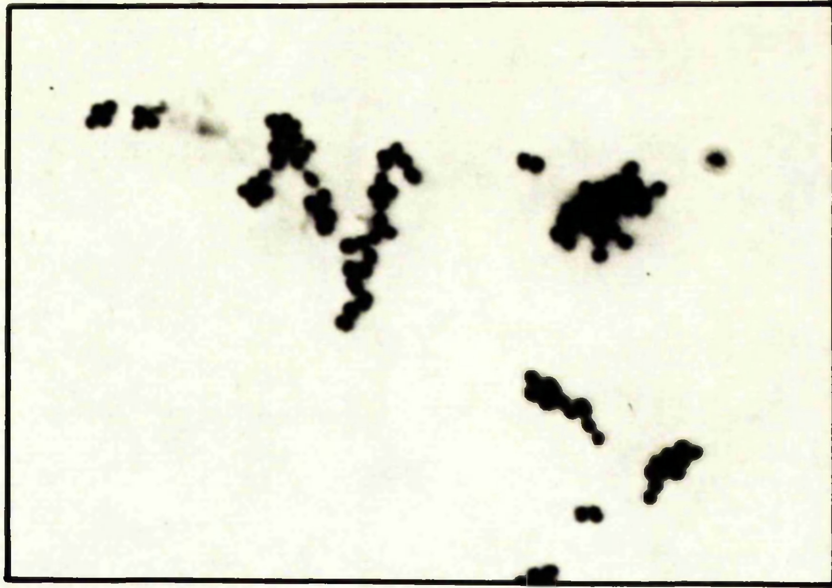
The occurrence of Gram positive cocci in sea water has previously been reported by ZoBell and Upham (1945); ZoBell (1946); Wood (1946, 1952, 1953); Brisou (1955) and Kriss (1962). These workers have attempted to ascribe their isolates to genera and have given them specific names.

Micrococci have usually been found to represent only a small proportion of the bacterial flora of sea water (ZoBell, 1946) but they formed nineteen per cent of the sea water isolates of Wood (1953), and Kriss (1962) states, that although micrococci were often present in small numbers they were occasionally the predominant group in sea water samples.

In this investigation, micrococci formed the largest group, equivalent to thirty-one per cent of the total isolates, though ninety per cent of these micrococci were isolated from sample C.

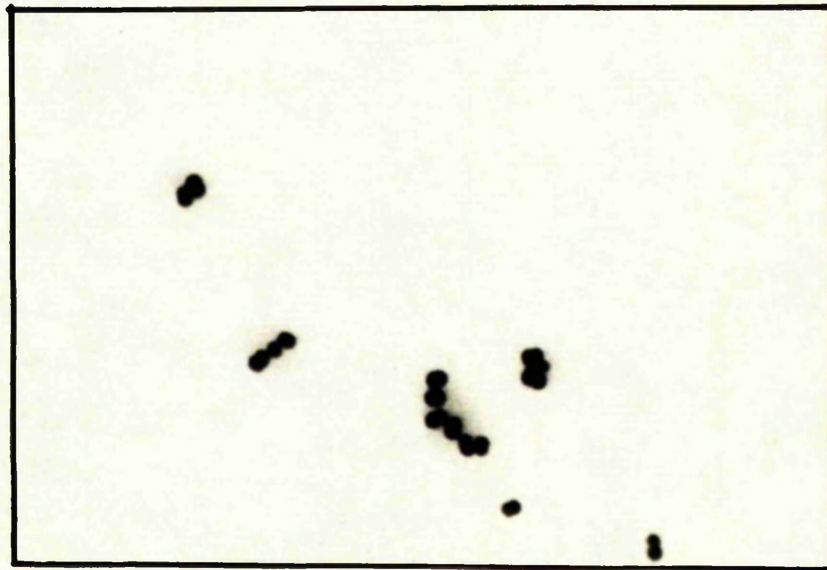
The classification and taxonomy of the micrococci is at present uncertain, many reports putting forward conflicting points of view (Hucker, 1924; Shaw et al., 1951; Van Eseltine, 1955; Hill, 1959; Pohja, 1960). Because of the confusion existing at present, the Gram positive, catalase positive cocci isolated in the present investigation have been placed together and designated "micrococci". These organisms were often irregular in size and could range from 0·5 to 5μ in diameter, within one culture. They occurred

Figure 23



MICROCOCCUS strain (oxidative) x 1,950

Gram's stain



MICROCOCCUS strain (fermentative) x 1,950

Gram's stain

Figure 24

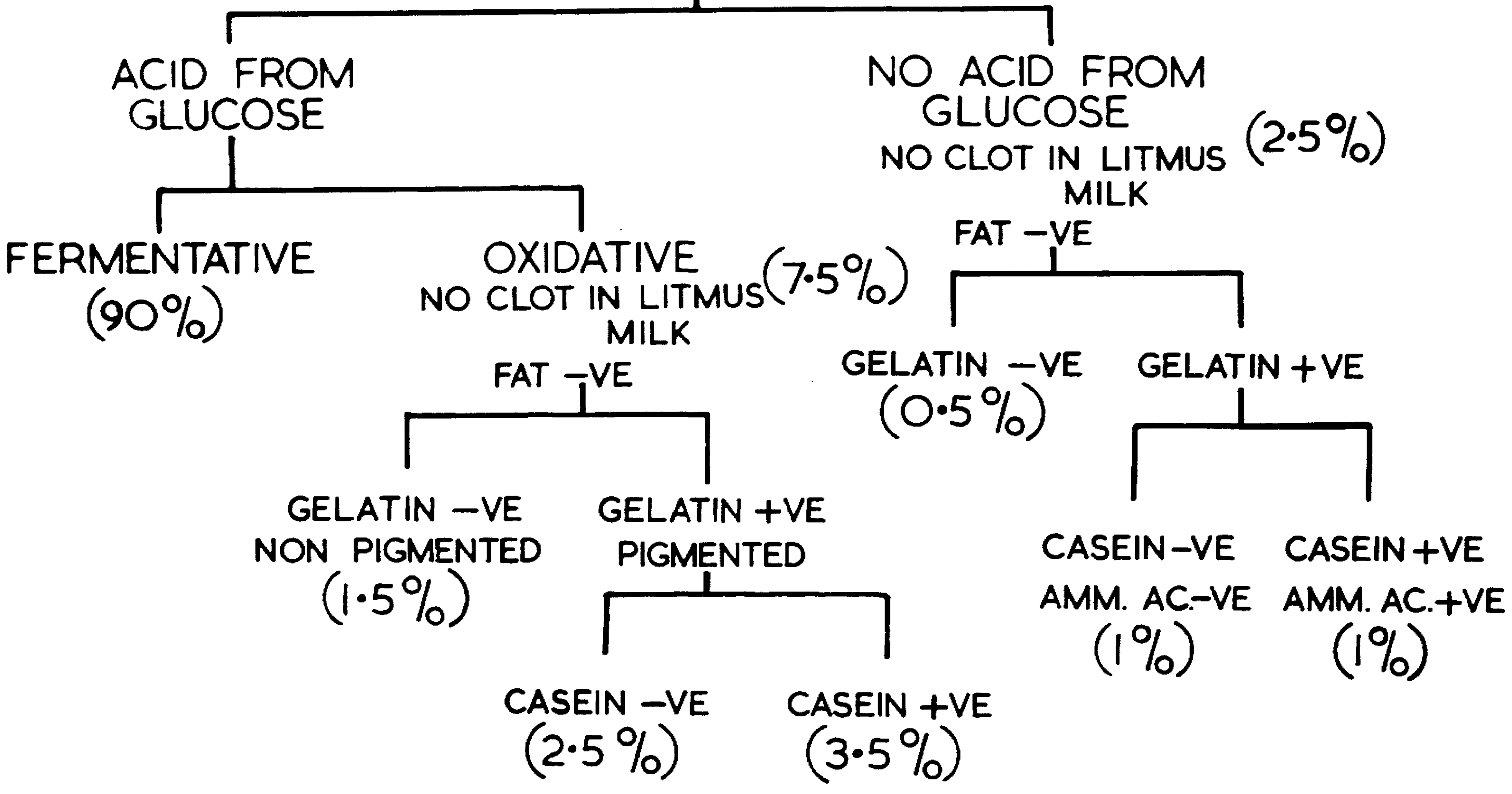
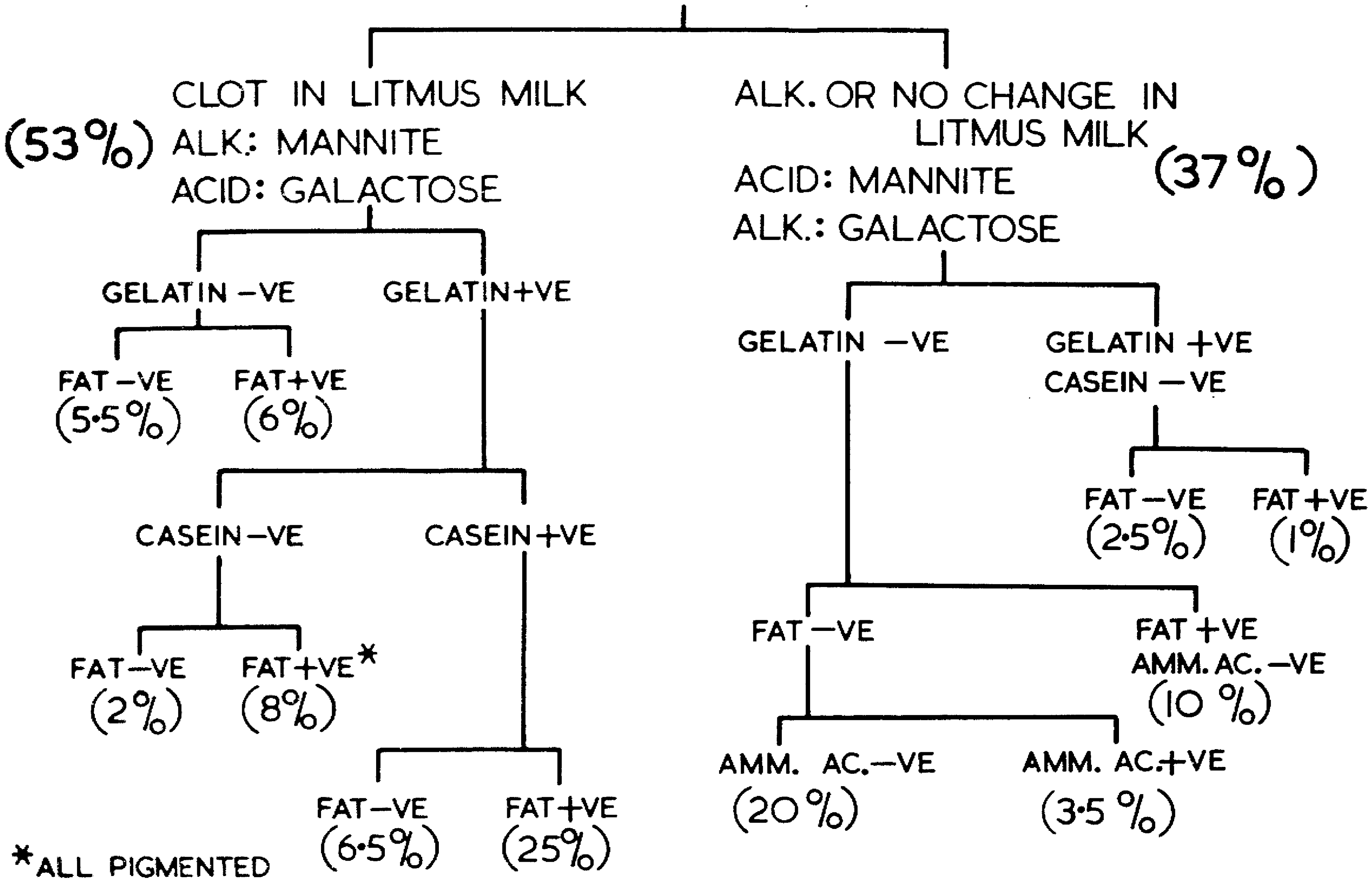


Figure 25



usually in irregular clumps or clusters and occasionally in pairs or tetrads. The fermentative cocci seemed to be more irregular in size than the oxidative cocci. A typical appearance is shown in figure 23.

The two hundred and five strains of micrococci isolated could be sub-divided into three major groups according to whether they produced acid from glucose oxidatively or fermentatively or not at all. It was then possible to further sub-divide these organisms into sixteen minor groups, as indicated in figures 24 and 25.

The fermentative cocci represented ninety per cent of the total and this group was divided into two sub-groups (figure 25). All the organisms in one of these groups produced a clot in litmus milk, a permanent acid reaction in galactose, and only a transient acid, with later alkalinity, in mannite. The other group failed to clot litmus milk, produced a permanent acid reaction in mannite, and only a transient acid in galactose. The validity of this sub-division is further emphasised by the fact that the strains constituting the former group were in general much more physiologically active than those in the latter group. Figure 24 shows how the oxidative and the non-saccharolytic micrococci could be grouped on a basis of gelatin hydrolysis and pigmentation but since there are only a few strains involved, these sub-groups are probably of limited significance.

Certain characters, which are presented in figure 57, proved to be of little value for systematic breakdown of the group. Thirty-six per cent of the micrococci had pigments usually belonging to pigment group 3 (Appendix 2). Pigmentation occurred irregularly throughout the sub-groups of the fermentative micrococci, except for one group which produced an acid clot in litmus milk, and

hydrolysed both gelatin and fat, but not casein, and which contained only pigmented strains.

Ninety-one per cent of all micrococci isolated, reduced nitrate, eight per cent could grow in the inorganic nitrogen medium, and four per cent hydrolysed starch. All were oxidase negative.

ACHROMOBACTER.

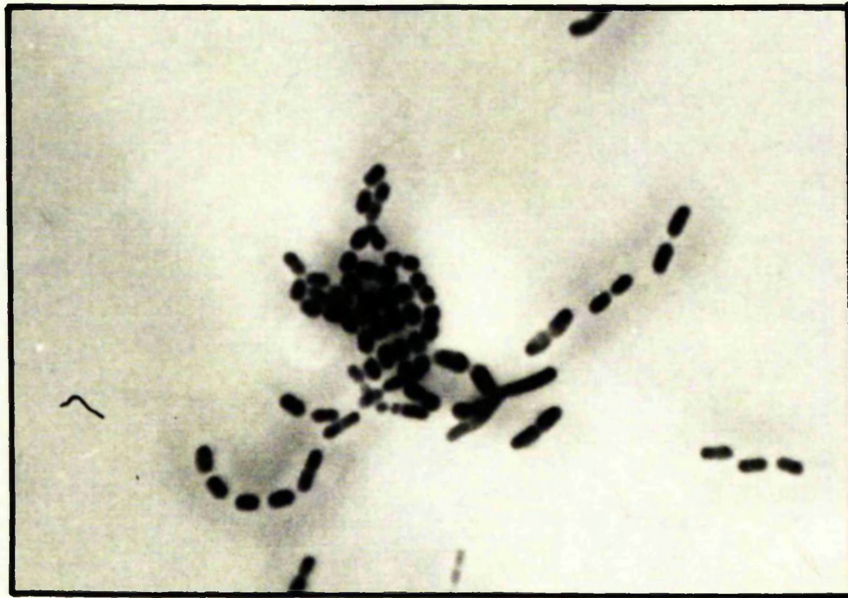
This genus has been defined for this investigation according to the proposals of Shewan et al., (1960a), i.e., Gram negative, short, stout or coccoid rods, non-pigmented, non-motile, and producing alkalinity or oxidative acid in sugar media. The organisms of this genus isolated during the present survey, were usually cocco-bacilli with some elongated cells, and stained intensely (figure 26).

The strains assigned to this group have been divided, as indicated in figure 27, into two major groups on a basis of their action in the glucose medium, in which some produced acid oxidatively while others produced alkalinity. Further sub-divisions were possible making use of fat hydrolysis and the ability to grow in the ammonium acetate medium. Other than the hydrolysis of fat by seventy-six per cent of the strains, and the reduction of nitrate by thirty-two per cent of the strains, this genus was largely inactive biochemically, as may be seen from figure 57.

CORYNEFORM GROUP.

ZoBell (1946) failed to report the presence of any members of the genus Corynebacterium in sea water, while Wood (1953) found that these organisms formed nine per cent of all his sea water

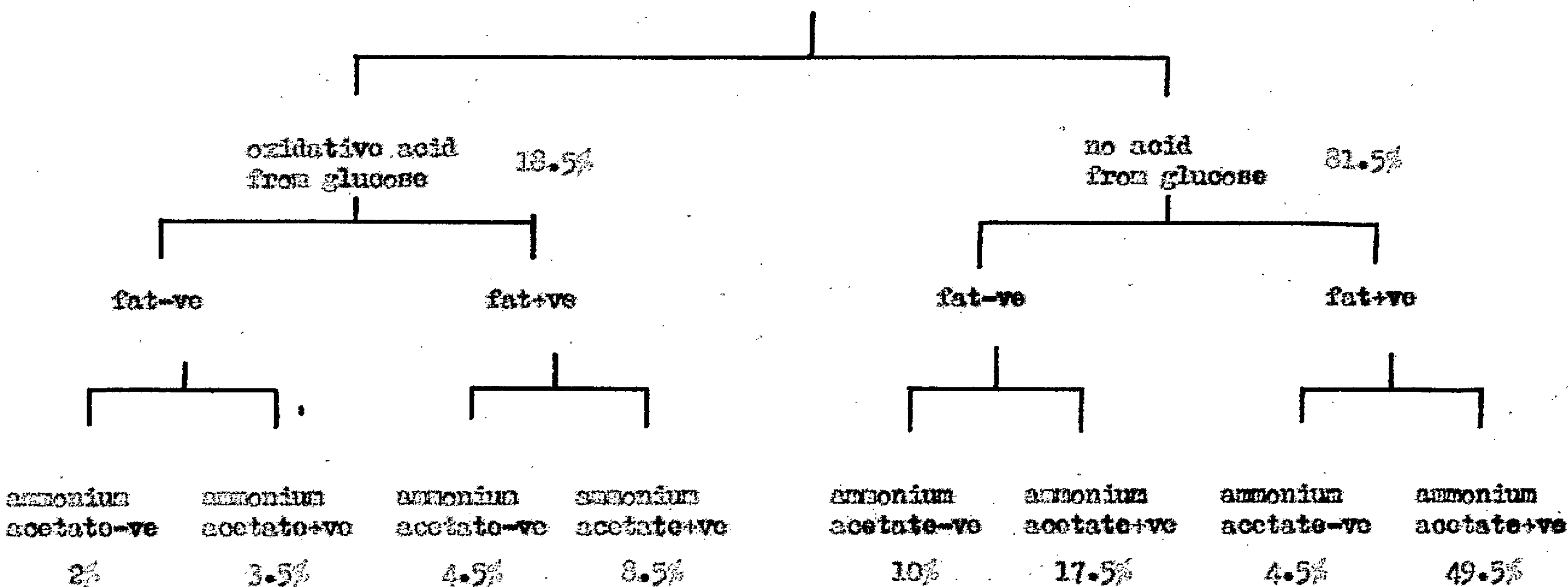
Figure 26



ACHROMOBACTER strain x 1,950

Gram's stain

Figure 27



isolates. Corynebacterium is considered by Wood (1955) to include "Gram positive rods, usually staining irregularly, not acid-fast, with snapping cell division".

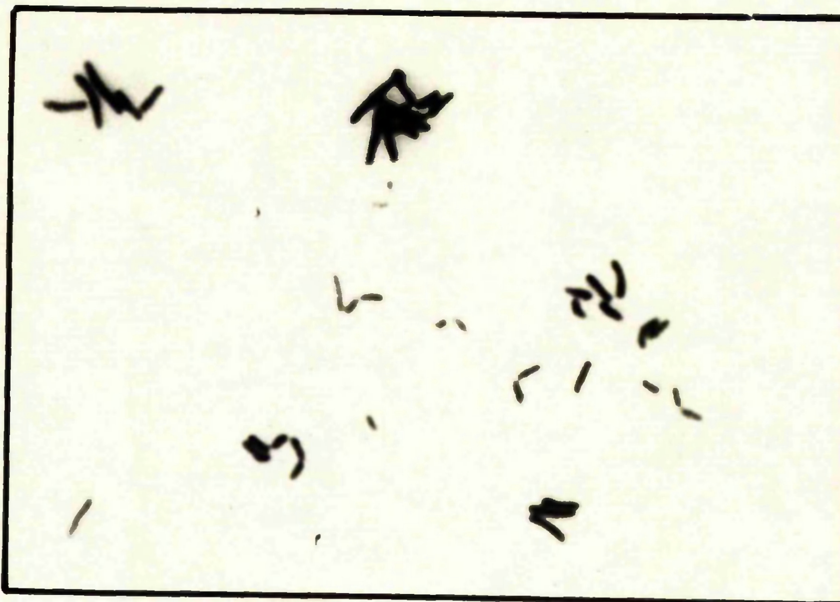
For the purposes of this investigation, all Gram positive rods which had no spores, never formed a mycelial type of growth, and were catalase positive, were classified as "coryneform bacteria". The difficulties of classifying this group have been pointed out by Jensen (1952), who states, "There are perhaps few groups of bacteria of which the typical representatives are easier to recognise and the aberrant types more numerous and more difficult to separate than those which originally constituted the genus Corynebacterium, and which we now may cautiously call the group of coryneform bacteria".

An attempt has been made by Advani and Iyer (1960) to sub-divide the coryneform bacteria into four groups on a basis of cell morphology. It was not possible to group the isolates from this study into four distinct morphological types, but they have been tentatively placed into two sub-groups: 1) large rods in typical "Chinese letter" formation, and 2) cocco-bacilli occurring singly or in clusters (figure 28).

The method of further sub-dividing these two groups can be seen from figure 29, and the characters and properties of this group as a whole are presented in comparison with the other groups or genera isolated during this study, in figure 37.

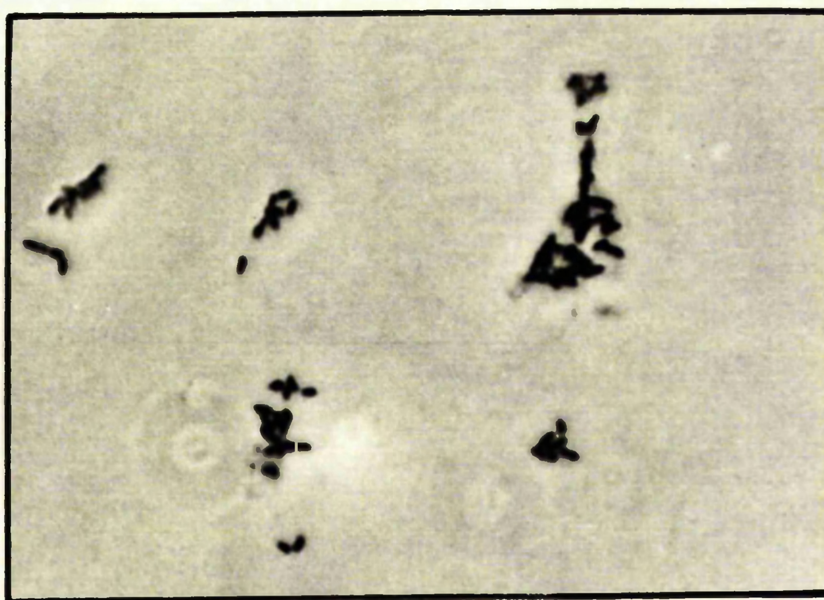
Eighty-five per cent of the coryneform strains isolated were classified as belonging to pigment group 5 (Appendix 2). The observation of Wood (1955) that there is a striking similarity

Figure 28



Large celled coryneform strain x 1,950

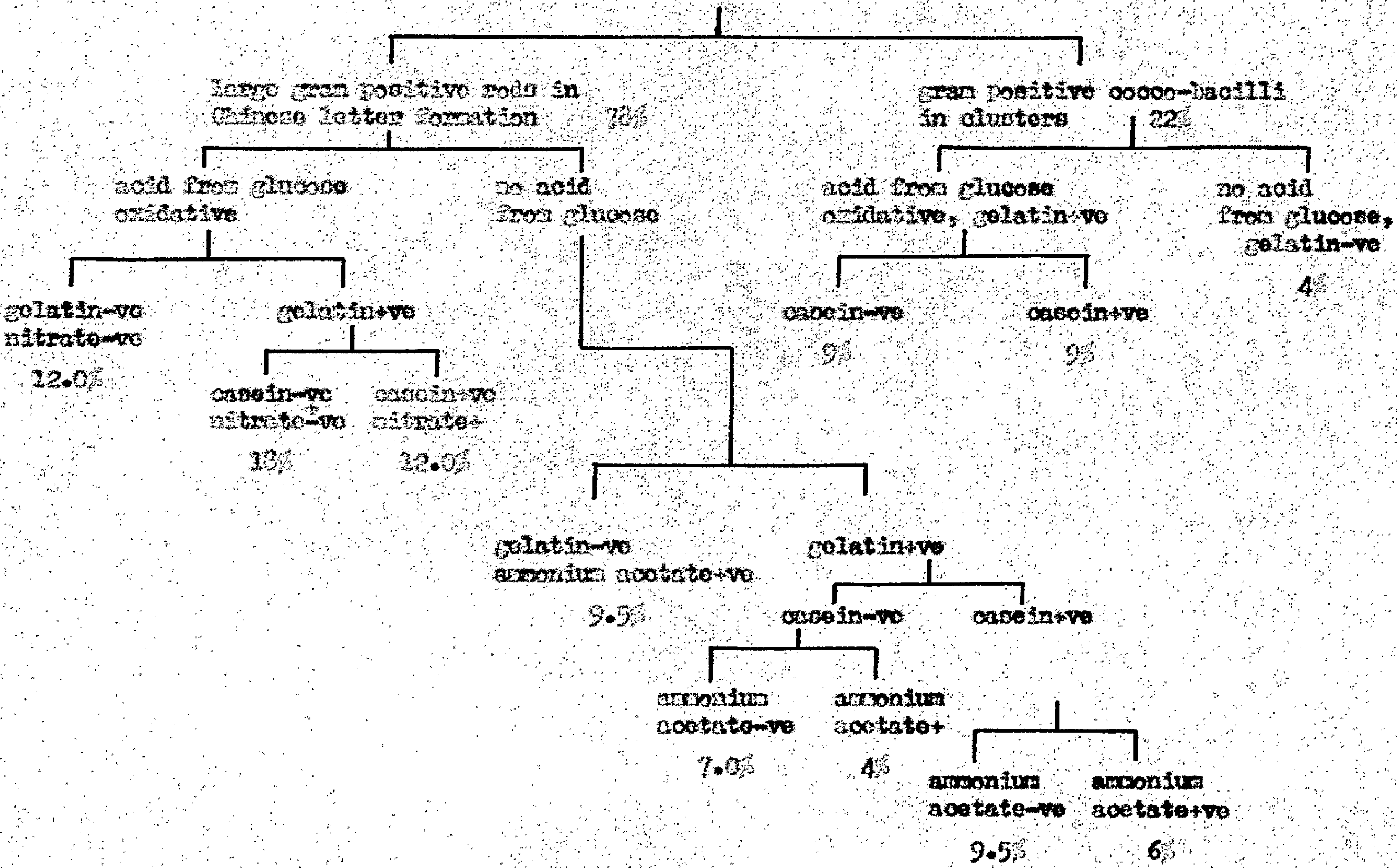
Gram's stain



Cocco-bacillary coryneform strain x 1,950

Gram's stain

Figure 29

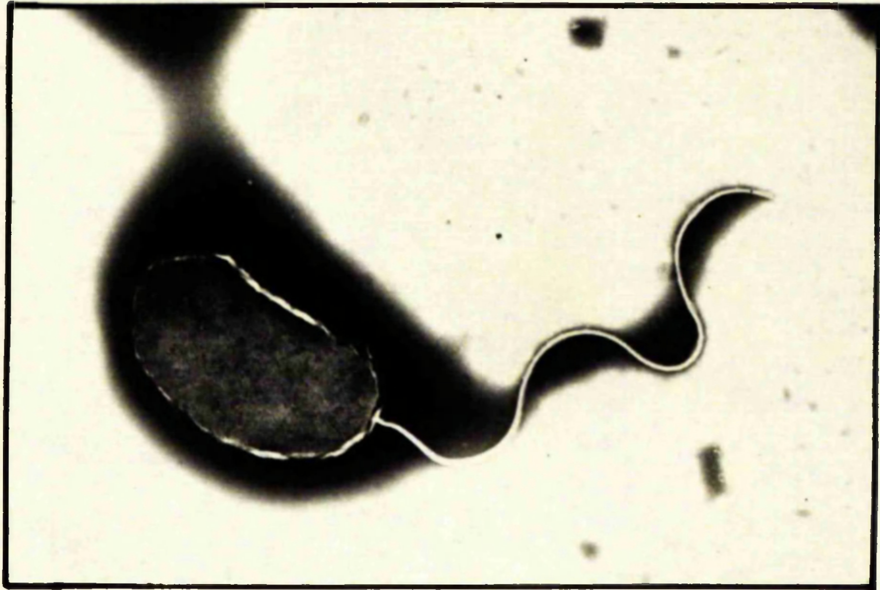


between the pigment range in the micrococcus and coryneform groups, has been borne out in the present study.

Reports of motility in the coryneform group have been reviewed by Jensen (1952) and it seems to be generally accepted that such organisms when motile have one or more polar flagella. When discussing motility in marine corynebacteria, Wood (1953) states "Some of Topping's strains were also motile, and motile forms with polar flagella are therefore also included in the present definition of Corynebacterium". It is uncertain why Wood should restrict this genus to non-motile or polarly flagellate strains apparently on a basis of Topping's (1928) results, since when discussing motility in a group of Gram positive, non-acid fast, non-spore-forming rods, Topping (1928) remarks that "short rod forms usually possess a single flagellum in an almost polar position. Longer rods and branched forms may possess several flagella, in which case they appear peritrichous".

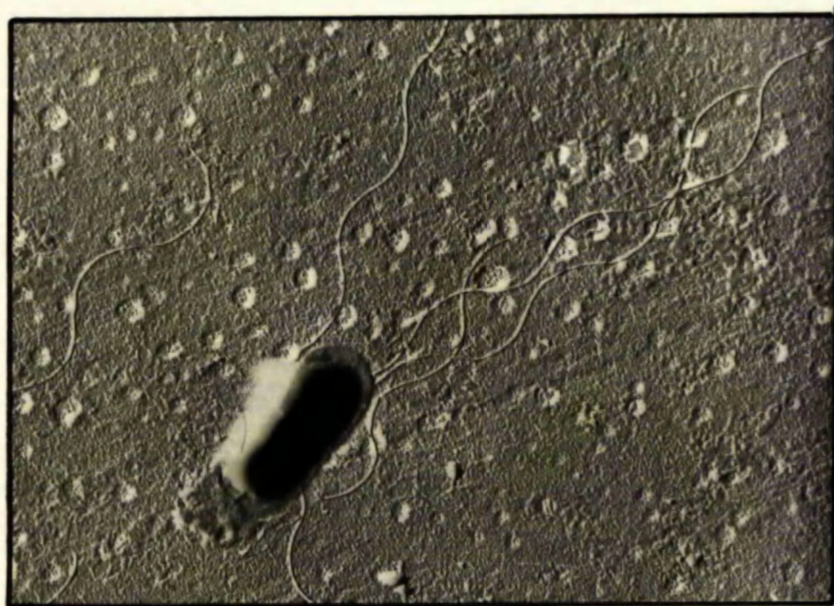
Only four strains of motile coryneform bacteria were isolated during this survey. Two of these were short rod forms and exhibit a single polar flagellum (figure 50) while the other two were larger rods in typical "Chinese letter" formation and possessed peritrichous flagella (figure 51). (Election micrographs were kindly prepared by Mr. W. Hodgkiss, Torry Research Station, Aberdeen.) The flagellar arrangement of these organisms is therefore in agreement with the results of Topping (1928).

Figure 31



Coryneform strain showing polar flagellum x 17,500

Figure 30



Coryneform strain showing peritrichous flagella x 16,000

PSEUDOMONAS.

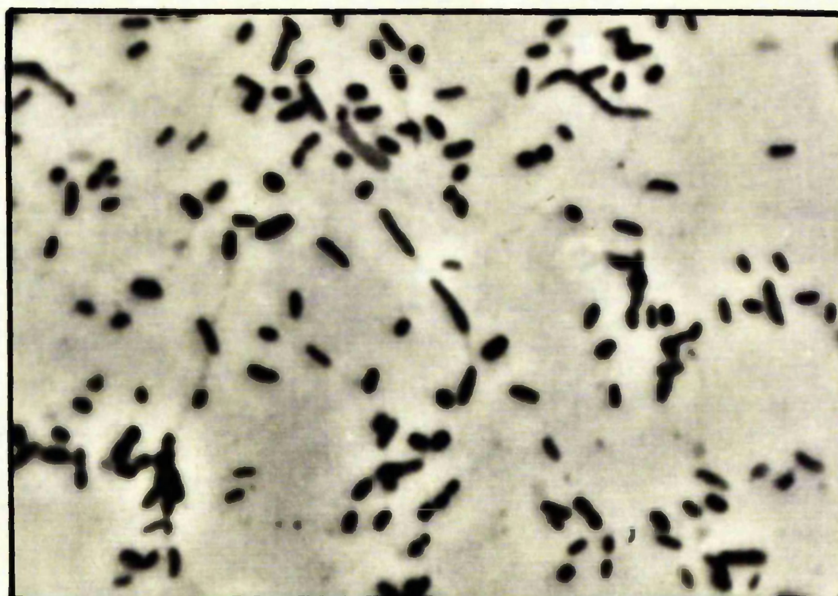
All isolates which were Gram negative rods, motile by means of polar flagella, oxidase positive, and produced oxidative acid or alkalinity in the glucose medium, were designated to the genus Pseudomonas (Shewan et al., 1960a). These organisms usually were small, irregular, and faintly stained rods (figure 32).

The members of this genus were divided into two main groups as a result of their action on glucose, and then further sub-divided on a basis of fat hydrolysis and ability to grow in the ammonium acetate medium (figure 35). Eighty-eight per cent of the strains producing acid from glucose also hydrolysed gelatin, whereas only thirty per cent of the organisms failing to produce acid from glucose had this ability. Thirty per cent of all Pseudomonas strains isolated were classified in pigment groups 2, 3 or 4 (Appendix 2). Most of these were in pigment group 2, but one group of seven organisms was "Eau-de-Nil" coloured and two strains were "primrose". All of these "pigmented" pseudomonads occurred in the group producing acid from glucose. None of the strains encountered produced a fluorescent pigment. The overall activities of this genus are presented in figure 37.

FLAVOBACTERIUM/CYTOPHAGA GROUP.

The genera Flavobacterium and Cytophaga are differentiated in Bergey's Manual 7th Ed. (Breed et al., 1957) on a basis of the ability of the Cytophaga to exhibit cell-flexing and gliding motility, properties which are absent from the genus Flavobacterium.

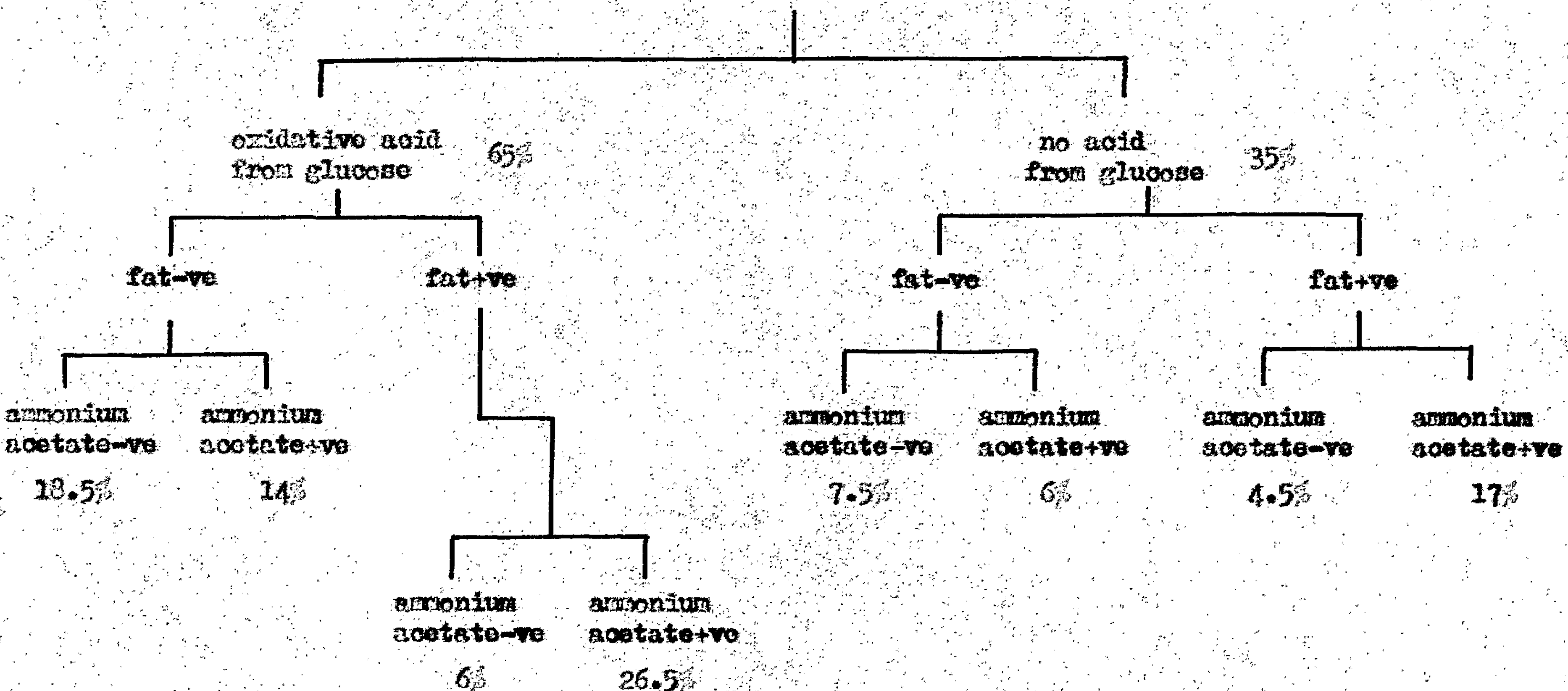
Figure 32



PSEUDOMONAS strain x 1,950

Gram's stain

Figure 33



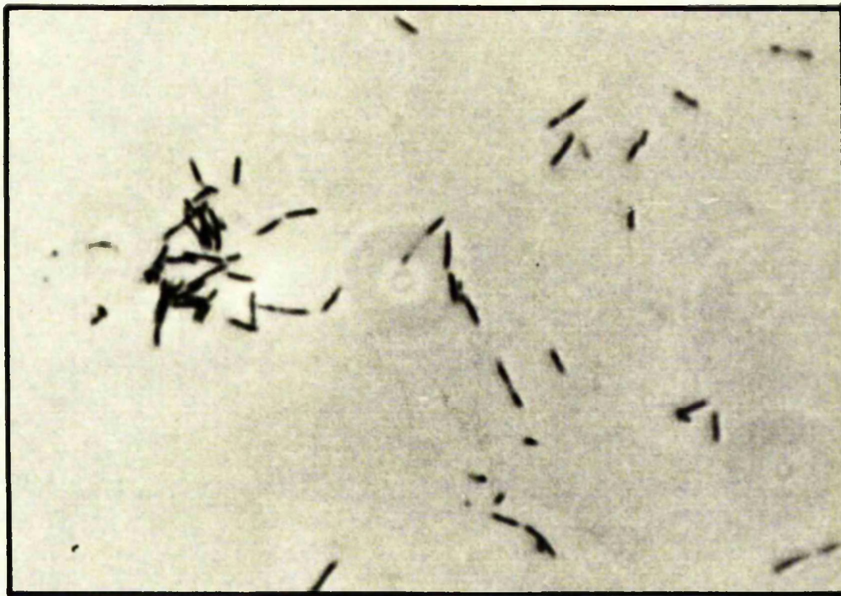
There is no difficulty in differentiating the large cell-flexing types of cytophaga from the small peritrichously flagellate flavobacteria, but these represent extremes neither of which was encountered during this investigation. Hayes (1960) has shown that the division between the small celled types of these groups is not clear cut, and since the strains isolated by the present author have all been small, slender, and non-motile Gram negative rods (figure 54), belonging to pigment group 5, the two genera have been linked together as the Flavobacterium/ Cytophaga group.

Of the forty-nine strains isolated, thirty-four produced acid from glucose oxidatively, three fermentatively, and twelve produced no acid. Such was the variety of physiological activities in this group that it was not possible to sub-divide strains as has been done with the groups previously described. However, it was possible to split the strains producing acid from glucose oxidatively, into two groups: 1) seventeen strains which tended to spread on the surface of agar, were oxidase negative, hydrolysed gelatin but not starch, and produced acid from mannite; and 2) seventeen strains which did not spread on the surface of agar, were oxidase positive, commonly hydrolysed gelatin and starch, but rarely produced acid from mannite. The overall activities of this group are presented in figure 57.

VIBRIO.

This genus has been defined as suggested by Shewan et. al. (1960a), as Gram negative rods, polarly flagellate, oxidase positive, and producing acid but no gas from a fermentative attack on glucose. The formation of spherical cells, the so called "round bodies",

Figure 34



FLAVOBACTERIUM strain x 1,950

Gram's stain

has been shown to be typical of members of this genus (Shewan *et al.*, 1960a).

The vibrios isolated during this investigation were small, irregularly shaped rods (figure 35), and often produced round bodies. They were all rapidly motile and had a single polar flagellum (figure 36).

This group of thirty-seven strains was not sufficiently large to merit further sub-division on a basis of correlative characters. Fifteen strains were "pigmented", eight of these belonging to pigment group 2 and seven to pigment group 5 ("primrose") (Appendix 2). The overall activities of this genus can be seen in figure 37.

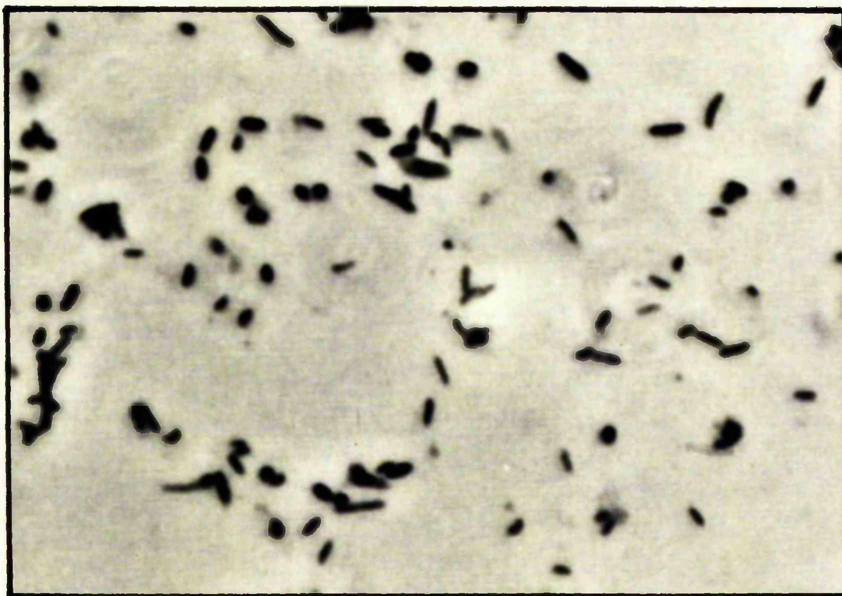
Having ascribed most of the isolates to one of these six groups it was apparent that there was a considerable variation in the percentage distribution of genera or groups among the three water samples. Figure 38 shows the percentage occurrence of the six major groups in each water sample.

MISCELLANEOUS GROUPS.

Eighty strains, representing twelve per cent of all isolates, did not fall into the six major groups already described. These included ten *Bacillus* strains, four *Nocardia*, two *Streptomyces* and seven yeasts. The remaining cultures could not be adequately classified using the available information and no further tests were carried out on them.

In order that the activities of those organisms which were not classified may be assessed, the characters of each strain are presented in Appendix 5.

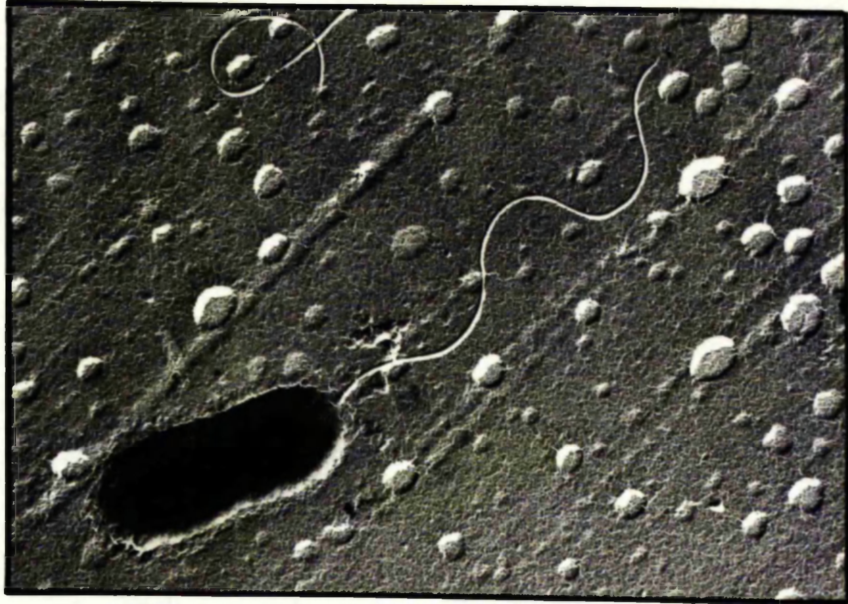
Figure 35



VIBRIO strain x 1,950

Gram's stain

Figure 36



VIBRIO strain showing polar flagellum x 15,000

5.3.6. A general comparison of the activities of the six major groups isolated.

Reference to figure 37 shows the different patterns of activity of the groups which have been previously defined and described. It can be seen that the micrococci and *Vibrio* strains were the most physiologically active groups, while *Achromobacter* was particularly inactive.

The breakdown of sugars to form acid was most common in the vibrios and micrococci though members of the genus *Pseudomonas* and the coryneform and *Flavobacterium/Cytophaga* groups were quite active in this respect. Starch was attacked to a significant extent only by members of the genera *Pseudomonas* and *Vibrio*, and to a lesser extent by the *Flavobacterium/Cytophaga* group.

Fat was hydrolysed by roughly fifty to sixty per cent of all isolates outwith the groups of *Flavobacterium/Cytophaga* and coryneform bacteria, where this activity was almost absent.

The hydrolysis of gelatin and casein was equally common among all groups except in the *Achromobacter*, where these activities were almost absent. The ability to grow in an inorganic nitrogen medium was common among the genera *Pseudomonas*, *Achromobacter* and *Vibrio*, and occurred to a much lesser extent in the coryneform group, but was very rare among the *Flavobacterium/Cytophaga* and micrococci groups. Denitrification was commonly found in all groups though the micrococci and vibrios were particularly active.

Figure 37

The distribution of characters within six groups of bacteria isolated from the North Sea

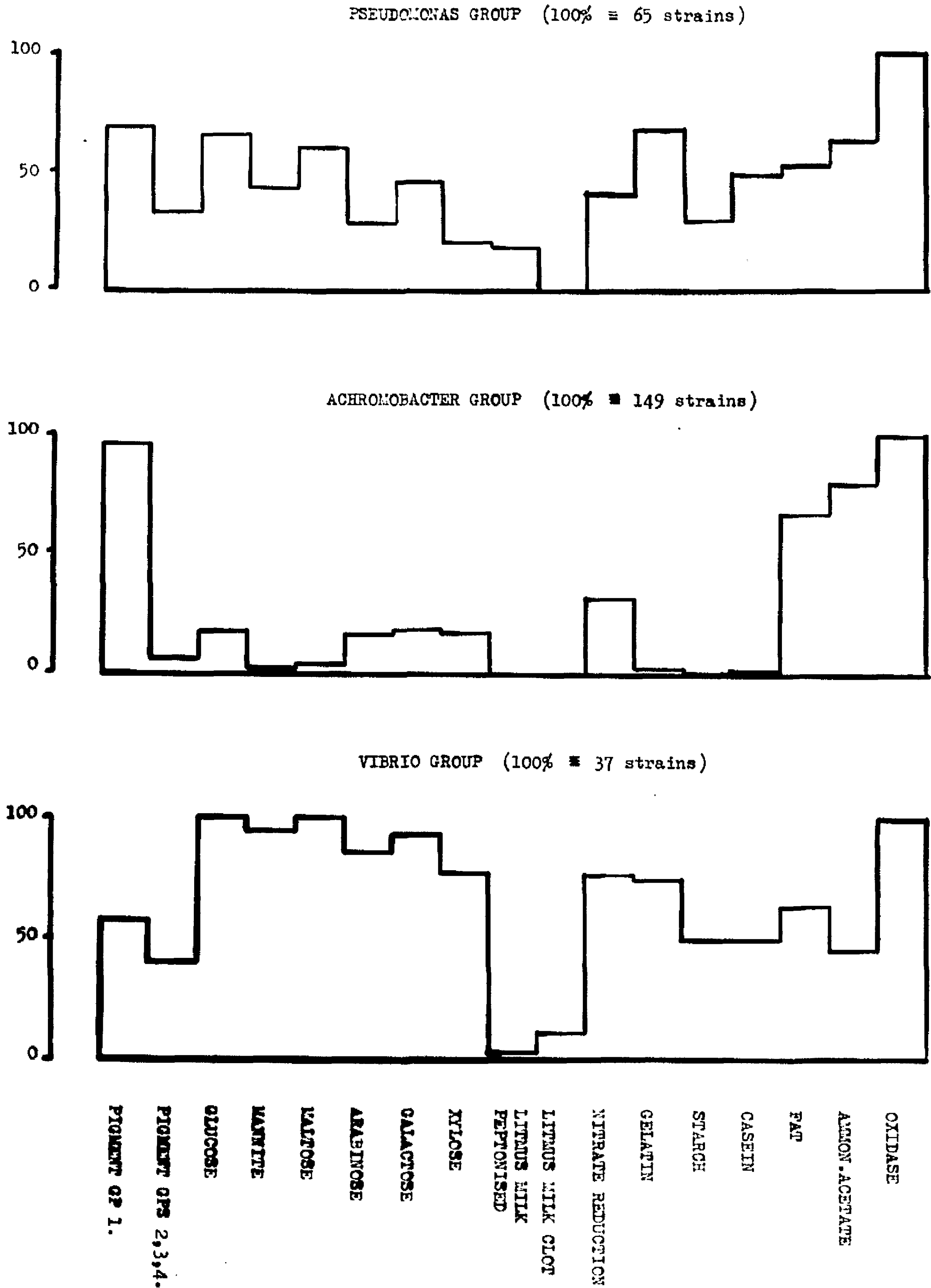
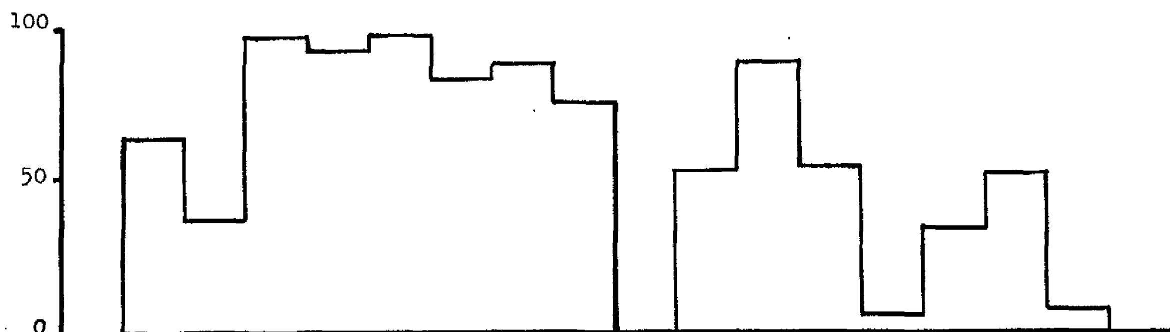
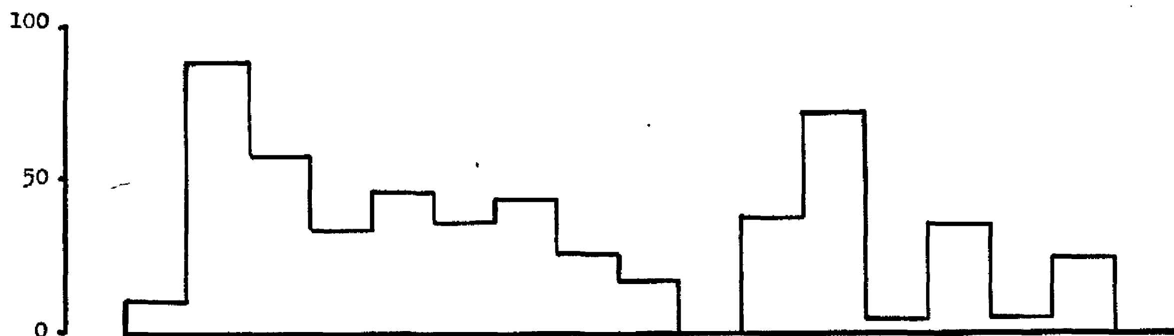


Figure 37 (continued)

MICROCOCCUS GROUP (100% ≡ 205 strains)



CORYNEFORM GROUP (100% ≡ 82 strains)



FLAVOBACTERIUM/CYTOPHAGA GROUP (100% ≡ 49)

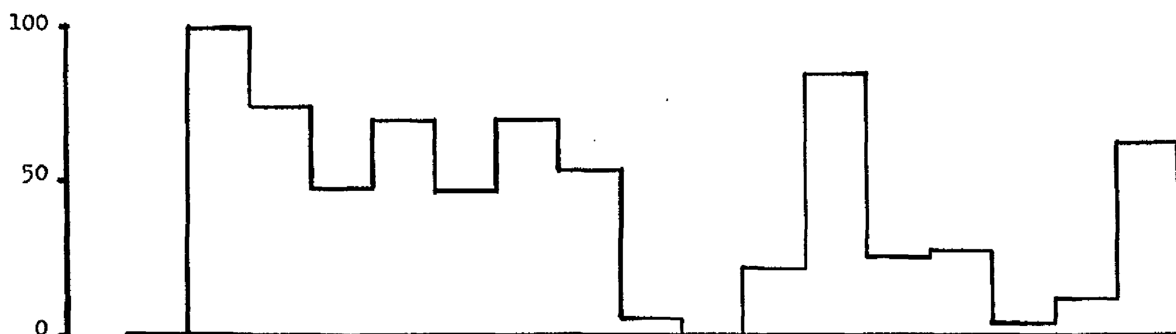


Figure 38.

The incidence of the six major groups in the three water samples examined (as %).

	Sample A	Sample C	Sample G
Pseudomonas	1	4	22
Achromobacter	50	5	27
Vibrio	1	5	9
Flavobacterium/ Cytophaga	11	< 1	13
Micrococci	4	69	4
Coryneform Bacteria	24	9	8
Miscellaneous Organisms	9	9	17

SECTION 4.

GENERAL DISCUSSION AND CONCLUSIONS.

4. GENERAL DISCUSSION AND CONCLUSIONS.

The years since the end of the Second World War have seen the expenditure of a great deal of effort in the field of marine microbiology, especially in Russia and the United States of America. The trend has two main causes: firstly, the world's oceans present an almost unexplored area of biological activity which is of great economic interest and, secondly, with the explosive increase in world population the time will come when the oceans will have to provide much more than the one per cent of the world's total food supply which they do at present. This latter point will require the organised fertilisation of large areas of sea water and this will be impossible without an understanding of the complex biological cycles which are carried out in the marine environment. A century ago, Eugene Noël showed a keen foresight when he wrote, "The ocean can be turned into an immense food factory. It can be made into a more fruitful laboratory than the earth. Fertilise it! Seas, rivers and ponds! Only the earth is cultivated. Where is the art of cultivating the waters? Hear, ye nations!"

Of central importance to the biological economy of the sea are the marine plankton and bacteria, and their interrelationships. Unfortunately there is a dearth of fundamental information about aquatic bacteria in general, and in particular knowledge of the microbial population of the North Sea is almost non-existent.

Marine bacteria can be grouped into two broad classes – the autotrophs and the heterotrophs – both of which are important

in any consideration of the physiological processes which are carried out in the sea. The work presented here was designed to give information about some of the physiological processes with which aquatic heterotrophs may be involved and to provide a basis for future work on the North Sea micro-flora. The main aims of the work were to develop cultural techniques, to assess the extent of sample to sample variation, and to evolve a picture of the nature of the bacterial flora existing in the North Sea.

The investigator embarking on a broad study of a particular environment requires isolation media which will a) enable him to grow the largest actual numbers of bacterialpresent and b) give him the most representative picture of the physiological types present, i.e. which will give him the best indication of the micro-biological potential of the environment.

The present investigation has led to the development of isolation media which are quantitatively superior to ZoBell's (1941) medium 2216 which has been most widely used in the field. These media (5 and 7, see section 2) have proved consistently superior throughout the study of several different water samples taken at different seasons.

If the purpose of an investigation is not to count the numbers of bacteria present but to estimate the range of physiological activities represented in the microbial population, the choice of isolation medium would appear, from the present work, to be immaterial since the various media gave the same overall pattern in any one sample. A point which emerges strongly, however,

is the very great qualitative differences in the flora derived from different water samples. This fact has not, on the whole, been sufficiently emphasised in previous work and it should be prominent in the minds of those engaged in the further development of this subject.

With the results of this investigation in mind it is pertinent to suggest ways in which our knowledge of the heterotrophic bacteria of the North Sea might profitably be extended:

From any one water sample a valuable picture of the physiological types of bacteria present could be obtained using medium 5 (figure 7) for isolation, and examining isolates by the scheme summarised in figure 13. Examination of the results obtained suggests that there is no significant difference in the picture emerging from a study of fifty or three hundred isolates from any one sample; a useful observation which would enable the investigator to extend the scope of his work by examining a limited number of isolates from each of a number of different water samples whilst retaining confidence in the validity of his results.

Using this approach it will be possible to investigate the fluctuations in microbial activity at a particular site throughout the year and to compare the nature of the bacterial populations in different areas. It must be emphasised, however, that such studies will inevitably involve the analysis of many samples over prolonged periods and that little useful information can be expected to emerge from a short term project.

Other important aspects of this are 1) the correlation of changes in the bacterial flora with local

changes in plankton populations and 3) the correlation of the marine bacterial flora with the flora developing on the surface of fish - a subject of obvious economic importance. These subjects should also be amenable to study along the lines suggested.

SECTION 5.

SUMMARY.

5. SUMMARY.

Marine bacteriology has received little attention in this country and no report, published during the past forty years, could be discovered where a British bacteriologist had investigated the bacterial flora of the water of the North Sea.

This investigation set out to develop methods for the study of the quantitative and qualitative nature of the aerobic heterotrophic bacteria in North Sea water.

Techniques for the isolation and further study of marine bacteria have been reviewed.

Seven media were compared with respect to their suitability for the isolation of bacteria from North Sea water, using several water samples collected at different seasons from a station ten miles E.S.E. of Aberdeen. Bacteria were isolated by plating 0·5 ml. aliquots of sea water on the surface of previously dried agar plates and incubating at 20°C. for fourteen days. One medium, containing 0·25 per cent peptone, 0·25 per cent yeast extract, 0·01 per cent ferric phosphate and 75 per cent "aged" sea water, gave consistently higher counts than the other media used.

Approximately seven hundred isolates were studied in detail using a new scheme for their characterisation and as a result it was shown that the influence of the composition of the medium used on

the qualitative nature of the flora isolated, was insignificant. There was, however, a striking difference in the bacterial populations isolated from different water samples.

The genera, or groups, of micro-organisms found in North Sea water have been: Achromobacter, Bacillus, coryneform bacteria, Flavobacterium/Cytophaga, micrococci, Nocardia, Pseudomonas, Streptomyces, Vibrio and yeasts.

APPENDIX 1.

INDIVIDUAL PLATE COUNTS FROM ALL WATER SAMPLES.

APPENDIX 1.

Individual plate counts from all water samples.

Sample A. (25/5/60) Plate counts per 1·0 ml. of sea water.

Medium 1

154	123	150	100	92
110	77	110	82	86

Medium 2

156	238	156	160	142
195	196	193	257	219

Medium 3

123	215	176	210	174
170	149	165	181	174

Medium 4

120	117	84	143	192
132	135	166	104	164

Sample B. (25/5/60) Plate counts per 1·0 ml. of sea water.

Medium 1

126	131	127	97	128
119	144	138	102	159

Medium 2

216	257	175	164	162
166	187	195	262	

Medium 3

181	157	157	150	151
155	250	185	168	159

Medium 4

161	121	262	168	268
185	250	118	225	122

Sample C. (14/12/60) Plate counts per 0·5 ml. of sea water.

Medium 1.

9 11 31 23 16

22 25 11 20 34

Medium 2.

14 16 20 16 20

16 25 22 18

Medium 3.

12 40 13 4 1

9 8 6 17 17

Medium 4.

30 30 13 19 32

18 42 25 20

Medium 5.

32 32 30 38 37

39 32 32 41

Medium 6.

10 5 17 8 6

10 15 6

Medium 7.

27 41 38 35 32

35 36 31 41

Sample D. (14/12/60) Plate counts per 0·5 ml. of sea water.

Medium 1.

49	21	12	45	47
44	24	49	40	

Medium 2.

26	53	55	15	59
41	40	54	30	26

Medium 3.

37	23	23	24	43
38	42	51	22	29

Medium 4.

41	20	53	56	45
50	38	53	57	

Medium 5.

35	42	50	31	47
45	52	39	40	49

Medium 6.

23	20	52	25	32
17	53	19	16	27

Medium 7.

26	50	36	50	37
29	55	59	32	38

Sample E. (3/10/61) Plate counts per 0·5 ml. of sea water.

Medium 1.

23	15	10	26	26
18	13	19	24	

Medium 2.

16	5	15	7	11
17	13	16	20	

Medium 3.

13	17	16	17	11
14	15			

Medium 4.

18	16	12	19	16
15	20	18		

Medium 5.

34	30	30	25	27
25	29	29	26	

Medium 6.

6	7	8	4	9
5	4	5	8	5

Medium 7.

31	29	34	30	35
35	34	35	27	37

Sample F. (3/10/61) Plate counts per 0·5 ml. of sea water.

Medium 1.

16   10   19   53   21
25   35   15   21   16

Medium 2.

24   55   16   17   25
11   20   22   24

Medium 3.

53   58   27   50   50
35   36   56   32   47

Medium 4.

23   20   25   15   15
16   27   15   20   22

Medium 5.

44   51   56   49   47
50   52   57   64   60

Medium 6.

20   11   25   15   17
14   12   23   12

Medium 7.

55   57   50   53   52
45   39   44   59

Sample 6. (3/10/61) Plate counts per 0·5 ml. of sea water.

Medium 1.

9	20	17	39	55
28	45	35	29	

Medium 2.

42	44	26	12	18
18	28	26	10	50

Medium 3.

25	8	25	20	12
22	24	11	30	

Medium 4.

25	52	25	26	24
27	51	21	55	27

Medium 5.

55	46	58	52	45
50	56	57	45	

Medium 6.

6	16	5	2	3
4	6	4	9	3

Medium 7.

69	46	56	65	59
45	42	58	55	

Sample H. (5/10/61) Plate counts per 0·5 ml. of sea water.

Medium 1.

53   40   58   57   76
57   62   65   66   68

Medium 2.

48   61   29   72   25
52   50   25   28   56

Medium 3.

85   51   56   67   89
67   62   76

Medium 4.

56   62   56   66   66
64   48   44   80

Medium 5.

73   73   82   75   86
81   74   73   66

Medium 6.

51   50   53   41   59
40   55   55   52   59

Medium 7.

70   70   60   62   48
46   50   69   86   48

Sample J. (9/10/61) Plate counts per 0·5 ml. of sea water.

Medium 1.

20 16 15 12 5
13 17 6

Medium 2.

14 8 18 17 11
8 9 15 11

Medium 3.

4 6 14 7 8
12 14 7 17

Medium 4.

8 7 5 10 10
8 9 10 9 7

Medium 5.

17 20 20 21 27
18 15 25 25 15

Medium 6.

2 5 0 6 1
6 7 1 7 1

Medium 7.

14 10 20 16 16
16 18 15 16

APPENDIX 2

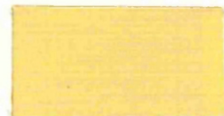
PIGMENT GROUPS

Pigment Group 1

Vellum

Portland stone

Light biscuit



Champagne

Sunshine

Beige

Pale cream

Pigment Group 2

Light buff

Middle buff

Deep buff

Light stone

Middle stone

Dark stone

Pigment Group 3

Traffic yellow

Light orange

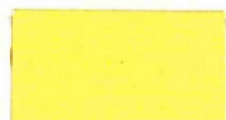
Golden yellow

Canary yellow

Lemon

Jasmine yellow

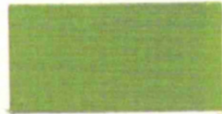
Primrose



Deep cream

0-004

Pigment Group 4



Eau-de-Nil

Poppy

Salmon pink

1-022

Appendix 2 (continued)

The figures presented here represent the number of strains having a particular pigment within each of the six major groups of isolates. Since there are only very slight differences in shades among Group 1 pigments these are not specified individually.

Pseudomonas

Pigment Group 1.		44 strains
Pigment Group 2		
	Light buff	1 strain
	Middle buff	2 "
	Deep buff	2 "
	Light stone	3 "
	Middle stone	1 "
Pigment Group 3		
	Primrose	2 strains
Pigment Group 4	Eau-de-Nil	10 strains

Achromobacter

Pigment Group 1		142 strains
Pigment Group 2		
	Light buff	2 strains
	Middle buff	2 "
	Light stone	3 "

Vibrio

Pigment Group 1		22 strains
Pigment Group 2		
	Light buff	4 strains
	Middle buff	3 "
	Light stone	1 "
Pigment Group 3		
	Deep cream	2 strains
	Primrose	5 "

Flavobacterium/Cytophaga

Pigment Group 3		
	Traffic yellow	3 strains
	Light orange	1 "
	Golden yellow	17 "
	Canary yellow	1 "
	Lemon	16 "
	Jasmine yellow	5 "
	Primrose	1 "
	Deep cream	5 "

Micrococci

Pigment Group 1		132 strains
Pigment Group 2		
	Light buff	5 strains

Micrococci (contd.)

Pigment Group 3

Golden yellow	13 strains
Canary yellow	2 "
Lemon	3 "
Jasmine yellow	14 "
Primrose	1 "
Deep cream	33 "
0-004	1 "

Pigment Group 4

Poppy	1 strain

Coryneforms

Pigment Group 1 — 10 strains

Pigment Group 2

Traffic yellow	11 strains
Light orange	1 "
Golden yellow	24 "
Canary yellow	10 "
Lemon	4 "
Jasmine yellow	16 "
Primrose	4 "

Pigment Group 4

1-022	1 strain
Salmon pink	1 "

APPENDIX 5.

All strains which were not classified into a genus or group are presented here with a list of their characteristics. For a key to the abbreviations used see page 119

Organism no.	41	51	57	62	74	204	219
Gram reaction	-	-	-	-	-	-	-
Morphology	SR	SRP	SRP	PF*	PF	CB	CB
Motility	-	-	-	-	-	-	+
Glucose	K	OxA	K	K	K	OxA	FAG
Mannite	K	A	K	K	K	A	AG
Maltose	K	A	K	K	K	A	AG
Arabinose	K	A	K	K	K	K	A
Galactose	K	A	K	K	K	A	AG
Xylose	K	K	K	K	K	K	A
Litmus Milk	-	-	-	-	-	-	-
Nitrate reduction	+	-	-	-	-	+	+
Gelatin hydrolysis	-	-	-	-	-	+	-
Starch "	-	-	-	-	-	-	-
Casein "	-	-	-	-	-	-	-
Fat "	-	-	-	-	-	-	-
Growth in ammonium acetate medium	+	+	-	-	-	-	-
Oxidase	+	+	+	+	-	-	-
Catalase	+	+	+	+	+	-	+
Pigment group	1	1	1	1	1	5	1

* See figure 59.

Organism no.	220	221	226	274	232	306	315	317
Gram reaction	-	+	-	-	-	-	-	-
Morphology	CB	CB	LR	SRP	CB	SRP	SRP	SR
Motility	+	-	+	-	-	-	-	-
Glucose	FA	FA	FA	FAG	FAG	FA	FA	FA
Mannite	A	A	A	AG	AG	A	A	A
Maltose	A	A	A	AG	AG	A	A	A
Arabinose	A	A	A	A	A	A	A	A
Galactose	A	A	A	AG	AG	A	A	A
Xylose	A	A	A	A	A	A	A	A
Litmus milk	-	-	A	-	-	-	-	-
Nitrate reduction	+	+	-	+	+	+	+	+
Gelatin hydrolysis	-	+	+	-	-	-	-	-
Starch "	-	+	+	-	-	-	-	-
Casein "	-	-	+	-	-	-	-	-
Fat "	-	-	-	-	-	-	-	+
Growth in ammonium acetate medium	+	-	+	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	+
Catalase	-	+	+	+	+	-	+	+
Pigment group	1	1	1	1	1	1	1	1

Organism no.	555	546	548	565	572	409	441	449
Gram reaction	-	-	-	-	-	-	-	-
Morphology	CB	SR	SR	F	OB	LR	F	OB
Motility	+	+	-	-	-	+	-	-
Glucose	FAG	OxA	K	OxA	FAG	FAG	K	OxA
Mannite	AG	A	K	K	AG	AG	K	A
Maltose	AG	A	K	A	AG	AG	K	A
Arabinose	A	A	K	K	A	A	K	A
Galactose	AG	A	K	K	AG	AG	K	A
Xylose	A	A	K	K	A	A	K	A
Litmus milk	-	-	K	-	-	-	K	-
Nitrate reduction	+	-	+	+	+	+	+	-
Gelatin hydrolysis	-	-	-	+	-	+	+	+
Starch "	-	+	-	+	-	+	+	-
Casein "	-	-	-	+	-	+	+	-
Fat "	-	-	-	-	-	-	-	-
Growth in ammonium acetate medium	-	-	-	-	-	+	-	+
Oxidase	-	+	+	+	-	+	+	+
Catalase	+	+	+	+	+	+	+	+
Pigment group	1	5	1	1	1	1	1	5

Organism no.	605	608	615	624	647	648	649	652
Gram reaction	+	-	-	-	-	-	-	-
Morphology	SR	SR	LR	LR	?	SR	SRP	SRP
Motility	-	-	-	+	-	-	-	-
Glucose	K	K	FA	FA	K	K	K	K
Mannite	K	K	A	A	K	K	K	K
Maltose	K	K	A	A	K	K	K	K
Arabinose	K	K	A	A	K	K	K	K
Galactose	K	K	A	A	K	K	K	K
Xylose	K	K	A	A	K	K	K	K
Litmus milk	-	-	-	-	-	-	-	-
Nitrate reduction	-	+	+	+	+	+	+	-
Gelatin hydrolysis	-	-	+	+	-	-	-	+
Starch "	-	-	+	-	-	-	-	-
Casein "	-	-	+	+	-	-	-	-
Fat "	+	-	+	+	-	-	-	-
Growth in ammonium acetate medium	+	+	+	+	-	-	-	-
Oxidase	+	+	+	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Pigment group	1	1	1	1	1	1	1	1

Organism no.	656	671	674	687	702	703	704	709
Gram reaction	-	-	-	-	-	-	-	-
Morphology	LR	SR	SRP	SR	SRP	OB	SR	?
Motility	+	-	-	-	-	-	-	-
Glucose	OxA	K	K	K	K	K	K	K
Mannite	K	K	K	K	K	K	K	K
Maltose	A	K	K	K	K	K	K	K
Arabinose	A	K	K	K	K	K	K	K
Galactose	A	K	K	K	K	K	K	K
Xylose	A	K	K	K	K	K	K	K
Litmus milk	-	-	-	-	K	-	-	-
Nitrate reduction	-	+	+	-	-	+	+	-
Gelatin hydrolysis	+	-	-	+	-	-	-	-
Starch "	-	-	-	-	-	-	-	-
Casein "	+	-	-	-	-	-	-	-
Fat "	-	-	-	+	-	-	-	-
Growth in ammonium acetate medium	+	-	+	-	+	+	+	-
Oxidase	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Pigment group	1	1	1	1	1	1	1	1

Organism no.	710	728	729	755	758	761	771	785	789
Gram reaction	-	-	-	-	-	-	-	-	-
Morphology	?	?	?	?	PF	SR	SR	LR	SR
Motility	-	+	+	-	-	+	-	-	-
Glucose	K	OxA	OxA	K	OxA	K	K	FA	K
Mannite	K	K	K	K	K	K	K	K	K
Maltose	K	K	K	K	A	K	K	K	K
Arabinose	K	A	A	K	A	K	K	A	K
Galactose	K	A	A	K	A	K	K	A	K
Xylose	K	A	A	K	A	K	K	A	K
Litmus milk	-	K	K	-	-	-	-	K	-
Nitrate reduction	+	-	-	+	-	+	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	+	-	+
Starch "	-	-	-	-	-	-	-	-	-
Casein "	-	-	-	-	-	-	-	-	-
Fat "	-	-	-	-	-	-	+	-	+
Growth in ammonium acetate medium	-	+	+	+	-	+	+	+	+
Oxidase	+	±	-	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Pigment group	1	5	3	1	1	1	1	1	1

Organism no.	795	794	811	826	862	864	866
Gram reaction	-	-	-	-	-	-	-
Morphology	SRP	SR	SR	SR	CDP	PF	SR
Motility	-	-	-	-	+	-	-
Glucose	FA	K	FA	K	K	K	K
Mannite	A	K	K	K	K	K	K
Maltose	A	K	A	K	K	K	K
Arabinose	K	K	A	K	K	K	K
Galactose	A	K	A	K	K	K	K
Xylose	A	K	A	K	K	K	K
Litmus milk	Pep	-	-	-	-	-	K
Nitrate reduction	-	+	+	-	-	+	+
Gelatin hydrolysis	+	+	-	+	+	-	-
Starch "	+	-	-	-	-	-	-
Casein "	+	-	-	-	-	-	-
Fat "	-	+	-	+	-	+	+
Growth in ammonium acetate medium	+	+	-	+	-	-	+
Oxidase	-	+	-	-	+	+	+
Catalase	+	+	-	+	+	+	+
Pigment group	5	1	1	1	1	1	1

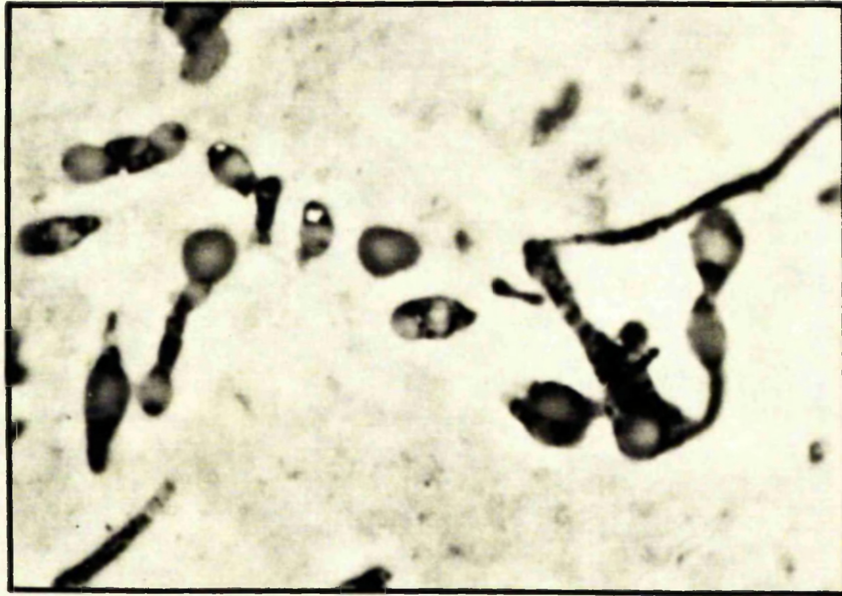
Abbreviations:

1) Morphology; SR = small rod; SRP = small pleomorphic rod; PF = irregular cells distorted by intracellular fat globules; CB = cocco-bacillus; LR = large rod; F = filaments; OB = oval bodies; CBP = pleomorphic cocco-bacilli.

2) Sugars; K = no acid; OxA = acid produced oxidatively; A = acid; FA = acid produced fermentatively; FAG = acid and gas produced fermentatively;

3) Litmus milk; K = alkaline; Pep = peptonisation.

Figure 39



Unclassified organism showing cell distortion by intracellular fat globules x 1,950

Stained with Sudan Black

SECTION 6.

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6. BIBLIOGRAPHY

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