MARINE MICROBIOLOGY

SYMPOSIUM

ON

MARINE MICROBIOLOGY

Compiled and Edited by
CARL H. OPPENHEIMER

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PREFACE

This *Symposium on Marine Microbiology* was the first attempt to bring together the leading investigators in this field from all parts of the world to exchange ideas, present research results, and discuss problems of mutual concern. It is hoped that a result of the Symposium and the publication of these reports will be increased interest in this important field, and that communications will be enhanced among marine microbiologists as well as investigators in collateral sciences.

It was by design that this meeting was held in conjunction with the 61st annual meeting of the American Society of Microbiologists in April 1961. By doing so, an opportunity was afforded for scientists, who did not have a primary interest in marine microbiology, to gain an understanding of its scope and importance. Interest in this field was evidenced by the large number of microbiologists who came to the Symposium prior to the opening of the ASM meetings. The success of the Symposium has led to the suggestion that future international conferences on marine microbiology be held at three or four year intervals—the next may be held in association with the Society for General Microbiology in England in 1965.

It is apparent from the scope of the papers, the discussion, and interests of participants, that the field of marine microbiology has grown tremendously in the last few years. The handful of people working in the 1930's has expanded to a composite group attempting to evaluate the role of microorganisms in many aspects of oceanography. It is also apparent that there are many gaps in our knowledge of microbial activities in the marine environment, and it is hoped that the Symposium has provided the personal interchange of ideas and the evaluation of small details of current research which will prove valuable in improving the

standardization of future research, and will aid in a cooperative effort to understand the many roles of microorganisms in the marine environment.

This Symposium was sponsored by the Microbiology Branch of The Office of Naval Research through a contract with the American Institute of Biological Sciences. The ASM contributed materially by scheduling the Symposium as a part of the annual meeting program. I should like to express my sincere thanks to the representatives of ONR, AIBS and ASM, and to those others who also contributed to the success of the Symposium and the publication of this volume.

Carl H. Oppenheimer

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MARINE MICROBIOLOGY

Chapter 1

Domain of the Marine Microbiologist*

Claude E. ZoBell

In order to set the stage for the following papers, my assignment is to make a few generalizations regarding the marine environment as a habitat for microorganisms. Among the many kinds of microorganisms considered are bacteria, actinomycetes, yeasts, fungi, blue-green algae, certain diatoms, dinoflagellates, microflagellates, some of the simpler protozoans, and viruses. Just as there is no well defined demarcation between microorganisms and macroorganisms, there is likewise a gradual merging of marine with brackish and fresh water environments, particularly in marshes, bays, estuaries, rivers, inland seas, and lakes. Most inland waters find their way into the sea, and information gained from the study of inland and coastal waters contributes to an understanding of conditions in the sea.

The sea has been described as a gigantic biochemical laboratory or chemostat in which a great diversity of organisms function together and in competition with each other for the sparsely concentrated nutrient substances. The kinds of organisms present, their abundance, characteristics, and activities are influenced by each other as well as by environmental conditions. As contrasted with other major habitats, the marine milieu is distinctive in its vast area and volume, its low content of organic matter, relatively high salinity, low temperature and high pressure at great depths.

The marine microbiologist is concerned with the qualitative and quantitative evaluation of the physiological activities of microorganisms that influence the state of carbon, nitrogen, sulfur, phosphorus, hydrogen, oxygen, and other elements in the sea

* Contribution from the Scripps Institution of Oceanography. New Series. This work was supported in part by grants from the Office of Naval Research, Task No. NR 103-020.

and its bottom sediments. Besides the basic task of studying microorganisms for their own sake, the marine microbiologist must also be concerned with (a) the effects of microorganisms on higher plants and animals and the productivity of the sea, (b) stimulating and inhibitory effects of microbial metabolites, (c) microbes as agents of disease, (d) their role as geochemical agents and in the diagenesis of marine sediments, (e) microorganisms as indicators of the origin, movement, and properties of water masses, (f) a study of the unique properties of marine microorganisms which might contribute fundamentally to a better understanding of the origin and processes of life, (g) problems of microbial corrosion, deterioration, fouling, and spoilage of materials of economic significance, (h) fate in the sea of microorganisms of sanitary significance, and (i) many other problems.

SALINITY OF NATURAL WATERS

Approximately 72 per cent of the earth's surface is covered with water. Exclusive of juvenile water in the primary lithosphere, more than 98 per cent of the world's water occurs in the sea or its sediments (18). About 1 per cent occurs in polar and continental ice. Inland waters (freshwater lakes, rivers, reservoirs, marshes, soil moisture, circulating ground waters) and atmospheric moisture account for less than 0.2 per cent of the world's water.

The salt concentration in the hydrosphere ranges from less than 1 ppm in certain alpine lakes, increasing in amounts in river mouths and estuaries to about 35‰ in the open ocean. Ten times this amount, or full saturation, occurs in certain landlocked salt water bodies. Remote from the influence of melting ice, large rivers, heavy rainfall, or excessive evaporation, the salinity of ocean waters ranges from 33 to 38 ‰. In many localized coastal areas ocean water gradually blends with fresh water from rivers or rain to become brackish. Excessive evaporation in other localized areas where there is little mixing may result in superhalinity as in salt marshes and salt lakes.

The majority of the microorganisms when taken directly from the open ocean (water as well as bottom sediments) re-

quire sea water or its equivalent. Most recently isolated species grow almost equally well at any salinity between 20 and 40 per cent. Fewer than half of such species grow well in nutrient media prepared with double strength sea water (S‰ = 70) or in media prepared with sea water diluted with distilled water to reduce the salinity to 10 ‰. In general, marine microorganisms tolerate hypotonicity much better than hypertonicity. Their tolerance increases during laboratory cultivation. The proportion of sodium, potassium, calcium, magnesium, and other major ions as well as the salinity and osmotic pressure of the milieu influence the growth and metabolism of marine bacteria (35, 36, 55).

Although there is some variation in the salinity of water in the open ocean, the ratios between the major ions are virtually constant. Tremendous differences in these ratios occur in different fresh, brackish, and superhaline waters depending upon the source and history of the water. The ratios of the principal anions (carbonates: chlorides: sulfate) and cations (calcium: magnesium: sodium: potassium) in sea water are quite different from these ratios in most fresh waters (Table 1). The ratios of most brackish waters are intermediate between those of fresh water and sea water. Evaporation of sea water results in marked changes in the proportions of ions owing to differential precipitation of salts.

TABLE 1

AVERAGE RATIOS OF MAJOR IONS IN OCEAN WATER COMPARED WITH THE MEAN VALUES FOR MANY RIVER WATERS AND SOME SOFT WATER WISCONSIN LAKES, COMPUTED FROM DATA OF CLARKE (7) AND HUTCHINSON (18)

Ratio of	*Ocean Water*	*River Water*	*Lake Water*
CO_3* : Cl	1 : 135	7.3 : 1	3.3 : 1
K : Na	1 : 28	1 : 4.6	1 : 2.2
Ca : Na	1 : 26	4 : 1	2.3 : 1
SO_4 : Cl	1 : 7.1	1.6 : 1	2.1 : 1
Ca : Mg	1 : 3.2	3.6 : 1	1.3 : 1

* Including HCO_3^- where present

CHEMICAL COMPOSITION OF SEA WATER

Ten major components (Table 2) constitute more than 99 per cent of the salts in solution in sea water. The remainder is made up of a large number of other elements, some in concentrations of less than 1×10^{-10} mg/liter. Many of the minor constituents are concentrated several hundredfold or thousandfold in certain marine plants or animals; thus their concentrations in different water masses depend upon the previous biological history of the waters (15). Most trace elements known to effect the metabolism of bacteria and higher organisms occur in sea water in concentrations ranging from 0.1 to 50 μg/liter.

TABLE 2

MAJOR CONSTITUENTS, EXCLUSIVE OF GASES, IN SOLUTION IN OCEAN WATER OF SALINITY ($S‰ = 35.00$ AT pH 8.0: COMPUTED FROM DATA OF LYMAN AND FLEMING (33)

Constituent	*Grams/kg*	*Grams/L at 20°C*	*Per Cent of Solids*
Chloride (Cl^-)	19.264	19.745	55.04
Sodium (Na^+)	10.714	10.981	30.61
Sulfate ($SO_4^=$)	2.688	2.755	7.68
Magnesium (Mg^{++})	1.291	1.323	3.69
Calcium (Ca^{++})	0.406	0.416	1.16
Potassium (K^+)	0.381	0.390	1.10
Bicarbonate (HCO_3^-)	0.142	0.145	0.41
Bromide (Br^-)	0.066	0.067	0.19
Boric acid (H_3BO_3)	0.026	0.027	0.07
Strontium (Sr^{++})	0.013	0.013	0.04
Micro-elements ca.	0.005	0.005	0.01
Totals	35.000	35.867	100.00

Concentrations as well as the molecular organization of carbon, nitrogen, oxygen, phosphorus, silicon, and sulfur influence and are influenced by biological activities; microorganisms are particularly important in this respect. The concentrations of plant nutrients, notably compounds of nitrogen, phosphorus, and silicon (required for the skeletons of diatoms and radiolarians), vary considerably in time and space (2), being affected by bio-

Chemical abundance in the marine hydrosphere

Compiled by Edward D. Goldberg and Claude Michaux*

	mg/L	*atoms/10^4 atoms Cl*		*atoms/10^4 atoms Cl*	
H	108,000.	202,000,000.	Ag	0.0003	0.005
He	0.00005	.0004	Cd	0.00011	0.002
Li	0.2	50.	In	<0.02	<0.3
Be			Sn	0.003	0.05
B	4.8	830.	Sb	<0.0005	<0.008
C	28.	4,300.	Te		
N	0.5	70.	I	0.05	0.7
O	857,000.	100,000,000.	Xe		
F	1.3	130.	Cs	0.0005	0.005
Ne	0.0003	0.03	Ba	0.0062	0.008
Na	10,500.	850,000.	La	0.0003	0.004
Mg	1,300.	100,000.	Ce	0.0004	0.005
Al	0.01	0.7	Pr		
Si	3.	200.	Nd		
P	0.07	4.	Pm		
S	900.	52,000.	Sm		
Cl	19,000.	1,000,000.	Eu		
A			Gd		
K	380.	18,000.	Tb		
Ca	400.	19,000.	Dy		
Sc	0.00004	0.002	Ho		
Ti	0.001	0.04	Er		
V	0.002	0.08	Tm		
Cr	0.00005	0.002	Yb		
Mn	0.002	0.07	Lu		
Fe	0.01	0.3	Hg		
Co	0.0005	0.02	Ta		
Ni	0.0005	0.02	W	0.0001	0.001
Cu	0.003	0.09	Re		
Zn	0.01	0.3	Os		
Ga	0.0005	0.01	Ir		
Ge	<0.0001	<0.003	Pt		
As	0.003	0.07	Au	0.000004	0.00004
Se	0.004	0.1	Hg	0.00003	0.0003
Br	65.	1,500.	Tl	<0.00001	<0.00009
Kr			Pb	0.003	0.03
Rb	0.12	2.2	Bi	0.0002	0.002
Sr	8.	160.	Po		
Y	0.0003	0.006	At		
Zr			Rn	9.0×10^{-15}	8.0×10^{-14}
Nb			Fr		
Mo	0.01	0.2	Ra	3.0×10^{-11}	2.0×10^{-10}
Tc			Ac		
Ru			Th	0.0007	0.006
Rh			Pa		
Pd			U	0.003	0.02

* *Geol. Soc. Amer. Mem.* 67, Vol. 1, p 346, 1957.

logical activities and also by the movements of water masses. Upwelling, eddy diffusion, and vertical convection are especially important in the transport of nutrient substances from deep water to the euphotic zone. Harvey (15) reports concentrations of some important nutrient elements in sea water as follows:

Constituent	μg/liter	Constituent	μg/liter
Ammonium-N	<5-50	Silicate-Si	1-3000
Nitrate-N	1-600	Phosphate-P	<1-60
Nitrate-N	0.1-50	Organic-P	0-16
Organic-N	30-150	Organic-C	200-5000

There is considerable variation in the quantity as well as in the kind of organic matter in sea water (9). The concentration of organic matter ranges roughly from 0.4 to 10 mg/liter in the open sea and up to 100 mg/liter in shallow productive bays. From 60 to 85 per cent of this exists in a colloidal or other finely particulate state, generally less than 25 per cent in a dissolved condition (13). Living cells, including plankton organisms, make up a small fraction (usually less than 10%) of the total organic matter, according to Fox *et al.* (12) who apply the term leptopel to the colloidally or otherwise finely particulate suspended detritus. Fox (11) estimates that the standing crop of organic leptopel in the world's oceans exceeds by at least tenfold (perhaps a hundredfold) the annual synthesis or standing crop of total living marine organisms.

An appreciable part of the organic leptopel consists of relatively inert substances such as humus, chitin, lignin, cellulose, complex proteins, and other refractory products along with partially decomposed cellular materials. Dissolved organic substances identified in sea water include traces of polypeptides, various amino acids, organic acids, certain sugars, biotin, carotenoids, enzymes, niacin, thiamine, and cobalamin (9, 56). The organic content of sea water is in a state of constant flux, being continually enriched by the organic remains, dissimilation products, and excretory materials from biota while losing organic substances through assimilation, oxidation, mineralization, and other processes.

Despite the low concentration of organic matter in sea water, it is a prime determinant of the growth of many kinds of micro-

organisms. It serves as a source of energy and building material for heterotrophs. Certain organic fractions stimulate growth or provide essential growth factors (1, 44, 51). Other organic fractions in sea water have inhibitory or antibiotic properties (32, 49, 56). A fourth possible role of organic matter is to influence the availability of trace metals by chelation, the effect of which may be beneficial or detrimental, depending upon the circumstances (47).

TEMPERATURE AND PRESSURE

Whereas pure water has its maximum density at 4 C and freezes at 0 C, sea water is most dense at its freezing point. The freezing point of sea water is a function of its salinity, being about −1.86 C at salinity 35‰. As sea water nears its freezing point in polar regions and becomes more dense, it sinks and fills ocean basins with cold water. This unique property of sea water has far-reaching effects on the movement, oxygenation, temperature distribution, and biology of water masses. At the ocean surface the temperature of water ranges from its freezing point to about 28 C in the tropics. Higher temperatures occur in shallow water in certain localized areas. Average surface ocean temperatures at different latitudes are shown in Table 3.

TABLE 3

AVERAGE SURFACE TEMPERATURE OF OCEANS FROM EQUATOR SOUTH TO POLAR ICE FRINGE: ADAPTED FROM SVERDRUP, JOHNSON, AND FLEMING, (54)

South Latitude	*Atlantic Ocean*	*Pacific Ocean*	*Indian Ocean*	*Antarctic Ocean*
	C	C	C	C
0-10°	25.2	26.0	27.4	—
10-20°	23.2	25.1	25.8	—
20-30°	21.2	21.5	22.5	—
30-40°	16.9	17.0	17.9	—
40-50°	8.9	11.2	8.7	—
50-60°	1.8	5.0	1.6	—
60-70°	−1.3	−1.5	−1.3	−1.4
70-80°	—	—	—	−1.5

The seasonal variation of ocean surface temperature is generally less than 10 C, being least in tropical waters and greatest in temperate zones. Below 200 meters ocean water has a nearly constant temperature at any specified depth (54). At depths exceeding 1000 meters temperatures are constant for large water masses at between −1.5 and 4.5 C; mostly between 2 and 3 C. More than 90 per cent of the marine environment by volume is colder than 5 C.

Besides affecting the activity rates of organisms and possibly their nutrient requirements (31), temperature is an important ecologic factor. In view of the relatively low temperatures characteristic of so much of the marine environment, one might expect psychrophilic microflora to predominate. Indeed, a good many of the bacteria taken from the sea grow slowly in nutrient media when incubated at 0 to 5 C, but virtually all of them grow much faster at 20 to 25 C. This generalization includes bacteria from hundreds of samples of water and deep sea sediments taken from places where the prevailing temperature is lower than 5 C. Ingraham and Stokes (19) point out that bacteria capable of growing slowly at 0 C or lower are commonly found in frozen foods, water, soil, and other materials, but most species of such psychrotolerant bacteria grow more rapidly above 20 C. Nearly half of the bacteria recently isolated from the sea will grow at 30 C; very few grow at 37 C or higher (62). Anomalously, however, a few thermophilic bacteria have been found in arctic and deep sea materials. In deep ocean bottom cores Bartholomew and Rittenberg (3) found from 100 to 800 bacteria per gram, which developed at 60 C but failed to grow at either 20 or 37 C. In arctic soil, peat, beach sand, and mud McBee and McBee (38) found numerous bacteria which formed colonies on nutrient agar incubated at 55 C.

Apart from these anomalous thermophilic bacteria and a few thermoduric sporeformers, most marine bacteria are thermosensitive, generally much more so than freshwater or soil bacteria. Only 7 to 18 per cent of the bacteria taken from the Philippine Trench survived ten minutes' exposure at 40 C (62). From 20 to 30 per cent failed to survive ten minutes' exposure at 30 C. Many of the bacteria occurring in sea water and marine mud

were killed by being plated with molten nutrient agar poured at 42 to 45 C (64). The maximum temperature for growth and the thermal death point for most marine bacteria are only a few degrees higher than their optimal growth temperature. The temperature optima and tolerance of microorganisms is influenced by salinity (45) and hydrostatic pressure (21, 65).

Hydrostatic pressure increases with depth in the sea almost exactly as a straight-line function, the pressure-depth gradient being about 0.1 atm/m. At the greatest known depths (60) pressures of nearly 1100 atm prevail. Approximately one-half of the ocean area, or one-third of the total area of the earth, is covered with 3800 or more meters of water, an aquatic environment characterized by pressures ranging from 380 to 1100 atm. Such pressures retard the growth of most surface-dwelling bacteria, yeasts, and fungi from terrestrial soil, sewage, freshwater bodies, and shallow (less than 3000 to 4000 m) layers of sea water. A good many, but not all, surface-dwelling microbes lose their viability after several days in nutrient media compressed to 600 atm (43). Few, if any, though, appear to be injured by a few minutes' compression to 1000 atm. In unenriched water and soil samples, surface-dwelling bacteria have been found to be viable after several days' compression to 1500 atm at 5 to 25 C. Such observations suggest that the pressure tolerance of bacteria depends in part upon the chemical composition of the milieu. Considerable difference has been observed in the pressure tolerance of different microbial species.

Many bacteria recovered from the deep sea floor (7,000 to 10,000 m) grow in nutrient media at ambient pressures, i.e., 700 to 1000 atm (25, 67). A few of these deep sea bacteria, to which the term barophilic has been applied, appear to grow only at pressures matching their native habitat (65, 66). But other deep sea bacteria grow as well or even better at 1 atm than when compressed (25, 67). Bacteria in this latter category (a) may be passive mutants which have come into being on the deep sea floor, (b) they may be shallow water forms which have settled and survived on the deep sea floor, or (c) they may be eurybaric species capable of growing throughout a wide range of hydrostatic pressure under certain conditions. Little is known

regarding the influence of nutrients, osmotic pressure, and other properties of the medium on the pressure tolerance of microorganisms, but such factors are believed to be important just as temperature is known to be (21).

Marked variations in morphology (68) are induced by incubating certain bacteria for a few days at hydrostatic pressures somewhat higher than their natural habitat. When thus compressed in nutrient media, cells of most species examined by Oppenheimer and ZoBell (43) grew to be somewhat larger in diameter and considerably longer. Similarly, Chumak (6) studied a barotolerant culture whose cells were 3.4 and 4.1 μ when incubated at 1 atm, but formed filaments 200 to 300 μ long when incubated under otherwise comparable conditions at 500 to 750 atm. Such compression was found to increase glucose utilization by this culture.

DISTRIBUTION OF MARINE MICROORGANISMS

Diatoms, dinoflagellates, blue-green algae, chlamydomonads, chrysomonads, cryptomonads, coccolithophores, silicoflagellates, and other photosynthetic microorganisms, including a few green and purple sulfur bacteria, are confined largely to the topmost layers of illuminated water or the surface of shallow sediments. The depth to which sunlight penetrates depends upon the transparency of the water and the intensity and angle of incident radiations, which vary seasonally and with latitude (17). The lower boundary of the euphotic zone, where there is sufficient sunlight to support photosynthesis, ranges from less than a meter in turbid estuaries to more than 125 meters in clear blue tropical waters. Some photosynthetic organisms sink below the euphotic zone and a specialized few are able to exist in the dark as heterotrophs (30). Only relatively small numbers of photosynthetic organisms occur at depths exceeding 200 meters, but some find their way to the deep sea floor (58).

Within the euphotic zone the abundance of photosynthetic organisms is influenced by the concentration and kinds of plant nutrients, organic metabolites, trace metals, illumination, temperature, pH, gas tension, turbulence, upwelling, eddy diffusion, pred-

ators, pathogens, and a tangle of other interrelated factors (15, 39, 44). There are diurnal, seasonal, and geographic differences in predominating species and population densities. The population of diatoms and dinoflagellates ranges from nil to 10^5 per liter. In certain waters the nannoplankton population reaches 10^6 per liter. Knight-Jones and Walne (23) found the small chrysomonad flagellate *Chromulina pusilla* in numbers ranging from 50 to 3500 per ml in English Channel waters. Rarely does the standing crop of phytoplankton exceed 0.2 mg organic carbon per liter in the open ocean (52).

The abundance, kinds, and products of photosynthetic organisms influence the occurrence of animals and bacteria in marine environments. Directly or indirectly, animals and heterotrophic bacteria depend for food upon organic matter synthesized by plants. Thus the greatest number of bacteria and animals are found in the topmost few hundred meters of water and on the sea floor to which the organic remains of plants and animals settle. Various workers (25, 57) have noted a parallelism between the abundance of bacteria and phytoplankton. It is an oversimplification of the situation, however, to say that the abundance of bacteria bears a direct relationship to the abundance of plankton. Bacteria are often most abundant in the wake of a plankton bloom rather than during the bloom. As stated by Harvey (15), the equilibrium between the standing crop of plants, bacteria, herbivores, and carnivores is continually passing in and out of balance. The annual production of zooplankton may be no more than one-tenth the annual production of phytoplankton, upon which they depend for food, but at times the standing crop of zooplankton may exceed the standing crop of phytoplankton. Similarly, the bacterial biomass sometimes exceeds the combined standing crops of phytoplankton and zooplankton. Bacteria are subject to predation by grazing zooplankton and a good many other animals.

Exceptionally large bacterial populations occur where land drainage, sedentary algae, coral reefs, plankton blooms, mass mortalities, convergences, or other conditions make for high concentrations of organic matter. Although dependent upon organic

matter for food, the well-being or survival of heterotrophic bacteria is influenced by predators, solid surfaces, and numerous other factors.

Unless otherwise stated, data on the abundance of bacteria are based upon the number which form visible colonies under laboratory conditions of cultivation. Since no one medium or combination of cultural conditions (temperature, oxygen tension, pH, etc.) provide for colony formation by all bacteria, colony counts generally represent only a small fraction of the total number of viable cells. Direct microscropic methods of observation often reveal from 10 to 100 times more bacteria than do cultural procedures (20).

In the littoral zone, where most observations have been made and where biological activity is usually most intense, colony counts commonly indicate the presence of from 10^2 to 10^3 bacteria per ml of water; the extremes range from <1 to 10^8 bacteria per ml. The scantest bacterial populations occur in the open ocean particularly at depths exceeding 1000 meters. So scarce are bacteria in many pelagic areas that from 50 to 1000 ml of water must be analyzed (either by concentration on membrane ultrafilters or by enrichment with nutrients) in order to demonstrate the presence of viable bacteria.

During the 1957-58 *Vitiaz* expedition Kriss (25) examined 1039 water samples collected at depths 0 to 9,000 meters in the central regions of the Pacific Ocean along the 172nd meridian east longitude and 174th meridian west longitude between 37° north latitude and 41° south latitude. Following filtration of 50 ml of water through membrane ultrafilters, the filters were superimposed on meat or fish peptone agar in Petri dishes. After three to four days, incubation at 25 to 30 C the colonies were counted. The number of colonies developing from 50 ml of water ranged from nil to a few hundred. There was a high order of variability in the abundance of bacteria vertically as well as horizontally. Great differences in the abundance of bacteria found in different areas were attributed to the concentration of easily assimilable organic matter. Considerable variation in the abundance of bacteria was also noted by Kriss (25) in the Indian Ocean where

1117 water samples from 54 stations at depths 0 to 5370 meters were examined on the *Ob* expedition. The number of colonies developing on membrane ultrafilters through which 40 ml of water had been passed ranged from nil to several hundred.

Beginning in 1954 Soviet microbiologists (25) worked on drifting ice stations in the vicinity of the North Pole (86 to 90° north latitude). Through holes cut in the ice a large number of water and bottom sediment samples were collected and glass slides were submerged for studying the relative abundance and growth rates of microorganisms (26). Although appreciable numbers of bacteria and a few yeasts appeared on slides during twenty-four hours submergence, some to a depth of 3450 meters, very few colonies developed.

BACTERIA IN BOTTOM SEDIMENTS

Bacterial populations ranging from <10 to 10^8 per gm wet weight of bottom sediment have been detected by cultural procedures (61). Mean values fall within the range of 10^2 to 10^5 bacteria per gram of sediment. Living bacteria have been demonstrated at depths as great as 10,000 meters (67) and at latitudes from the Equator to the North Pole (25). Bacteria are most abundant in the topmost layers of bottom sediments under shallow organic-rich waters. The abundance of bacteria falls off fast with increasing core depth.

At many mid-Pacific Ocean stations (depth 1700 to 5900 m), Morita and ZoBell (40) found only 10 to 10^4 bacteria per gm of the topmost 10 cm layer of pelagic sediment and none at the bottom of certain cores 300 to 600 cm long. Kriss (25) reports finding from 1000 to 40,000 heterotrophic bacteria per gram of surface layers of sediment from deep water (2000 to 8000 m) areas of the Okhotsk Sea adjacent to the Pacific Ocean. At the bottom of cores about a meter long, bacteria were found in numbers of less than 100 per gm. These low numbers may be contrasted with bacterial populations of 16^6 per gm of surface sediment in shallow marine bays; 10^4 at the bottom of 75 to 130 cm cores (41). In the surface layers of freshwater lake sediments Hayes and

Anthony (14) often found 10^6 bacteria per gm, 10^4 to 10^5 per gm at core depths of 0.5 to 1 m, and 10^2 per gm down to 6 m.

BACTERIAL BIOMASS

The bacterial biomass is computed from data on the abundance of bacteria and their mean weight. The volume of most marine bacteria falls within the range of 0.1 to 1 μ^3. Since smaller cells predominate, 0.2 μ^3 is taken as the mean volume of marine bacteria for purposes of estimating their biomass. The weight of such a bacterium is about 2×10^{-10} mg, its dry weight 4×10^{-11} mg, and its organic carbon content about 2×10^{-11} mg. From these values bacterial biomasses are calculated for different population densities. The second and third columns in Table 4 represent the standing crop or standing stock. Of greater significance is the quantity of organic carbon cycled annually through bacterial cells. The annual production of bacterial carbon is derived by multiplying the standing crop values by the number of bacterial generations per year. The fourth column in Table 4 gives orders of magnitude of bacterial carbon for different population densities based upon an assumed generation time of twenty-four hours or 365 generations per year. (See Chapter 65) (Zhukova).

TABLE 4

Bacterial Biomass Per Cubic Meter of Water For Different Population Densities and the Annual Production of Bacterial Carbon if the Bacteria Reproduce Every Twenty-Four Hours

Bacteria per ml	*Bacterial Biomass per Cubic Meter of Water*		*Annual Production Per Cubic Meter*
	mg wet wt.	mg carbon	mg carbon
10	0.002	0.0002	0.073
10^2	0.02	0.002	0.73
10^3	0.2	0.02	7.3
10^4	2.0	0.2	73.0
10^5	20.0	2.0	730.0

The generation time of marine bacteria varies from less than an hour to several days or maybe many months, depending upon

species, temperature, nutrients, and other factors. In large volumes (5 gal) of natural sea water maintained in glass bottles at 12 C, the bacterial generation time during the first few days was found to range from ten to twenty hours. During the first two days' storage at 22 C the generation time was only three to six hours. But for various reasons marine bacteria are more active under laboratory conditions than *in situ*.

By noting the increase in the number of cells in microcolonies developing on glass slides submerged in the sea, Kriss and Rukina (28) estimated the bacterial generation time to range from 2.0 to 3.4 hours during the first eight hours submergence and from 6.8 to 18.7 hours during twenty-five hours submergence. From the rate at which microcolonies grew on submerged slides, Kriss and Markianovich (27) estimated the bacterial biomass in the Caspian Sea to increase 13 to 80 per cent per day. Employing similar techniques, Kriss and Lambina (26) estimated a daily increase of 12 to 72 per cent in the bacterial biomass in Arctic Ocean water near the North Pole. Assuming a steady state in which bacteria die off at the same rate at which they reproduce, a 12 per cent increase in bacterial biomass is equivalent to a generation time of 8.3 days or about 44 generations per year; an increase of 80 per cent in biomass is equivalent to a generation time of 1.25 days or 292 generations per year. In some ways the submerged slide technique approximates natural conditions, but bacteria may develop more rapidly on such solid surfaces than when free-floating in water.

There is extreme patchiness in the abundance of bacteria in the sea and probably even greater variability in their reproductive rates. The abundance as well as the rate of reproduction of bacteria is influenced by various environmental conditions—especially by available organic matter which limits the bacterial biomass. The net production of organic matter by photosynthetic organisms in the sea ranges roughly from 0.03 to 10 gm of organic carbon per square meter per day, the exact amount depending upon season, locality, and a multiplicity of factors affecting the growth of plants (46, 53). The mean net production by phytoplankton is estimated to be from 35 to 350 grams organic carbon per square meter per year, an amount equivalent to 1.2

to 12×10^{16} gm per year for the hydrosphere, or from 0.008 to 0.08 gm per cubic meter. This amount must be many times more than the quantity of organic carbon cycled annually through the cells of heterotrophic bacteria, because much of the phytoplankton crop is consumed by zooplankton and other grazing animals.

From all available data on the abundance of bacteria in different water masses, the amount of organic carbon in the standing crop appears to range from as little as 1×10^{-9} gm per cubic meter in many areas of the open ocean to as much as 10 gm per cubic meter in certain coastal waters. Unfortunately, quantitative data are too fragmentary to warrant making an estimate of the average value; even less is known about the rate of reproduction or number of bacterial crops per year. Kriss (24) estimates the mean range in the standing crop of bacteria in different natural bodies of water as follows:

Water Body	Bacteria per ml	Biomass per m^3
Freshwater lakes	23×10^3 - 12×10^6	9 - 5,000 mg
Rivers	25×10^3 - 15×10^5	10 - 600 mg
Inland and shallow seas	3×10^3 - 25×10^5	0.6 - 340 mg
Open oceans	10 - 8×10^3	.002 - 1.6 mg

These values may be conservatively small since the bacterial populations were determined by cultural procedures which ordinarily detect only a small percentage of the viable cells. Employing direct microscopic methods for enumerating bacterial populations in shallow marine bays, Oppenheimer and Jannasch (42) found the bacterial biomass to be from 0.18 to 0.9 gm per cubic meter of clear water and up to 10.4 gm per cubic meter of turbid water.

From the abundance of bacteria in the topmost 0.5 cm of marine mud, Mare (34) calculated the average bacterial biomass to be from 0.07 to 0.36 gm per square meter. It is interesting to note (Table 5) how this compares with the biomass of other categories of organisms observed in the mud samples. Such data would be more meaningful if we knew the generation time and metabolic rates of the organisms. There may be a hundred or more generations of bacteria per year and only one generation of some of the larger organisms.

TABLE 5

AVERAGE NUMBER AND BIOMASS (WET WEIGHT) OF DIFFERENT KINDS OF ORGANISMS PER SQUARE METER OF MARINE MUD IN THE TOPMOST 0.5 CM: COMPILED FROM DATA OF MARE, (34)

Kind of Organisms	*Average Number/m²*	*Biomass/m²*
Large macrobenthos	2.8	3.75 g
Small macrobenthos	230	3.30
Meiobenthos	146,000	1.15
Protozoa	283,000,000	0.02
Diatoms	590,000,000	0.06
Bacteria	355,000,000,000	0.07

IMPORTANCE OF MICROORGANISMS IN THE SEA

Other papers in this symposium series give more details on microbial populations and how they influence conditions in aquatic environments. Unquestionably the most important function has to do with the modification of organic matter, its formation as well as its mineralization. Virtually all kinds of organic matter are susceptible to microbial attack under certain conditions. The end products of decomposition directly or indirectly provide essential plant nutrients (ammonium, nitrate, phosphate) and carbon dioxide. Under ordinary conditions of decomposition, from 60 to 80 per cent of the carbon in organic matter assimilated by bacteria is liberated as CO_2 (complete mineralization). The remaining 20 to 40 per cent is converted into bacterial cell substance. This is the major source of bacterial biomass, much of which is devoured by animals. Heterotrophic bacteria contribute substantially to the nutrition of aquatic animals. On the deep sea floor, animals may get much of their food from bacteria which develop there at the expense of dissolved, colloidal, and detrital organic matter conveyed there by ocean currents or sedimentation (4, 67).

Unfortunately, data are not available to permit the appraisal of the possible importance of autotrophic bacteria as food for aquatic animals. There are only fragmentary accounts of marine bacteria which derive their energy from the oxidation of ammonium, nitrite, methane, molecular hydrogen, and hydrogen sulfide. Likewise, very little is known regarding the abundance or nutritional value of photosynthetic bacteria in marine environ-

ments. Small naked flagellates sometimes constitute a major bulk of the biomass (52). Such nannoplankton appear to be very important as primary producers and as food for aquatic animals (5).

Autotrophic as well as heterotrophic microbes catalyze countless chemical reactions which influence the states and cycles of carbon, nitrogen, sulfur, phosphorus, oxygen, and other elements in the sea. Some of these reactions influence the pH, Eh, and other environmental conditions, particularly in sediments and microzones. Bacteria are believed to be among the most important dynamic agents in the diagenesis of marine sediments where they play a role in the origin of oil.

Although preponderantly beneficial, there are many ways in which marine microbes are deleterious to the economy of the sea or to the welfare of man (59). In this latter category is the microbial spoilage of fish and the deterioration of man-made structures. Many kinds of marine microorganisms contribute to the fouling of submerged surfaces. Certain marine plants and animals have been shown to be injured by microbial diseases and maybe all higher aquatic organisms are susceptible to microbial infections. In localized environments microbes sometimes vitiate water by depleting dissolved oxygen or by producing hydrogen sulfide or other toxic substances.

CONCLUDING COMMENTS

Such glimpses as we have gained of marine microorganisms indicate that many kinds occur in aquatic environments where they influence biological, chemical, and physicochemical conditions. An accurate assessment of their significance awaits much more information on their ecology, physiology, *in situ* reaction rates, and other characteristics. New methods and new approaches are needed to realize the great contributions that microbiology has to make to oceanology and limnology, as well as to basic biology. This symposium has already done much to take an inventory of what is known and to delineate many of the unsolved problems. It is hoped that these sessions will catalyze more active interest in all aspects of aquatic microbiology and help pave the way for worldwide cooperation in studying the sea around us.

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Chapter 2

Geological Activities of Marine Micro-organisms

Wilhelm Schwartz

I do not intend to give a summary of the papers presented in session B2 of our symposium, as I believe, if one is interested, it is better to read the papers themselves. It seems to be more important to discuss the general aspect of the relations between microbes and geochemical and geological processes under marine conditions, at least for some problems, because it is impossible to discuss all the topics within the frame of our symposium.

In this discussion it will soon become obvious that we are just at the beginning of an understanding of geomicrobiology. Of course, we know already a great many facts about biocenosis and biotopes, about mutual relations of microbes among themselves or between microbes and other organisms in the sea, and in marine sediments; and about biochemical activities under laboratory conditions and sometimes under natural conditions.

Also one should not neglect to compare marine, brackish and limnic conditions if the specific features of life in a marine environment are to be understood. Often the distinct environments are quite obvious, but by no means always.

The following are but a few of the important facts and suppositions which we have to consider when discussing geomicrobiological activities. In my laboratory we are studying, under marine conditions, the activity of microbes in relation to sedimentation, diagenesis and weathering, including: the effects of organic and inorganic compounds in the biotope; of high and low temperature; of osmotic pressure and water pressure and so on; to the cycle of some elements and chemical compounds which are important for life processes and at the same time significant for geochemical or geological processes, e.g., Fe, Mn, S, Si, CO_2,

and carbonates (problems of genesis of deposits of sulfur, of sulfidic ores of iron and some other metals, the precipitation of dense limestone, and the weathering of rocks and minerals). A very important problem is to determine the role of microbes in genesis, preservation and accumulation of liquid hydrocarbons.

During the deposition of salt beds microbes may be active until the mother-brine is evaporated. These salt deposits in some cases are significant to the accumulation of bituminous substances, to the precipitation of sulfur, iron oxide, or iron sulfide, so that various microbial activities may occur under various specific conditions, e.g., of pH or Eh. Finally, living halophilic microbes interfere with the crystallization process. They are incorporated into crystals of Halite or other salt minerals or in brine drops within the crystals. For some time the bacteria may be alive, then they die and are preserved in the salt where we find not only common bacterial cells but also the threads of *Chlamydobacteria* (type *Leptothrix*) and hyphae of fungi.

Geomicrobiological research along these lines can be conducted in two ways. The first is concerned with observations of recent conditions in the field or in model experiments. We can investigate, for example, the behaviour of microbes in sediments. We can determine the species living in sediments, their microhabitats, how long they can live during the diagenesis, how they are transported within the sediment, how their metabolism proceeds under natural conditions, and their age and origin of population in deeper and older layers of a sediment.

The second is more difficult. It is concerned with the reconstruction of special events in earlier geological periods, events in which microbial activities are assumed or open to question. Microbes of the type *Bacteria* and Cyanophyceae are in a phylogenetical sense the oldest organisms we know. We are allowed to assume that they have been "ready" in general at least during the Archaicum so that we can count on their activity during the later geological ages. We suppose that the factors governing life conditions have been essentially the same as they are, in general, today.

The geological processes in question take place with other time scales than current biota life span, thus we have to accelerate

our experiments in one or the other way by using more favorable conditions, or we have to restrict ourselves to reproduce and to join single episodes of a long geological process.

It is obvious that these methods are more speculative and that the conditions of a model experiment must be discussed in every respect. On the other hand, we cannot renounce these methods. The value of geomicrobiological ideas can only be established if we try to take both paths.

Chapter 3

Ecology of Algae, Protozoa, Fungi and Viruses

E. J. Ferguson Wood

I have been asked to review the situation in marine microbial ecology as it is evinced by the papers we have heard, and as a reviewer, my own idiosyncracies may intrude. I shall not hesitate to call on ideas I received in other sessions or in post-sessional discussions. This review will not absolve you from the necessity of reading the papers. As our field is so wide and important, it proved impossible in the time allotted to the session to cover the field completely, so the ciliates and rhizopods were completely omitted, while the small, naked flagellates have not received justice. The ciliates, except for the Tintinnids, are a sadly neglected group, which is frequently numerous, almost always present in the phytoplankton and in benthic environments, and can grow and multiply under a wide range of conditions including redox potentials from -0.25 V to $+0.45$ V. They are voracious scavengers, but from the few records I have seen, and from my own observation, they seem to be rejected from the food chain. Rhizopods also are probably more important than is recognized. They can frequently be cultured from plankton samples, especially neritic ones.

COMPOSITION OF THE PHYTOPLANKTON

Braarud (2) gives to the macroscopic, benthic algae, 2 per cent of the area of the oceans, and this seems reasonable when we consider that the macroscopic algae are practically confined, apart from the Sargasso Sea, to a strip along the shore line in temperate waters, with incursions into boreal regions, while in the tropics the dominant macroscopic algae are the corallines.

In tropical waters the smaller flagellates are relatively im-

portant, especially in the open ocean. They may outnumber, and at times exceed the mass of the so-called "net phytoplankton," i.e., the diatoms and dinoflagellates. In his paper Holmes suggests that we may have to accept the existence of "less-than-1μ flagellates," as carbon assimilation can be demonstrated from material and retained on a 0.22μ pore filter which passed through a 0.45μ pore filter. This could, of course, be due to still-active fragments of organisms which were destroyed in filtration on the larger membrane. In the open ocean near Australia, diatoms are predominant in the phytoplankton close to shore in the tropics, more especially close to the shores of the island chains of Indonesia, New Guinea, the Solomon Islands and the New Hebrides. Chrysomonads and cryptomonads are abundant, but coccolithophores are relatively sparse in the Coral, Arafura, Timor, and Tasman Seas, but become more important at the western end of the Timor Sea and east of the Coral Sea towards Fiji. Farther west in the Indian Ocean, Bernard reports an abundance of Coccolithophores and blue-green algae (chiefly Nostocaceae). *Trichodesmium*, especially *T. erythraea* occurs in large sheets and windrows in the tropical Pacific and Indian Oceans and their offshoots and in the Sargasso Sea. As Watson pointed out in the discussion, Dugdale has found that *Trichodesmium* can fix dissolved nitrogen, so that these blue-green algae form a vast potential for nitrogen fixation in tropical waters, especially as their power to fix nitrogen is stated not to be so dependent on the absence of other forms of nitrogen as in the case of the bacteria that have been investigated. The ability of *Trichodesmium* to fix nitrogen would supply the nitrogen requirements during the red tides caused by this organism in the open ocean far from land. The concentration of nutrients for such large concentrations of organisms is difficult to visualize otherwise. It would be interesting to know the phosphate requirements of these algae, and whether they are auxotrophic. Despite their quantitative importance in the tropics, little is known about their biology, or ecology.

In the waters south of the ill-marked sub-tropical convergence in the south Pacific and Indian Oceans, diatoms predominate in the phytoplankton, and become still more dominant in the Antarctic. Many species such as *Rhizosolenia curvata, Fragilar-*

iopsis antarctica, Biddulphia weissflogii and *Synedra pelagica* are restricted to waters south of the subtropical or of the sub-antarctic convergence. Upwellings of sub-Antarctic and Antarctic waters can sometimes be clearly indicated by the presence of certain diatom species outside their expected range. Antarctic species have been collected in upwelling water off the east Australian coast, and current movements are such that these must have survived (presumably as spores) for at least a year at high pressures in darkness.

A group of organisms which has rarely been recorded from the oceanic phytoplankton, but which is generally present is the Euglenidae, particularly *Eutreptia viridis*, and *Euglena* spp.

Wood recorded the frequency of occurrence of large numbers of colorless flagellates amongst the phytoplankton, and that in fact they may be the dominant microorganisms in the plankton. As such organisms are obligate heterotrophs, and as chloroplasts are difficult to see in the smaller photosynthetic forms, it is easy to draw false conclusions regarding the numbers of photo-synthonts from samples studied by the Utermohl technique of phytoplankton counting. They may also assist in giving the negative results sometimes found in the C^{14} assimilation techniques used for productivity studies.

EVIDENCE OF HETEROTROPHY IN PHYTOPLANKTON

Bernard has found Coccolithophores and blue-green algae at depths of more than 1,000 m, i.e., far below the photic zone, let alone the compensation depth. I have found diatoms (6) which are believed to be functional in bottom deposits at depths to 10,000 m, and one can usually find pennate and discoid species (*Navicula, Diploneis, Synedra, Frustulia, Coscinodiscus* particularly *C. marginatus, C. lineatus* & *C. centralis*, and *Melosira polymorpha* subsp. *granulata*) at or near the surface of bottom deposits. They do not contain chlorophyll and are brown in color. On the continental shelf, below the photic zone (1% surface light), I have frequently found naviculoid species (*Pleurosigma* spp., *Navicula* spp., *Diploneis, Cocconeis, Mastogloia, Frustulia*) with green chloroplasts and exhibiting active motility and phototropism. Bursa (Chapter 58) has found diatoms growing in the

dark under large ice masses. In the laboratory, Joyce Lewin has demonstrated that a number of naviculoid diatom species can grow in the dark with glucose as a carbon source, but she failed to grow many of the centric forms in this way. This is difficult to reconcile with Bursa's findings, so it looks as though heterotrophic growth in phytoplankton may occur by metabolic pathways not yet known. I regard Dr. Lewin's findings as very important.

Osnitskaya (Chapter 33) has demonstrated heterotrophic growth among the purple sulfur bacteria, and has shown that a *Chromatium* could utilize lactic and propionic acids with minimal illumination. She suggests that these organisms use a photoheterotrophic nutrition when light becomes poor. This would explain the occurrence of purple sulfur bacteria below deep algal mats in estuaries.

From the papers given in this symposium, we must recognize facultative heterotrophy as a common phenomenon in the sea, spread over a wide range of photosynthetic organisms. The question is to what extent does heterotrophy occur in the sea among chlorophyll-bearing organisms, and as Provasoli suggested to me, do they form at times a metabiotic system in which several heterotrophic organisms combine to produce a system which is autotrophic overall? Parker and Bold (5) have shown a carbon-dioxide-oxygen interchange between *Chlamydomonas* and *Streptomyces* which could suggest a mechanism for this. One might get some leads from thermo-dynamic considerations, especially as hydrostatic pressure may considerably alter energy relationships. Osnitskaya's work raises the thought that photoheterotrophic assimilation of carbon might take place in the algae as well as in the purple sulfur bacteria, especially in regions of minimal light intensity.

PRODUCTIVITY STUDIES

The study of what is called "productivity" is made more difficult by a lack of precision in our terminology. Apart from that, the measurement of productivity is extremely difficult as so many illunderstood factors are involved. The most modern concept of measurement is "carbon assimilation" using added C^{14} to estimate this. Chlorophyll determination still has its adherents,

and formulae have been devised to convert chlorophyll units of standing crop into dynamic units. However, the C^{14} method is more spectacular, and should, in theory, determine rate of assimilation. Various techniques are employed, including incubation for a period (usually 4-6 hours) in a light bath, incubation in sunlight with a range of filters to simulate depth, and incubation in the sea at the depth from which the sample was taken, a method which can involve the experimenter in unrehearsed aquatic sports. From correlations with fluorescence counts of phytoplankton made recently over fourteen stations at depths from surface to 150 m, it seems possible that the C^{14} technique measures standing crop rather than the rate of production, or that, in uniform water masses, standing crop may relate directly to rate of production.

Such measurements, moreover, take no account of the physiological state of the organisms, or of reproductive rhythms. It is not sufficient to know that a phytoplankton sample is assimilating at a given rate. One must know the rate of change of that rate, as blooms in the sea are often of very short duration, and, in mixed waters, may be limited in space. To my mind, the biology of phytoplankton is the most important study at the present time.

Motoda and Kawamura (Chapter 26) consider from their studies that phytoplankton adapts itself so that its maximum rate of photosynthesis corresponds to the light intensity of the area; in more northerly waters, the maximum rate will be at a lower light intensity than in more tropical waters with a higher light intensity. This is an interesting possibility.

EXTERNAL METABOLITES IN THE SEA AND SUCCESSION

The study of external metabolites in the sea is rather popular at the present time, since the suggestion by Lucas (3, 4) of their importance and particularly the importance of exocrines. There is no doubt that extracellular substances are produced by marine organisms, but their importance as stimulative or repressive agents in phytoplankton succession is yet quantitatively unknown. Smayda gives his reasons for doubting their importance, but a great

deal of work has yet to be done before we can assess the theories on this subject. Smayda's reference to the importance in biological succession of rhythms and physical and chemical factors is timely, but I do not think we know enough about any of them to place them in order of importance. It may well be that some external metabolites are effective in the heterotrophic growth of phytoplankton below the photic zone, and we have seen in the various papers of this session some of the ways in which this could happen. And remember that persistence in a relatively unfavorable environment is a powerful weapon to an organism struggling to maintain its position in a succession.

In theory, a dearth of inorganic nitrogen compounds should encourage the growth of nitrogen-fixing species, and may well account for the massive blooms of *Trichodesmium* in the tropics and of the Nostocaceae recorded by Bernard. But we have no information regarding the nitrogen content of these waters due to the reluctance of hydrologists to analyse for anything other than nitrates and nitrites. As many phytoplankton organisms grow better when amino acids are present in addition to inorganic nitrogen sources, it is probable that such amino-acids are extensively utilized. Do "red tides" of *Trichodesmium* become monospecific by monopolizing available food, by deterrent exocrines or by spatial crowding? Speculation cannot avail us here. Observation and experimentation are required to solve this problem, the answer to which may have extensive application.

Inhibition of growth by metallic ions may be controlled by the ability of certain microorganisms to chelate these substances, e.g., the chelation of iron in the pectinoid sheaths of diatoms as observed by Baas Becking and Mackay (1).

SORPTION

Smayda and Wood, Chapter 27, 24, both referred to sorption phenomena in somewhat different connections. It is not always possible to demonstrate the surface phenomena associated with adsorption when two organisms or an organism and a particle adhere to each other. At the same time, there is evidence that adsorption is an important phenomenon in the oceans, and that, when the smaller phytoplankton elements adhere to particles,

adsorption of the excreted metabolites and possibly also of nutrients occurs. Since according to Wood, particles (leptopel) are often 100 to 1000 times as numerous as phytoplankton, adsorption may be highly important in the dissemination of nutrients as far as the smaller microorganisms are concerned.

THE DISTRIBUTION OF PHYTOPLANKTON

Bernard (Chapter 22) has demonstrated large occurrences of Coccolithophores and blue-green algae far below the photic zone in the Mediterranean Sea and in the western Indian Ocean. As it has been generally considered that phytoplankton is limited to the photic zone during its active state, a search at greater depths than 150 meters has rarely been made by other phytoplanktologists. I have failed to find any phytoplankton elements at 300 m in the Coral Sea, in the few samples that I have taken, but have made no observations below this depth. It is necessary for the phytoplanktologist to probe deeper into the ocean to determine the quantitative significance of Bernard's findings. In my experience, the phytoplankton in the South West Pacific and North East Indian Oceans shows a maximum growth in the vicinity of 75 m depth, often expanding in daylight upwards to 50 m and downwards to 100 m, the maximum occasionally occurring at 100 m and very rarely at 150 m. At night, phytoplankton is concentrated usually at or near the 75 meter level. In the southern Tasman, but still in the East Australian Current, and in the sub-Antarctic waters, the maximum growth occurs usually between the surface and 50 m. The total phytoplankton population of the photic zone is lower at night than in the day, and the diurnal fluctuation is more marked at the surface. This diurnal fluctuation does not show the same pattern every day, and maxima and minima may occur at different times, so it is not possible to allow for this variable by collecting samples at a fixed hour and applying a conversion factor. We have confirmed this by counting phytoplankton, total organisms and estimating the assimilation of C^{14}, and the same story is told in each case. This limits the value of quantitative studies made at a series of stations in the ocean, but diurnal variation is likely to be much lower

than the change in population due to a change in physical, chemical or other biological conditions produced by a change in water mass or by mixing.

Diurnal variation may be caused by grazing, diurnal reproductive rhythms or in part by vertical movements of phytoplankton. It is one of the immediate problems of marine microbiology.

Horizontal distribution seems from Wood's results to be surprisingly uniform to the extent that, in a uniform water mass, stations up to 60 miles and at times up to 300 miles apart may show a variation of surface phytoplankton numbers which is of the order to be ascribed to diurnal variation. This would appear to apply both in oceanic waters and in neritic regions. This means that we can assess the significance of samples taken two or three times a day from a ship steaming at say 12 knots. Qualitative studies can also be invoked to extend our knowledge of the uniformity or heterogeniety of samples, and to increase the validity of the quantitative results.

In estuaries and close inshore, especially in areas of turbulence, horizontal variation is greatly increased, and it becomes more difficult to assess the validity of sampling programs.

Motoda has shown that maximum photosynthesis did not always coincide with maximum light intensity, and suggests that, in cooler waters, there is a compensating mechanism which permits of a more efficient use of light energy.

MARINE FUNGI AND YEASTS

The importance of fungi and yeasts in the ocean has not really been assessed, though work is now going on, particularly in the United States and Germany to fill this gap in our knowledge. Kohlmeyer (Chapter 30) has attempted with some success the difficult task of assessing the criteria for a marine fungus. He points out that, while marine Ascomycetes usually possess spore appendages which assist in flotation or attachment, marine Deuteromycetes as a group do not. He therefore considers that the latter are less truly marine. He shows also that the marine Ascomycetes have genera that are wholly marine, while the Deuteromycetes do not. The physiological adaptation of fungi to the

marine environment is not so clear, although Ritchie has shown that *Zalerion* at least has an osmotic reaction to the NaCl content of the medium and to temperature.

There are in the sea a number of parasitic Phycomycetes. Myers (Chapter 31) has listed a number of algal species parasitized by Ascomycetes, while some of the estuarine and neritic sea grasses are also attacked. Algae washed ashore are rapidly attacked by autochthonous fungi.

A great deal of work on the rotting of wood and cordage has been recorded by Meyers, Kohlmeyer, Höhnk and others. While this is important from a commercial point of view, and necessary to give a true picture of estuarine phenomena, it has tended to divert attention from studies of the role of fungi in the open ocean. There seems to be little or no evidence of the role of fungi in the destruction of phytoplankton or zooplankton, though it may well be that chitinoclastic fungi are important in the destruction of skeletons of copepods, euphausids and other planktonic crustacea. Because of the lack of lignin and lignocellulose in the open sea, fungi attacking these materials would not be expected there.

Yeasts, on the other hand, appear to be widely distributed, (although in small numbers) even in the open ocean, and have been recorded incidentally by several authors from fish, sea water, estuarine muds etc. Fell and van Uden (Chapter 32) have studied in more detail yeasts from Biscayne Bay, from subtropical areas on the Atlantic coast of the United States and from the Californian coast. They conclude that the majority of these yeasts are physiologically similar to their terrestrial counterparts, but that there are species of *Rhodotorula* and *Candida* that may be truly marine. These are important considerations and suggest that a wider study of the yeasts to be found in marine environments may be profitable.

VIRUSES IN THE SEA

The existence of viruses in the sea has been the subject of speculation since the demonstration of bacteriophage by d'Herelle. However, it is only recently that we have been able to assess the likelihood and importance of viruses in marine environments. Phages active against human intestinal bacteria have

been identified from polluted areas in sea water, but cannot be regarded as specifically marine, as they do not occur in oceanic environments. However, more recently, phages active against marine bacteria have been demonstrated from estuarine and neritic waters and muds, and as recently reported by Spencer, from the open sea. Spencer (Chapter 34) describes the methods successfully used by him to isolate marine phages, and has also very usefully characterized these phages. He points out that phages from marine sources are usually inactivated at 55 C in one hour while non-marine phages may resist temperatures up to 60 C for that period. Also, while marine phages were not inactivated in sea water, they were inactivated by NaCl isotonic with sea water, i.e., inactivation is chemical rather than osmotic, a very different reaction from that reported by Ritchie for the fungus *Zalerion*. Marine phages were also active at lower temperatures than terrestrial phages appear to be, they remain active far longer in sea water than their terrestrial counterparts, and the monovalent-divalent cation ratios which they require are also different.

It would appear, that not only do phages, active against marine bacteria, occur in sea water but that they have characteristics which distinguish them from terrestrial phages.

It is interesting to consider that, while phages, bacteria and Ascomycetes can with some certainty be classified as terrestrial (including aquatic) or marine, other fungi (Deuteromycetes) and yeasts cannot. These differences may be due to different methods of adaptation e.g. osmosis, tolerance to Na^+, to cation ratios etc.

Lewin recorded the presence of a virus active against colorless algae related to the Oscillatoriaceae and derived from marine muds. Moewus (Chapter 35) demonstrated the possibility of the existence of viruses in the sea by showing that such a virus could exist in a ciliate *Uronema* associated with tumors of the sea horse. This would provide a vector whereby marine viruses, if they exist, could be transferred from one host to another, and also be a means of searching for such viruses.

GENERAL CONSIDERATIONS

It was difficult in my section to do justice to the several disciplines involved, Phytoplankton, Mycology and Virology, but

the papers have one thing in common. They point out the enthusiasm of marine microbiologists throughout the world, and the paucity of our knowledge. Every paper, though it denotes important progress, finished by raising questions rather than answering them.

Such questions are;—

1. How do we explain the existence of large quantities of phytoplankton organisms at depths far below the photic zone, whence do they derive their energy and what is their carbon source?

2. What is the actual importance of heterotrophy in phytoplankton organisms within the photic zone?

3. Do we know enough of the biology of phytoplankton to supply factors for dark fixation of carbon, reproductive and grazing rates and other biological data in the productivity equation?

4. What are the factors governing the succession of phytoplankton, the occurrence of monospecific and polyspecific blooms in oceanic waters and what promotes or limits phytoplankton populations?

5. What is the significance of fungi, yeasts and viruses in the oceans?

These are only a few of the questions that arise in our minds when we read the papers given in my session. You will think of many more.

All our speakers show an increasing awareness of the complexity of our problems and a determination to proceed towards a solution of them. It is not long since there were only one or two voices crying in the wilderness of marine microbiology, particularly those of Waksman and ZoBell, and it is only very recently that it has been recognized as a discipline which should have support and in which one could profitably work, and in fact maintain as a life-work. I can only hope that this symposium will stimulate the interest of microbiologists in general, and speed up the tempo of research in this absorbing subject. Now that we have enthusiastic workers, especially among the younger generation, we need only the facilities to extend our research. These facilities include still more enthusiastic people, and also finance

(since even marine microbiologists like the objects of their researches must live and reproduce, and since their families demand a normal life and opportunities).

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Chapter 4

Distribution and Function of Marine Bacteria

F. R. Hayes

The present section of the Symposium set out to consider the distribution of bacteria in the world oceans and in special situations therin; to describe some special substrate capacities peculiar to the marine environment, and to introduce arctic microbiology. On the last mentioned topic, only two papers have been forthcoming, one on the successions of microorganisms in the extreme South, and the other on successions of phytoplankton in Northern zones. There is also a paper on sub-arctic sediments. Evidently what we call in Canada "the vision of the North" has not yet generated large numbers of research publications in microbiology.

The opening paper of the meeting was submitted by Professor Kriss of the Institute of Microbiology, Moscow (Chapter 46),who sent regrets that, owing to ill health, he was unable to come in person. His paper was on the immediate tasks of marine microbiology, which has seriously lagged behind other oceanographical disciplines. A major failure has been the basing of work on isolated stations with limited forays, instead of traversing world oceans at all seasons and with vertical as well as horizontal distribution studies. Studies of standing crops of microbial population must be supplemented by quantitative information on rate of reproduction which has hitherto been unobtainable in the open habitat. However, Kriss and his associates have now worked out a method to measure this. Hence it is now possible to elucidate the process of turnover of living microbial matter, the mineralization rate and the utilization of microorganisms by acquatic animals. For example, in the Caspian Sea about half of the microbial cells found in the mud are digested by *Nereis* during the passage of mud through the alimentary tract of that worm. This is a link

in the food chain, microorganisms-nereids-fish. The application of the author's submerged slide method which allows one, as it were, to descend with a microscope to the required depths, has revealed organisms previously unknown. Electron microscope studies suggest that ultramicroscopic non-cellular forms of life exist in the seas and oceans.

The second paper was given by Dr. Shewan (Chapter 47) of the Torrey Research Laboratory on the troublesome question of how to separate marine genera of *Pseudomonas, Achromobacter, Aeromonas, Vibrio* and *Flavobacter.* Shewan described his scheme, evolved over a number of years, for rapid differentiation of these groups.

Anthony (Chapter 48) has conducted counts on the bacteria of marine sediments with the object of determining the precision of replicate estimates, and whether sediments of different regions could be distinguished from one another on the basis of membrane filter counts. The expression of accuracy was one which had been developed for lake counts, namely the condition under which four trips are made, and upon each trip, four sediment cores are taken, and from each of the cores, four filters are prepared for counting. With lakes, the standard error of the mean had been about ±35 per cent, but in the marine habitat, it was reduced to something like ⅛ of that value. An encouraging feature was that sediment samples could be frozen and held at −20 C for over 6 months without change in the counts; this may be of some advantage for sea cruises, where rough weather makes certain techniques difficult. Direct counts, made with the acridine orange fluorescence technique of Strugger, were not in satisfactory agreement with the membrane filter results. On the basis of filter counts, three regions proved to differ significantly in the bacterial counts of their sediments. Maximal was a strait where the water is warmed through to the bottom in summer, and drains a good farming area. Coastal waters draining inhospitable granite-slate areas were appreciably lower. Finally, the continental shelf waters were only about 1/10 those of the strait. It should be noted that all counts were so low as as to constitute near sterility.

Sieburth (Chapter 49) reported on Antarctic microbiology, a topic on which there are relatively few studies. He suggested that

previous failures to cultivate bacteria there were due to lack of knowledge of regulating mechanisms rather than to an actual scarcity of organisms. For instance, a particular group of penguins, which feed on euphausids, did not contain, by culture tests, any detectable microflora in their anterior gut segments. Direct microscopic examination however, revealed an abundance of organisms. Eventually an antibacterial substance was traced, first to the diet-organisms, then to their stomachs, and finally to phytoplanton blooms in which a particular alga dominated, on which the euphausids had been feeding. The principle was isolated and identified as acrylic acid and its antibiotic effect on *E. coli* has been verified. Penguin faeces which form guano deposits at rookeries apparently undergo bacterial decomposition at the ambient temperature of 5 C to yield a "humus" which supports a bryophytic flora. In an attempt to characterize bacterial changes, penguin guano was examined in various stages of decomposition. Media inoculated with freshly voided faeces initially became alkaline, and then with further decomposition an acid reaction set in, eventually reaching pH 4. With time there was a gradual transition from a faecal to a soil microflora.

Lebedeva and colleagues from the Sevastopol Biological Station (Chapter 50) have made cultures of the micro-organisms from different regions of the Mediterranean Sea. About 40 ml of water are passed through a membrane filter, and eventually cultured on a medium of agar and fish meal hydrolysate which had been digested with trypsin. This brings up colonies capable of decomposing albuminates. Regional differences were found, the largest counts being recorded from the Strait of Otranto and the Strait of Tunis. The various seas in the central part of the Mediterranean gave lesser values. No relation could be found between the quantitative distribution of microorganisms at different depths and the vertical distribution of temperature and salinity. The distribution of maximal and minimal counts of bacteria with depth suggests that the whole water mass of the Mediterranean has a general pattern of stratification. Very high counts were observed at stations near the mouth of the Nile, and it was possible to follow the penetration of Nile water out into the open sea for at least forty miles, and to trace its north-easterly direction, on

the basis of the microorganisms present. Comparing the open waters of the Mediterranean with other parts of the world, the counts of heterotrophes are higher than those of the Antarctic and Arctic regions of the world ocean. They are slightly below those of the sub-tropics and the tropics of the Indian Ocean and of the Black Sea. Since the river discharge into the Mediterranean is small, it must be assumed that the source of nutrients for saprophytic bacteria is the phytoplankton produced in the sea itself. Thus the considerable number of saprophytic bacteria suggests that the Mediterranean Sea is not as poor in plant life as had sometimes been suggested, and in this conclusion the authors support recent direct observations on phytoplankton productivity, which show that the southern part of the Mediterranean has phytoplankton equal to that of the oceans generally.

In several subsequent papers attention was turned to the capacity of sea water to support or inhibit micro-organisms, and to additives which might be favourable for growth. Because of technical difficulties, little is known about the growth of bacteria in extremely dilute media like sea water. The establishment of satisfactory systems for steady state enrichment culture should provide an optimum system for the development and calibration of methods for enumerating and isolating significant micro-organisms. This approach also shows promise in investigation of the interactions of bacteria with phytoplankton and zooplankton, and in relating bacterial numbers to their activities under natural conditions.

Palmer and Ordal* grew *Sphaerotilus* abundantly in a perfusion of tap water containing glucose, mineral salts and ammonia. *Thiothrix,* however, would not grow in a perfusion similar to its own spring water. As to marine organisms, although the continuous cultures bring up forms which look like nitrifying bacteria, the selection of characteristic population types has not so far been very successful.

Jannasch (Chapter 51) has applied the continuous culture procedure of Novick and Szilard, using both dilute artificial media and natural sea water. The organism was a *Spirillum,* isola-

* Paper presented - Steady state enrichment cultures of acquatic and marine microorganisms.

ted from decaying sea weed, and on it tests were made to assay the effect of progressive withdrawal of nitrogen on the population level. The source of nitrogen was asparagine and the carbon source, lactate, was kept in surplus. The technique was to take a flourishing colony of *Spirillum* and prefuse it with dilute medium until the population declined to a new lower plateau; then a further dilution was applied and so on until a wash-out of the entire colony occurred with (in theory) eventual extinction. In this way it was possible to establish the relation of the level of nitrogen to the population which could be supported. Unfortunately, efforts to extend the method to natural sea water have so far been unsuccessful. Neither the polluted water of Naples harbour nor offshore water would serve unless supplemented with pieces of sea weed and nutrients. Thus, this promising technique may require the selection of some other test organism before it can yield direct measurements on natural productivity.

Turning to another topic, it is well known that although great numbers of bacteria enter the ocean by way of surface drainage and sewage outfalls, a rapid decrease occurs as one proceeds outward from land. It has also been reported that the most important factor contributing to the decline is the bactericidal action of the water. Pramer and colleagues (Chapter 52) dealt with the phenomenon, repeatedly observed, that this bactericidal action of sea water tends to be abolished by autoclaving. In a typical experiment by Pramer *et al.*, the survival of *E. coli* was followed in untreated control, in filtered and in autoclaved sea water. In all six tests, autoclaving greatly increased the number of survivors. Membrane filtration also promoted survival, though to a lesser extent.

Working in the same field, Jones (Chapter 53), reported on the terminal growth of several organisms. Normal sea water, supplemented with a complex medium was quite inhibitory, more so, for example, than 16 units of penicillin G per ml to susceptible organisms in artificial sea water. The inhibitory effect varied considerably with place of collection and depth; samples adjacent to the bottom were more toxic than those near the surface. Toxicity declined gradually with storage. The general conclusion from both papers is that the inhibiting effect resides with inorganic salt

balances rather than with specific organic substances, and may involve such factors as redox potential, concentration of dissolved gases, and ionic equilibria.

Of possible relevance to the foregoing was a paper by Demny and colleagues,* who have made a survey of antibiotic-producing microorganisms in salt marshes, sea water, shore, bay and deep core habitats. Out of nearly 1500 isolates about ten percent exhibited antibiotic activity. The main types were streptomycin and streptothricin which were observed in each of the five areas surveyed. Xanthomycin yielders were also frequently encountered. There was no evidence to suggest that the marine antibiotic producers are necessarily different from those seen in soils.

An interesting trio of papers was given on organisms with very special nutrient requirements. Hutner and colleagues (Chapter 54) reported on a photosynthetic bacterium, *Rhodopseudomonas,* which turned up in a diversity of substrates. All strains so far examined have required para-aminobenzoic acid, the need for which, surprisingly enough, could be satisfied by sulfanilamide. The significance of this finding for problems of assay will be obvious.

Yaphe (Chapter 55) discussed the difficulty in classifying those microorganisms which utilize the polysaccharides of sea weeds. There are essentially marine bacteria, since the polysaccharide substrate is characteristic of marine plants. The word agar-agar is used to describe various gelatinous materials and cannot be chemically defined. The author urges that commercial preparations should state the botanical and geographical source of the parent alga. Obviously confusion will arise when attempts are made to classify bacteria on their ability to degrade agar. Yaphe commented on Bergey's classification of the group in question and proposed modifications to take in forms which utilize carrageenan in addition to or instead of agar and alginic acid. He is not prepared to advocate the creation of a genus on the basis of ability to digest this or that polysaccharide.

Dealing with another group of chemical specialists, Lear (Chapter 56) discussed bacteria capable of digesting chitin

* Paper presented. Occurrence of variety of Actinomycetes isolated from marine materials (with I. M. Miller and H. M. Woodruff).

which it was hoped might serve as an indicator group to study the loci of nutrient cycles in the sea. Chitin-destroying bacteria are very rare in sea water but occur attached to zooplankton organisms; thus it appears that the bacterial cycling occurs on the bodies of individual organisms, from which the nutrients are directly released to the water. The zones or layers of bacterial decomposition thus correspond roughly with the places of zooplankton abundance. The gut contents of certain fishes were found to contain populations of chitin-destroying bacteria, as might be expected if these fish are feeding on zooplankton.

In its final portion the meeting heard three reports on specialized problems of distribution. Liston and Colwell (Chapter 57) have investigated the question of whether bacterial populations were specifically associated with marine animals, i.e., does the species of marine animal and its environment determine which bacteria are present? Swab samples were taken from fish or invertebrates for culturing. Collections from both north and south Pacific showed *Pseudomonas* and *Achromobacter* types to be of overwhelming importance, the two together accounting for over half of the total flora. Species of microorganisms having similar physiological characteristics tended to predominate in each particular environment provided by the body of the host animal. Bacteria from vertebrates tended to be more active biochemically than those from invertebrates. There was on the whole little evidence of any taxonomic difference between the bacterial populations of vertebrates and invertebrates, or among populations derived from different geographical areas. However, bacteria did exhibit the adaptation which has been described for higher organisms, of being acclimated to the temperature of their environment.

The cycling of microorganisms in Arctic seas was described by Bursa (Chapter 58), who has repeatedly observed a spring maximum of Pennatae under the April ice, followed in August by the characteristic summer Centriceae and often dinoflagellates.

In the final paper of the session, Pratt and Austin (Chapter 59) described the effect on growth rate of microorganisms by dilution of sea water, and of substitution of ions, such as might be found in estuarial and other situations. Sulphate was found to be

capable of substitution for chloride and other ions and sugars could to some extent take the place of sodium, although the latter was always required in considerable quantities.

Chapter 5

Marine Bacteriology and the Problem of Mineralization

Sydney C. Rittenberg

With the first international symposium dedicated to marine microbiology approaching completion, it might be considered presumptuous to ask whether we are justified in speaking of marine bacteriology as a branch of bacteriology. We should not ignore, however, the view of Winogradsky expressed in relation to soil microbiology some 33 years ago on a somewhat similar occasion. Looking back over a much more voluminous body of literature than has as yet accumulated for marine microbiology, Winogradsky (29) had the following to say, "The question as to whether we are justified in speaking of soil microbiology as of the youngest branch of microbiology has repeatedly been asked and differently answered. The writer is of opinion, already formulated in his address in Rome and elsewhere, that such a branch, if born, is yet in its infancy. What we call "soil microbiology" is not more than a chapter of general microbiology treating of microorganisms isolated from the soil and hypothetically admitted to be taking part in some processes which are characteristic of this natural medium. Remarkable work has been done in this direction by numerous investigators in many countries, and the accumulated knowledge of 35 years work must be regarded as acquired scientific knowledge. Without doubt, it forms a necessary introduction to soil microbiology, *but it cannot be taken for soil microbiology itself*. The general topics of the two are too widely different to be considered under the same heading.

"In fact, the subject of the general microbiologist is the study of the morphology and physiology of species which have been chosen by him or which have in some way fallen into his hands, whereas the aim of the soil microbiologist is to study the

biological agents of soil processes, *such as they are given in nature,* in their original soil and under the special conditions of that soil. The former is free to use in his experiments all means suggested by certain standard programs or by his own ideas; but the latter has to pursue his investigations, as exactly as possible, in the boundaries placed by nature itself."

Winogradsky's statement, properly paraphrased, also gives, I believe, an accurate appraisal of the current situation with respect to marine bacteriology. Understanding of mineralization in the marine environment illustrates his thesis, and the title of this paper might well have been reversed to read "Mineralization, and the Problem of Marine Bacteriology."

Mineralization in the sense of the symposium just completed is defined as the metabolic conversion of organic molecules to the inorganic forms utilized as plant nutrients, and it is thus concerned with the completion of the nutrient cycles. Although the concept of the cycling of nutrients in nature can be traced back at least to the time of Lavoisier, it was the genius of Pasteur who first clearly conceived the full significance of microorganisms in the mineralization process. Generalizing from the results of his studies on fermentation, Pasteur (15) was able to express the full import of microbial activity in one brief, elegant sentence. "Sans eux, la vie deviendrait impossible, parce que l'oeuvre de la mort serait incomplete."

The realization that soil fertility and microbial activity were intimately connected soon led to a study of the microorganisms of the soil. Quantitative laboratory investigations of the mineralization of complex organic matter commenced with the work of von Wollny (30) and Laurent(14). However, it was the efforts of Winogradsky and of Beijernick and their development of the selective or enrichment culture technique, starting in the mid 1880's, that paved the way to the discovery and description of the various bacteria involved in the more specialized steps of mineralization. In particular, the isolation of the nitrifying organisms (28) was a major milestone not only because it identified the agents required for the completion of the nitrogen cycle but also because it showed the marked specificity of function to be found in the microbial world.

In the succeeding two decades, a large number of metabolically specialized bacteria of potential significance in mineralization were uncovered, as for example the nitrogen fixers, various sulfur oxidizers, cellulose and agar digesters and so on. By the early 1900's, schematic nutrient cycles, of varying degrees of complexity for the individual key elements were included in standard textbooks. The physiologically unique microorganisms described during this period proved to have a fascination for succeeding generations of microbiologists who have continued to study them as interesting entities in themselves without necessarily relating their investigations to the problems of the soil.

Marine bacteriology had its beginnings in the same era. Concomitantly with the study of the soil microorganisms, a lesser effort was expended in the description of the marine microflora. Using techniques already applied to the soil, Certes (4), Fischer (9), Russel (21) and other pioneers demonstrated the existence of a general heterotrophic flora, very similar to the heterotrophic bacteria of the soil, in sea water and in marine sediments.

By applying specific enrichment culture procedures, also borrowed from soil studies, the activities of physiologically specialized forms was also demonstrated. Nitrification, denitrification, nitrogen fixation, agar and chitin digestion, sulfide oxidation and sulfate reduction, among other processes were shown to occur in the enrichment cultures. In many investigations, it was tacitly assumed that the bacteria involved were the marine counterparts of the previously described soil forms and the experimenters were content to demonstrate only the chemical change. A careful reading of the literature leaves the impression that the agents responsible for many of the important transformations in the oceans, including nitrification, are yet to be described and suggests that even the descriptive phase of marine bacteriology is far from complete.

Not only were individual mineralization steps demonstrated in the laboratory, but also, if only in rough form, complete cycles. The classical paper of von Brand, Rakestraw and Renn (1), showing the successive formation of ammonia, nitrite and nitrate from decomposing plankton, is a most elegant example. Since the chemical oceanographers had demonstrated the same se-

quence of appearance of these molecules *in situ*, as for example in the English Channel (5), it was not difficult to accept that the nitrogen cycle in the oceans is essentially the same as that on land.

As knowledge of the potential role of bacteria in the oceans increased, attempts were made to explain oceanographic phenomena in terms of bacterial processes. The most prominent early attempt was that of Brandt (2) who attributed the low productivity of tropical seas to the release of molecular nitrogen by denitrifying bacteria. The hypothesis was supported by the ease with which bacteria, that denitrified under laboratory conditions, could be isolated from sea water. This hypothesis led to a very active controversy and ultimately to the rejection of the idea on two main grounds; Gran's (10) conclusion that denitrifying organisms do not use nitrate if oxygen is available (which is in accord with modern findings), and the insufficiency of organic matter in the water to provide for the activity of these organisms (32 p. 155). It is important to point out here that no attempts were made until very recently, to demonstrate denitrification *in situ* which, of course, gets at the crux of the problem.

The situation is comparable in much of the work concerned with mineralization in the marine environment. Investigators have been content to demonstrate a potential organism for a process or the process itself in laboratory experiments under artificial conditions and then extrapolate the results to the oceans. Unfortunately, with enough effort, almost any kind of bacterium can be isolated from marine sediments and even from sea water itself, but this is not enough to demonstrate the significance of the process catalyzed by the bacterium. What is lacking in general, is both the demonstration of the process *in situ* and *the quantitative assessment of its significance.*

The laboratory approach has sometimes led to oceanographically unintelligible results, as in the case of nitrification. Enrichment cultures inoculated with sea water generally fail to nitrify, whereas those inoculated with marine sediments usually do (26, 3, 32 p. 151-153). However, the physio-chemical nature of the two environments, as well as the distribution of ammonia, nitrite and nitrate in the oceans, suggest that the water is the

major site of nitrification. The calculations of Gunnerson (12) based on chemical analyses of water in Santa Monica Bay, not only support the thesis that active nitrification is occurring in the water but also provide a magnitude for the process in the area studied. It must be admitted that the data on which the calculations are based could be more extensive, and also that the environment involved is not a normal marine area because of the massive discharge of domestic sewage received. Nevertheless, areas such as the Santa Monica Bay which are numerous all over the world, deserve the attention of marine bacteriologists because the normal mineralization processes must be accentuated therein. They provide *in situ* experimental situations amenable to direct investigation by the microbiologist. The demonstration of nitrifying organisms, either of the classical soil type or of distinct new types, whose distribution decreases outward from the point source of the substrate, the sewage outfall, in a manner consistent with the distribution of ammonia and nitrate around the outfall, would be, I believe, a contribution to marine microbiology in the sense that Winogradsky would have approved.

Returning to the problem of denitrification, mentioned earlier, available oceanographic data suggests that profitable microbiological work remains to be done. Some six years ago my colleagues and I (6) attempted to draw up budgets for three major nutrients in the oceans, nitrogen, phosphorus and silicon. Our results are briefly summarized in Table I. Considering the uncertainties in the data available, we were agreeably surprised to find that a reasonable balance was obtained between the phosphorous and silicon input from the land and the loss to the marine sediments, as should be the situation if the oceans are in a steady state condition. However, the same was not true for the nitrogen calculation which showed an excess input of about 7×10^7 metric tons per year. To balance the budget, we had to postulate denitrification as a mechanism for the escape of nitrogen from the sea. Assuming denitrification to be exclusively an anaerobic process (see for example, Skerman and MacRae, 23), one would think first of the sediments of high organic content, such as those of the neritic basins and estuaries, as its site. However, calculations based on the decrease of nitrogen with depth

of burial in the sediments indicated that a maximum of about 8.6×10^6 metric tons per year could be denitrified annually in such areas which is only 10 per cent of that needed to balance the budget. Three possible answers to the dilemma were considered: 1) the data used for calculating the budget were grossly in error, 2) anaerobic microenvironments exist in the water column, as for example within the test of a decomposing planktonic organism or 3) bacteria exist which denitrify in the presence of oxygen. Although the last alternative might not appeal to the modern bacterial biochemist, in light of the data then available, the search for such bacteria would have made sense to marine microbiology.

TABLE 1

NUTRIENT BUDGET OF THE OCEANS*

	Millions of Metric Tons		
	Nitrogen	*Phosphorus*	*Silicon*
Reserve in ocean	920,000	120,000	4,000,000
Annual use by phytoplankton	9,600	1,300	——
Annual contribution			
by rivers, dissolved	19	2	150
by rivers, suspended	0	12	4,150
by rain	59	0	0
Annual loss to sediments	9	13	3,800

*From Emery, Orr and Rittenberg, (6)

That faulty data are not completely responsible for the lack of a balance is suggested by a recent contributition by Erickson (8). Using a different approach to calculate nitrogen balance, he arrived at a value of 10^8 metric tons per year for the magnitude of denitrification, essentially the same as the previously mentioned value. Searching for the site of this process, he suggested the possibility that "there are organisms that can carry out denitrification inside their bodies. It may be possible that at least in some species denitrification occurs as a by-product in the normal reduction of nitrate to ammonia which has to be carried out in the assimilation of nitrate by plants." Again, although such an explanation might be classified as heresy by the modern bacterial

biochemist with his credo of comparative biochemistry and his respect for the precision of nature in performing biosynthesis, unless alternative ways out of the dilemma are presented, a search for such a biochemical mechanism would make sense for a marine microbiologist.

Actually, we are not yet forced into an examination of the heresies suggested above. Reasoning that metabolically released nitrogen might have a different isotopic composition than atmospheric nitrogen with which the ocean nitrogen is in equilibrium, Richards and Benson (17) measured the N^{14}/N^{15} ratios of the molecular nitrogen present in the anaerobic waters of the Cariaco Trench and in the open ocean outside. The great difference in the ratios led them to conclude that denitrification is an active process in the Cariaco Trench.

The significance of this outstanding contribution is several fold. In the first place, it provides a tool whereby the marine microbiologist, who should also be a chemical oceanographer of sorts, can look for dentrification *in situ*. Secondly, it delineates a type of area where the search for, and the description of, denitrifying bacteria would be meaningful for marine microbiology. It should not be inferred that the findings of Richards and Benson answers the dilemma posed by the nitrogen balance. Actually it presents the marine microbiologist with a third chore, the development of some method that will measure the *in situ* rate of denitrification in environments like the Cariaco Trench. This is the most difficult task of all, but only when it is accomplished will marine microbiology know whether the books are balanced or whether viewpoints that might currently be considered heresies must be brought into a state respectability.

The mention of denitrification almost automatically brings into focus the counterprocess of nitrogen fixation. To the extent that this process occurs, the nitrogen budget of the oceans would be thrown further out of balance. This in itself does not rule out nitrogen fixation as being significant in the marine environment. It does point out, however, that the demonstration of potential nitrogen fixing organisms in the marine microflora has essentially no significance to marine microbiology unless at the same time

some means is devised to show the occurrence of the process *in situ*.

Turning from specific processes to the overall quantitative aspects of mineralization, we again find that marine bacteriology has provided little information of value. In the discussion of this point I will substitute the term regeneration for mineralization since it cannot be implied that complete mineralization of organic matter takes place or is necessary.

Regeneration and productivity are of course intimately related and in a steady state system, must be in balance. A great deal of experimentation has been carried out in an effort to arrive at a value for the productivity of the oceans, and although the various estimates do not completely agree, a reasonable value that is probably not in error by as much as a magnitude may be assumed. A value of 150 gm of carbon assimilated annually per square meter was chosen to calculate the budget mentioned earlier which is intermediate between the estimates of Trask (25) and Riley (18) on the one hand and that of Steeman-Nielsen (24) on the other. Assuming that the standing crop of cellular matter is reasonably constant then the above value may also be taken for the annual regeneration of carbon. The questions arise as to where the regeneration takes place and to what extent bacterial processes are involved.

Based on the distribution of oxygen, phosphate and nitrate in the Atlantic Ocean, Riley (19) estimated that 90 per cent of the organic matter formed is utilized in the upper 200 meters of water, most of the remainder being regenerated in the deep water and sediments. In terms of rate, this represents the regeneration of about 0.1 μg carbon per hour per liter of water in the upper 200 meters. Assuming that the organic carbon is oxidized to CO_2, the corresponding oxygen demand is 0.25 μg per liter per hour. Using ZoBell's (31) estimate of 2×10^{-8} μg oxygen consumed per cell per hour, a steady state bacterial population of over 10^7 cells per liter would be required if the bacteria were exclusively responsible for mineralization.

This required quantity of bacteria is far out of line with current estimates of average bacterial populations in the upper 200

meters of sea water. Even the highest estimates based on viable counts would be two to three magnitudes too small. If one utilizes population values derived from direct microscopic counts, the discrepancies disappear to a considerable extent but such data must be viewed with suspicion. The counting errors involved in the technique and its failure to distinguish between active and inactive cells needs no discussion. It is more important to point out that one can calculate the oxygen demand of certain typical basin sediments from the decrease in organic matter with depth in the sediment (7) and then convert this to standing crop of bacteria by assuming a "reasonable" value for the respiration rate of a bacterial cell in the sediment. If this is done, one finds that the calculated standing crop falls well within the range of viable counts obtained for such sediments. In the sediments, therefore, there is no need to postulate more active bacteria than can be found by viable counting procedures. Although there may well be nutritional differences between the bacteria in the water and in the sediments, as is indicated by the data of Skerman (22), there is no reason to suspect that a significantly lesser per cent of the active water flora should be detected by the plating procedures commonly employed in current marine investigations.

This reasoning leads to the conclusion that bacteria play a very minor role in the regeneration of carbon in the upper layers of the ocean where the bulk of the process is going on. This conclusion is not new of course, having been arrived at earlier by Riley (19) who ascribed most of regeneration in the upper and mid depths to the activities of the phytoplankton and the animals. If the above arguments are valid, they also imply that the standard minerlization cycles as worked out for the soil cannot be applied *a priori* to the marine environment.

What applies to carbon also applies to nitrogen. The increase in C/N ratio between the plankton and the organic matter in the surface of marine sediments indicates that nitrogen must be regenerated relatively faster than carbon, so that the assumption of 90 per cent regeneration in the upper 200 meters appears reasonable. Following the same pattern of calculations used above, a release of roughly 0.02 μg nitrogen per liter per hour is found. If all the released nitrogen were nitrified before being

used by the phytoplankton and if respiration of marine nitrifiers proceeded at the previously assumed rate of 2×10^{-8} μg O_2 per cell per hour, then a standing crop of nitrifiers of more than 10^6 cells per liter is required. As previously mentioned, standard enrichment procedures as borrowed from soil microbiology usually fail to detect any nitrifiers at all in the ocean waters.

The existing lack of information about nitrification allows ample room for speculation. One can postulate inadequate techniques or nitrifiers of an undetected nature; one can postulate that the bulk of the nitrogen is only ammonified before reuse; or one can even postulate that a significant portion of the nitrogen is not mineralized at all but is reused by the plankton in the form of soluble organic nitrogen. It would be possible to support all of the postulates above by reference to results of laboratory investigations (see for example papers by Watson (27), Guillard (11), and Pintner and Provasoli (16), this symposium) but a quantitative assessment of their significance is not possible. It is obvious, however, that we are not yet privileged to assume for the oceans the same cycle of nitrogen as in soil.

In light of the material presented, it seems fair to say that few of the oceanographically intelligible questions that might be asked of a marine microbiologist about mineralization seem answerable at this time. Thus, perhaps, it was not so presumptuous to pose the question that opened this paper. Unlike Winogradsky, however, I have no methodology to present that is completely adequate to remedy the situation. It is my feeling that *in situ* studies are necessary. For such studies more than the laboratory techniques of bacteriology are needed: knowledge of oceanography, sedimentology, chemistry and biochemistry must also be applied.

It is much simpler to deal with marine sediments than with sea water. This is perhaps why most of the contributions to the mineralization session of this symposium were concerned with microbial activity in sedimentary material. There are several reasons why this should be so, among the most important being that in general, once deposited, sediments stay put. Applying the steady state generalization to a sediment, one has only to measure the change of some parameter with depth in a sediment to have

unfolded before him the results of an experiment stretching not over hours or days but over millenia of time (7). The data of such an "experiment" conducted *in situ* under natural conditions, and interpreted in the light of existing knowledge of the various disciplines that must be brought to bear, can yield quantitative knowledge about microbial processes *in situ*. Using this approach for the sediments of a marine basin, it was possible to determine the rate of release of nitrogenous compounds from the sediment, as well as its oxygen demand (20). A similar approach should yield significant data for other types of sediments or for other facets of the mineralization process.

To suggest an equally effective approach to the understanding of mineralization processes in the water is, at the moment, very difficult. Unlike the sediment, waters in the ocean do not stay put. The technique of adding radioactive tracers to the water and following their fate over several days, which has been so fruitful in limnological investigations (13 and others) appears at the moment to be inapplicable to the open ocean at least. Similar studies in more confined area of the sea, like bays and fjords, may be worthwhile, however. The magnitude of the changes taking place in the water appears from calculations to be too small to detect by direct measurements over periods of time that one might hope to keep track of a tagged body of water. Areas may exist, where, for natural or man-made reasons, the mineralization process is so magnified that direct measurements of rates of individual processes become possible and such areas deserve exploration. For most of the oceans, however, there currently seems to be no alternative to the detailed and painstaking investigations of the oceanography and chemistry of an area. The total data, brought into proper focus, may then delineate the individual processes taking place. In a generalized manner, this was the approach of Riley (19) whose conclusions were referred to earlier; this also was the approach of Gunnerson (12), in a more restricted manner, in the material he presented at this symposium.

Regardless of approach, the goal should be the integration of microbiological phenomena with the existing knowledge of physical, chemical and biological oceanography.

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Part 1
The Producers and Their Relation to the Chemical and Biological Environment

Chapter 6

Cultivation and Properties of *Thiovulum majus* Hinze*

J. W. M. La Rivière

The study of the physiology of the colorless sulfur bacteria has been seriously hampered by the technical difficulties involved in their isolation and cultivation, *Thiobacillus* (19) being the one outstanding exception. Consequently their role as chemo-autotrophs and as participants in the sulfur cycle is far from satisfactorily defined and needs further investigation; this should be rewarding also from a comparative point of view because of the close morphological relationships between this group and some organisms known to be heterotrophic or photosynthetic (15, 18).

Chemo-autotrophy has been firmly established for *Thiobacillus* species, and claimed for *Beggiatoa* and *Thiothrix* (2, 9), though this has never been confirmed. The isolation of heterotrophic *Beggiatoa* strains by various workers (*cf.* 6) has cast doubt on the validity of the earlier claims.

From ecological observations and culture experiments it seems probable, however, that many of the larger colorless sulfur bacteria require both H_2S and O_2 in low concentrations. As a result conventionally prepared media, initially satisfactory for growth, rapidly become inadequate through auto-oxidation and microbial oxidation of the sulfide. Available evidence also indicates, that in contrast to *Thiobacillus,* they cannot use thiosulfate as oxidizable substrate. Hence their cultivation calls for special techniques, aimed at maintaining the requisite H_2S and O_2 concentrations. This is more easily accomplished by the use of liquid than of solid media, with the consequence that one must resort to washing and dilution procedures for their isolation. Such methods

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have been developed for the cultivation of *Thiovulum majus* Hinze, a colorless sulfur bacterium at present included in the *Thiobacteriaceae*.

Early descriptions and illustrations of *Thiovulum* have been given by Müller (13), Warming (20), Hinze (8), Lauterborn (12) and Bavendamm (2). The cells are round to ovoid, measuring from 5 to 25 μ in diameter. They normally contain sulfur inclusions, frequently concentrated at one end, but sometimes filling the cells almost entirely. The cells multiply by longitudinal fission preceded by constriction. The organism is extremely motile and rotates around an axis coinciding with the path of travel. It is strongly chemotactic and as a result the cells concentrate in white, sharply defined veils, consisting of separate, ever moving cells. By staining fixed specimens, Hinze (8) demonstrated peritrichous flagellation with great difficulty. This has not been confirmed by subsequent authors, including Fauré-Fremiet and Rouiller (5), who in a cytological study discovered a polar cytoplasmatic organelle and structures resembling an endoplasmatic reticulum. The importance of these findings for comparative cytology was discussed by Murray (14).

The first physiological studies on *Thiovulum*, conducted at the Hopkins Marine Station by van Niel, Wijler and Lascelles, (unpublished) showed that it is extremely microaerophilic, and that, like in *Beggiatoa*, its sulfur inclusions are lost soon after the medium has become devoid of H_2S, whereupon the organisms perish. Taking advantage of the chemotaxis of *Thiovulum*, Wijler developed a method for its enrichment. A layer of decaying algae *(Ulva)*, placed on the bottom of jar filled with sea water, served as a continuous source of H_2S, and a slow trickle of seawater, introduced near the bottom, provided a continuous supply of O_2. The overflow flushed out many contaminating organisms, and *Thiovulum* grew in characteristic veils in areas where the optimum H_2S and O_2 concentrations prevailed. Massive transfers to a similar jar without algae could be maintained by adding small amounts of H_2S once or twice daily.

Part of this work has been recorded by van Niel (16); the present study is a further extension of it.

METHODS

Enrichment Propagation of Crude Cultures

Primary elective cultures were prepared by Wijler's method. The trickle of seawater was initiated after about one to two weeks, when the algae had started to decay and a thick film of bacterial growth had developed on the surface; *Thiovulum* usually appeared during the third week. Transfers were made to jars of 5-10 L capacity to which, depending on the condition of the culture, 5-20 ml of a saturated H_2S solution were added once or twice daily. For propagation on a smaller scale a 10 L reservoir with an aerated solution of 0.005% NH_4Cl, 0.005% KH_2PO_4 and 0.005% Na_2CO_3 in seawater, pH 8, was attached to a continuous culture device as described by Bulder (4), set at a delivery rate of 20-35 ml/hr. The medium flowed through a 1 L bottle without air space to which 2-5 ml of a saturated H_2S solution were added daily from a burette, permanently connected to an H_2S generator. It then entered at the bottom of a 100 ml Erlenmeyer flask provided with a side tube near the top; the overflow dripped into a second similar flask, and from here into a sink. The flasks served as culture vessels. The entire system was placed in a 15 C incubator.

Purification of Inoculum

About 50-100 ml of a *Thiovulum* suspension, obtained from veils in a primary or secondary culture, were rapidly filtered through a thin layer of cotton into a 100 ml glass cylinder. After five to fifteen minutes the cells settled in a characteristic pattern on or near the bottom. They were removed in as small a volume as possible, and transferred to a test tube with 15 ml sterile seawater where, in another five to fifteen minutes, they formed a new web near the bottom. Subsequent transfers, with sterile pipettes, completed the operations. A healthy culture could be so "washed" up to ten times. Visual observation of the settling process was facilitated by lateral illumination with a point source. Plating of 1 ml samples from successive tubes in 0.1 per cent peptone seawater agar, incubated at 22 C, served as a check on the efficiency of the procedure in the elimination of heterotrophs.

Stationary Pure Cultures

In a 500 ml Erlenmeyer flask, 250 ml of the following sterile agar medium was allowed to solidify: seawater, 2 per cent agar (Bacto, Difco), 0.01% NH_4Cl, 0.01% KH_2PO_4, 0.01% Na_2CO_3, 0.02% H_2S, pH 8.0. The H_2S was added as a saturated solution in sterile seawater; Na_2CO_3 was autoclaved separately as a 1 per cent solution; both ingredients were added to the agar just before it had gelled. After the agar had solidified, 250 ml sterile seawater was added; the flask was closed with a sterile rubber stopper containing an outlet with a cotton plug and a device for localized aeration, consisting of a cotton-plugged Pasteur pipette with a glass tube sealed around its drawn-out end. The glass tube was open underneath and had a hole above the water level (Fig. 1). When a slow current of air was passed through the pipette, the resulting aeration was restricted to the liquid in the tube, the dissolved oxygen slowly diffusing into the bulk of the medium. The device, by preventing turbulence, provided for the establishment of a concentration gradient of both H_2S and O_2, the former being highest at the agar surface, the latter at the aer-

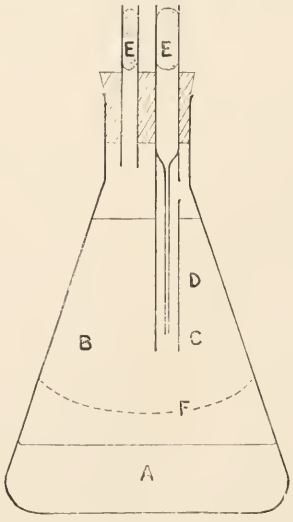

Fig. 1. Schematic drawing of culture vessel for stationary cultivation of *Thiovulum*. A = sulfide-containing agar layer; B = mineral seawater medium; C = protective tube for localised aeration; D = Pasteur pipette; E = cotton plug; F = position of *Thiovulum* web in initial stage of cultivation.

ation tube. This was confirmed by adding some thionine to the liquid phase. Without aeration the entire liquid remained colorless. After aeration through an unprotected pipette, the redox dye was oxidized to its colored form throughout the liquid. With localized aeration a colorless zone of about 1 cm above the agar surface could be observed for several weeks.

The culture flasks were allowed to equilibrate under localized aeration overnight at 12-15 C before inoculation with 0.1-0.3 ml of a purified cell suspension. The purity of cultures obtained in this manner was checked by inoculating 1 ml samples into the following liquid media: (a) seawater, 0.1 per cent peptone; (b) the medium of Baalsrud and Baalsrud (1) for the cultivation of *Thiobacillus,* made up with seawater; (c) seawater, 0.1 per cent sodium acetate, 0.05% NH_4Cl, 0.05% K_2HPO_4; and (d) seawater, 0.1 per cent yeast autolysate, 0.1 per cent glucose. Duplicates of these cultures were incubated aerobically at 12 C and at room temperature. Anaerobic cultures were also prepared with the glucose medium.

X-ray Diffraction, Electron Microscopy, Photography

The x-ray diffraction pattern of the sulfur inclusions of the cells was determined on material taken from an enrichment culture. The cells were washed twice in seawater, centrifuged, and distilled water added. After recentrifugation at low speed the sediment was dried and used for the x-ray diffraction study. For electronmicroscopy (3) cells from crude cultures were used after a one-step purification and fixation with osmium tetroxide. The preparations were shadowed with platinum and examined in a modified Philips E.M. 100 electron microscope. Pictures of living, motile cells were taken by means of a Zeiss photomicroscope with a flash of 3×10^{-3} second (Fig. 2, 5-7).

RESULTS

The methods developed by Wijler for the cultivation of *Thiovulum* were reproducible and dependable when repeated at the Hopkins Marine Station; no specific inoculum was necessary to obtain good cultures. But similar experiments performed in Holland with seawater from the Dutch coast, taken at Scheveningen or at den Helder, were unsuccessful unless some mud was

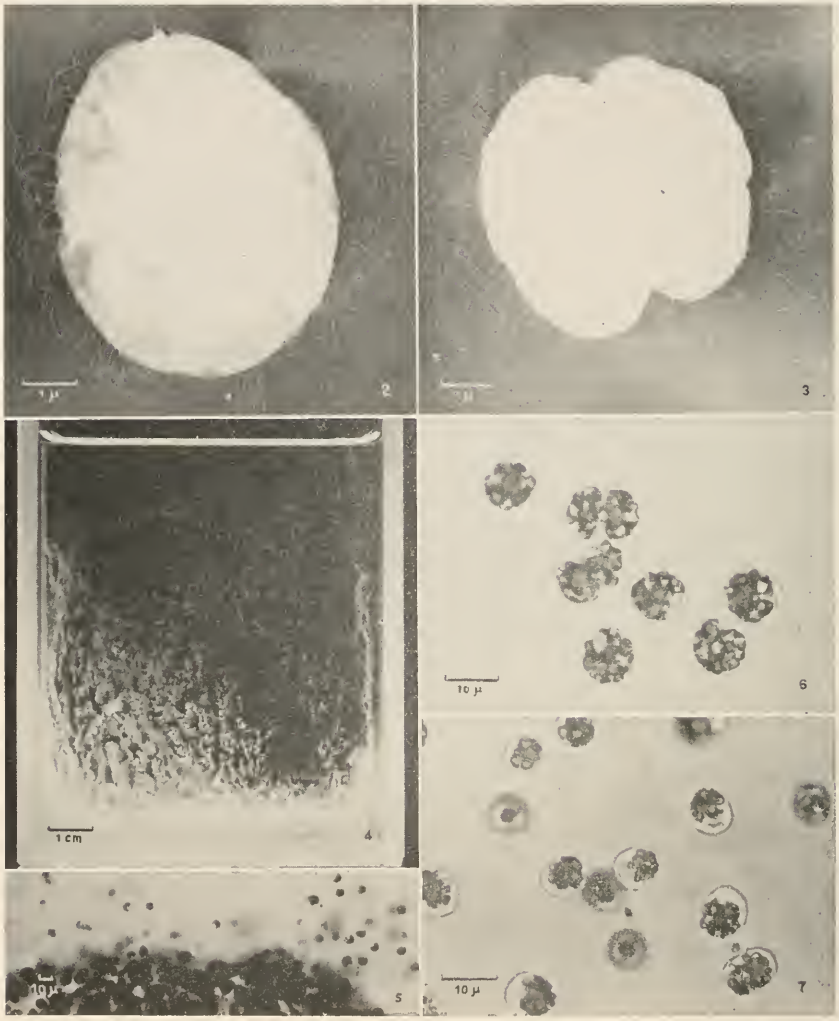

Fig. 2. (2) Electron micrograph of *Thiovulum*, (3) Electron micrograph of *Thiovulum* in the stage of constriction, (4) A veil of *Thiovulum* cells formed in a cell suspension in a cuvette. Photograph taken with reflected light, (5) Micrograph of the edge of a mass of chemotactically aggregating cells, (6) and (7) Micrographs of *Thiovulum* cells, containing various quantities of sulfur.

added from a lagoon near Bergen op Zoom, Holland, in which *Thiovulum* occurs. The cultures obtained were, however, much less vigorous and more heavily contaminated than those grown at the Pacific coast. Nevertheless, crude cultures, derived from this material, could be maintained for a period of three months in the above described small-scale continuous culture system, which alleviated the strain on the limited seawater supply at Delft. Cells from this culture did not survive a sufficient number of purification steps to yield pure cultures but provided suitable material for electron microscopy.

Numerous experiments were carried out with cells from the Pacific Grove enrichment cultures to develop methods suitable for isolation and stationary cultivation. Since it proved easier to maintain a concentration gradient rather than constant concentrations of sulfide and oxygen for a prolonged period of time, and a gradient method had successfully been employed for cultivating *Gallionella* (10), the agar block method combined with localised aeration appeared promising in view of the strong chemotaxis of *Thiovulum,* which could be relied upon to lead the organisms to the area of optimal concentrations. The sterile media were inoculated with *Thiovulum* suspensions purified by seven to nine consecutive passages through sterile seawater; this seemed adequate because it had been found that six such passages sufficed to eliminate contaminants.

After a few hours, the inoculated cells were usually observed to have settled in a sharply defined zone above the agar. The cells were crowded together in small agglomerates, apparently creating an optimal micro-environment. Within the following days the number of separate agglomerates increased until a complete, sharply defined web was formed at about 1 cm above the agar in the otherwise clear liquid; with lateral illumination individual, sulfur-stuffed cells could be seen dancing around in the web. On prolonged incubation the web rose slowly because of increasing oxygen demand; when the available sulfide was nearly consumed the web moved downward, but by this time signs of decay appeared in the form of white strands hanging down the sides of the flask, consisting of dead cells adhering to each other. For three weeks after inoculation successful transfers to similar flasks could be performed. Cultures failing to produce any growth

in the media for detection of contaminants, were considered pure. These cultures were also characterized by the absence of turbidity outside the zone of growth of *Thiovulum*. Isolation and subsequent cultivation were repeated successfully several times with material from different enrichment cultures. Cultivation on a somewhat larger scale in a 3 L Fernbach flask, provided with an agar-layer and localised aeration proved entirely satisfactory. As incubation temperature, 12-15 C proved most suitable, but it has not yet been determined whether this corresponds to the optimum temperature for the organisms or renders the culture conditions more stable.

Addition of 0.005 per cent yeast autolysate to the agar block did not appreciably affect growth of pure cultures. When special Agar (Noble, Difco) was substituted for Bacto Agar in the normal medium, development was somewhat slower. No growth occurred in the medium suitable for *Thiobacillus*, with thiosulfate as hydrogen donor. Artificial seawater prepared according to Pringsheim (17) and Lyman and Fleming (11) could not replace natural seawater, the cells dying off within three days.

The morphology of the organisms studied in California and in Holland was similar. In accordance with the results of Fauré-Fremiet and Rouiller (5) the sulfur inclusions were found to be birefringent when examined in the polarizing microscope; material derived from cells subjected to osmotic shock by immersion in distilled water furnished an x-ray diffraction pattern similar to that given for orthorhombic sulfur by Hanawalt *et al.* (7).

Organisms from the Pacific as well as from the North Sea proved to be gram-negative and lacked catalase, which may explain their sensitivity to high oxygen concentrations.

The puzzling failure to detect locomotor organs in so motile an organism, reported by Fauré-Fremiet and Rouiller (5), invited reinvestigation; but no flagella could be observed by staining or dark field and phase-contrast microscopy. Electron microscopy also failed to reveal flagellation when specimens were prepared by allowing drops of cell suspensions to evaporate on the membranes followed by washing. However, cells previously fixed by means of osmium tetroxide invariably showed a large number

of flagella, distributed at random over the greater part of the cell surface. The length of the flagella was about one-third of the length of the cells; their width measured between 100 and 150 A, which is comparable to that of flagella of other bacteria. A more detailed account on the electron microscopy of *Thiovulum* will be published by de Boer *et al.* (3).

DISCUSSION

The necessity of using a specific inoculum for successful enrichments with sea water from two different spots at the Dutch coast suggests that the occurrence of *Thiovulum* is restricted to a definite ecological zone, as may well be expected from its extreme sensitivity to environmental conditions.

The rediscovery of flagella, first observed by Hinze in 1913, solves the problem of the mechanism of locomotion. The negative findings of previous authors may be explained by a high sensitivity towards osmotic changes and other adverse conditions since no flagella were observed in unfixed preparations even if centrifuging had been omitted. The identity of the organism studied by Fauré-Fremiet and Rouiller with that studied by us seems to be well-established by the unique characteristics they have in common.

Our results have demonstrated the possibility of isolating *Thiovulum* and of growing it in stationary cultures. The success of the method depends on the one hand on the degree of contamination of the starting material which determines the number of successive washings needed to achieve purity; and on the other hand on its capacity to survive these treatments. The reason why enrichments with water from the North Sea were unsatisfactory in these respects is not yet understood and is being further investigated.

The evidence obtained thus far is consistent with the assumption that *Thiovulum* is a chemo-autotroph but far from sufficient to prove this. Whether or not the oxidation of sulfide fulfils the energy requirements of the cell, it is certain that sulfide and oxygen are of great significance in its metabolism. The sensitivity of the organism to changes in concentration of these sub-

stances demonstrates the importance of chemotactic locomotion for survival.

The principle of the enrichment technique developed by Wijler resembles closely that of the method used by Winogradsky (21) for cultivating *Beggiatoa* in a flow of liquid medium. It is therefore probable that the application of conventional continuous culture procedures will also be fruitful for the cultivation of other colorless sulfur bacteria, particularly of those which are chemotactic or sessile; stationary cultivation of purified material may then prove feasible for chemotactic and activily motile organisms by means of the gradient method here described for *Thiovulum*.

SUMMARY

Thiovulum cells obtained by means of Wijler's enrichment technique could be freed from contaminants by repeated migration through sterile seawater. Stationary cultivation of purified cell material was realised at 12-15 C in flasks where a concentration gradient of H_2S and O_2 was established between a sulfide-containing agar layer and an overlaying seawater medium aerated locally near the top. In this manner cultures were obtained which were considered pure since no contaminants could be detected by subculturing in various liquid media. The success of the described method depends on the degree of contamination of the enrichment material and the capacity of the cells to withstand a sufficient number of purification steps.

The sulfur inclusions of the organism proved to be orthorombic. The cells were gram-negative and devoid of catalase. Electron micrographs showed the presence of numerous flagella.

ACKNOWLEDGMENT

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Chapter 7

Autotrophic Nitrification in the Ocean*

Stanley W. Watson

Nitrification is one of the most important but least understood phases of the nitrogen cycle in the oceans. Our knowledge is limited to shallow coastal waters, where the existence of the process has been demonstrated, as reviewed by Carey (2) and ZoBell (8). Although nitrification has been worked on by more than 12 investigators, only Thomsen (6) has succeeded in culturing nitrifying bacteria from a marine habitat, but he could find these only from areas between the shore and 1,200 m from land. In open ocean waters verification of nitrification by indigenous populations is entirely lacking, although we know that nitrate must be regenerated because it is constantly used in the euphotic zone as a source of nitrogen for marine plants.

Very little is known about the site of nitrification in the oceans. Carey (2), after finding active nitrifying populations in sediments but not in surface waters, concluded that nitrification occurs primarily in the bottom sediments and that the nitrates are returned to the photic zone by mixing. Her hypothesis may apply to shallow coastal waters, but it does not appear realistic for oceanic waters. If such a hypothesis is applied to the open sea, the maximum nitrate concentrations would be expected just above the ocean floor. In reality, the maximum concentration exists at mid-depths between 250 and 800 m (5).

It would also seem probable that organisms similar to those responsible for nitrification on land would play a role in the

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oceans. *Nitrosomonas* and *Nitrobacter* are generally accepted as chiefly responsible for nitrification in soil; the former oxidizes ammonia to nitrite, the latter nitrite to nitrate. They obtain energy for growth and CO_2 fixation from the energy released by the reactions. Both are considered obligate autotrophs, since they cannot use organic compounds as an energy source.

My objectives in this investigation were 1) to learn more about the site of nitrification in the oceans, 2) to culture the bacteria responsible for these reactions, and 3) to study the physiology of these bacteria and to compare them with known cultures of *Nitrosomonas europaea* and *Nitrobacter agilis*.

METHODS AND MATERIALS

Bottom sediments were collected in September 1957, October 1957, and January 1958 from 21 stations ranging in depth from 50 to 2,000 m, off New York. Sediments were obtained by means of a Phleger coring tube or a small bucket.

Immediately after collection, 2 to 5 g of each sample were inoculated into 50 ml of sterile sea water enriched with 50 μM $(NH_4)_2SO_4$ or 100 μM $NaNO_2$. The samples were returned to the laboratory, incubated on a mechanical shaker at room temperature for 60 days, and analyzed periodically for nitrites and nitrates. Cultures in which 50 μM NH_4^+-N or 100 μM NO_2^--N had been oxidized were scored as indicating nitrification.

Water samples were also collected and 100 ml of each were immediately dispensed in a 500-ml Erlenmeyer flask enriched with 1 μM K_2HPO_4 and 50 μM $(NH_4)_2SO_4$. The samples were incubated for 60 to 90 days on a mechanical shaker at room temperature.

On three cruises in 1959 and 1960, water samples were obtained from the continental shelf, in slope water just off the continental shelf, in the Gulf Stream, and in the Sargasso Sea. Five to ten liter samples were collected by means of Van Dorn bottles and enriched immediately with 50 μM $(NH_4)_2SO_4$. The samples were incubated in aerated 15-liter carboys at 20 C for two to four months. Periodic assays were made for nitrites and nitrates. The cultures were scored as positive when nitrite or ni-

trate increased by 100 μM. When nitrification did occur, appropriate amounts of ammonia were added to replace that lost through aeration and oxidation.

Ammonia-oxidizing bacteria were subcultured by inoculating one liter of sterile medium with 100 ml of a sample taken when nitrites were increasing exponentially. The medium contained, per liter of distilled water: NaCl, 25 g; $(NH_4)_2SO_4$, 3.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; chelated iron ("Atlas EDTA"), 0.1 mg. In addition, 1 g of $CaCO_3$ was included if solid substrate was required. The pH was adjusted after sterilization to 7.5 by adding 1 N NaOH. NaCl and $CaCO_3$ were added to the water and sterilized by autoclaving. The remaining salts were sterilized separately by Selas filtration and added aseptically. As ammonia was oxidized from the enriched cultures, more was added aseptically.

The marine bacteria that oxidized nitrite were subcultured in the medium of Aleem and Alexander (1) with the addition of 2.5 per cent NaCl and, if solid substrate was wanted, 1 g/L of $CaCO_3$.

Serial transfers were made using two methods: a 10 per cent inoculum was transferred into a medium free of $CaCO_3$, or a subculture containing $CaCO_3$ was filtered through an AA Millipore filter and the residue transferred to fresh medium. The latter method had two advantages: All undesirable end products, such as nitrite and soluble organic matter, were eliminated from the inoculum, and all the bacteria were retained.

Terrestrial *Nitrosomonas europaea* (obtained from Dr. David Pramer) and *Nitrobacter agilis* (obtained from Dr. Martin Alexander) were cultured, using the methods of Aleem and Alexander (1) and Engle and Alexander (3).

A polarographic oxygen electrode (4) was used for respiration studies. Marine nitrifying bacteria grown in a medium with $CaCO_3$ were filtered on a Millipore filter, washed with sterile medium, and resuspended in sterile medium. The cultures were diluted to give oxygen uptake values of about 5 ml/L/hr. Cultures of terrestrial nitrifying bacteria grown in a soluble medium were centrifuged at 34,800 x G and washed with 0.05 M K_2HPO_4

buffer until less than 1.25 μM NH_4^+-N or NO_2^--N remained. The cell paste was resuspended in sterile medium and diluted to give oxygen uptake values of about 5 ml/L/hr.

The respiration chamber was a 250-ml graduated cylinder shortened to 150 ml. The mouth was sealed off tightly with a rubber stopper; care was taken to prevent the inclusion of air bubbles. The oxygen electrode, pH electrodes, a thermometer, and a capillary tube extended into the chamber through the stopper. The culture was stirred by a Teflon-coated magnetic bar. The pH was monitored with a Zeromatic pH meter, and the oxygen consumption recorded continuously on a Leeds and Norththrop recorder using a 1 mv sensitivity. The substrate concentration could be altered by adding $(NH_4)_2SO_4$ or the pH readjusted by injecting 1 N HCl or NaOH via the capillary tube. The temperature was held at 24.8 C $\pm$ 0.01° and the pH was controlled to within 0.05 units.

EXPERIMENTAL RESULTS

Ammonia was oxidized in cultures inoculated with sediment from nine out of the fourteen stations less than 100 m deep; nitrite was oxidized in all bottom samples from the seven shallow stations. Nitrification was not demonstrated in mud collected from stations over 100 m deep. Nitrification did occur in fifteen out of the 32 water samples collected in 5- to 10-liter quantities, but not in any of the 100-ml samples. The fifteen samples were from 10 m over the continental shelf, from slope waters at depths of 10, 50, 300, 1300, and 1500 m, and from the Sargasso Sea at a depth of 10 m.

In the samples enriched with ammonia, nitrification began after a long lag of 20 to 40 days (Fig. 1). Once nitrification had started, nitrites and nitrates increased almost simultaneously until each reached concentrations of 20 to 60 mM.

Subcultures of ammonia- and nitrite-oxidizing microorganisms were obtained while nitrites and nitrates were increasing exponentially. Both physiological types of microorganisms have been maintained in the laboratory for the last eighteen months by subculturing them periodically.

Media with $CaCO_3$ were superior to completely soluble

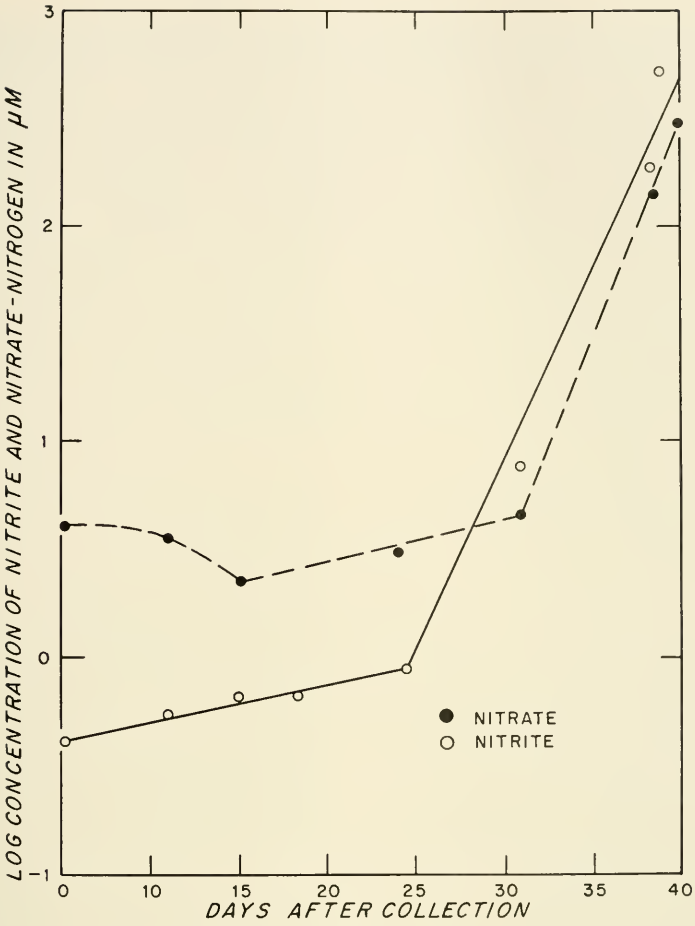

Fig. 1. Increase of nitrites and nitrates in slope water enriched with ammonia.

media for growth of marine nitrifying bacteria. Generation time for terrestrial nitrifying bacteria was less than twenty-four hours and cell concentrations reached 2×10^8 cells/ml. Marine nitrifying bacteria grown in similar media without $CaCO_3$ had a generation time of twelve days and cell density never exceeded 2×10^7; in the presence of $CaCO_3$ generation time, as judged from the increase of nitrite or nitrate, was shortened to two days.

An attempt was made to grow both terrestrial and marine

nitrifying bacteria in media free of NaCl and in media containing 2.5 per cent NaCl. Terrestrial bacteria grew best in media free of NaCl, whereas the marine forms generally fared better in media containing 2.5 per cent NaCl. In media containing NaCl both *Nitrosomonas europaea* and *Nitrobacter agilis* grew, but generation time was prolonged from one to eight days and the cultures failed to divide after about five generations. Both failed to survive after more than two serial transfers in the presence of NaCl. Experiments with marine nitrifying bacteria were highly variable. In some experiments the organisms grew equally well in the presence or absence of NaCl, in others no growth occurred in the absence of NaCl. It was impossible to maintain stock cultures of marine nitrifying organisms for prolonged periods in the absence of NaCl.

Although both *Nitrosomonas europaea* and *Nitrobacter agilis* were obtained in pure culture by streaking on Noble's agar enriched with ammonia or by making serial dilutions, the marine organisms could not be isolated using similar techniques. Thus the present studies were conducted with mixed cultures of marine microorganisms. However, the ammonia-oxidizing bacteria were successfully separated from the nitrite-oxidizing bacteria.

Several morphologically different microorganisms persisted in the marine cultures, although ammonia or nitrite served as the sole source of substrate. None morphologically resembled any described species of *Nitrosomonas* or *Nitrobacter*. All were markedly different from *Nitrosomonas europaea* and *Nitrobacter agilis*. *Nitrosomonas europaea* is an ellipsoidal rod 0.6 - 1.9 by 0.7 - 1.0 μ and frequently occurs in pairs (Fig. 2a); *Nitrobacter agilis* is a rod 0.6 - 1.0 by 2.0 μ (Fig. 2b). The most prevalent bacterium in the marine cultures was a rod 0.3 by 1.0 - 2.0 μ (Fig. 2c). There was also a protozoan-like organism (Fig. 2d) in all cultures where ammonia was oxidized to nitrite where it constituted the greatest cell volume and often the greatest cell number. The cells were 5 - 6 μ in diameter immediately after division but elongated to 10 - 12 μ prior to division (Fig. 2e). Often in old cultures the cells hypertrophied to two to three times their normal size (Fig. 2f). Although cells were motile, flagella were not visible upon examination with a phase-contrast microscope.

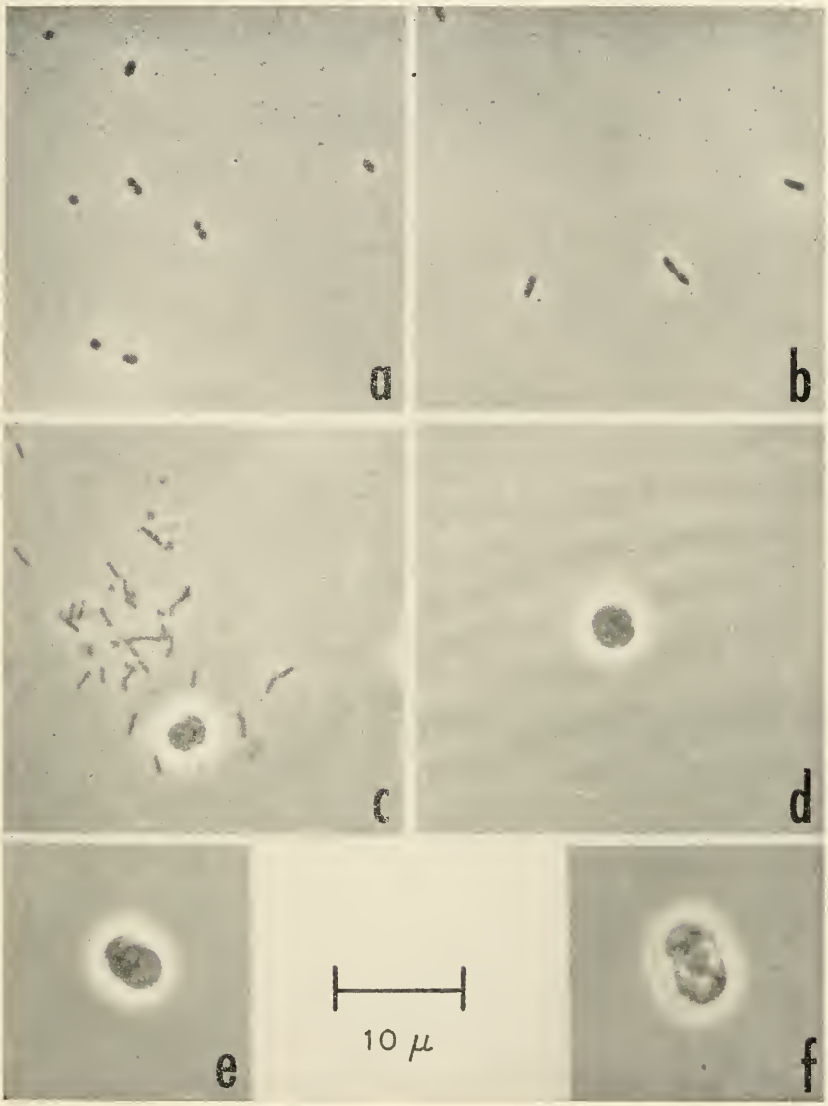

Fig. 2a. Nitrosomonas europaea.
Fig. 2b. Nitrobacter agilis.
Fig. 2c. Marine nitrifying bacteria.
Fig. 2d. Protozoan-like cell just after division.
Fig. 2e. Protozoan-like cell just prior to division.
Fig. 2f. Hypertrophied protozoan-like cell from senile culture.

Dense cultures of both *Nitrosomonas europaea* and its marine analog are yellowish-green, but when they are concentrated by centrifugation, reddish-brown pellets are obtained. Examination of intact cells of both forms in the Carey recording spectrophotometer shows absorption maxima in the 520 and 550 mμ

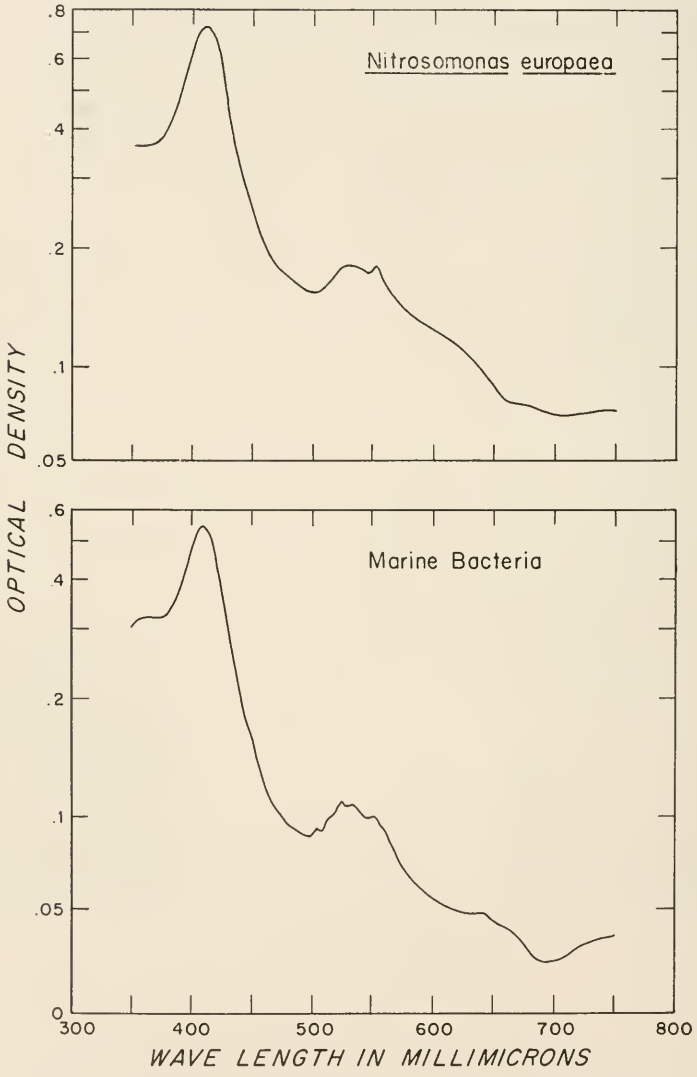

Fig. 3. Light absorption by ammonia-oxidizing bacteria

regions, probably the alpha and beta peaks of cytochrome c (Fig. 3).

Marine and terrestrial bacteria that oxidized ammonia responded almost identically to varying concentrations of ammonia. Maximum growth rates were obtained for both in media containing 25 - 100 mM $(NH_4)_2SO_4$. Maximum respiration rates were constant for both over a range of ammonia concentrations of 2 - 250 mM. Above 250 mM the rate decreased rapidly and in a 1 M solution oxygen uptake decreased by 80 per cent.

Optimal pH for respiration for *Nitrosomonas europaea* and its marine counterpart varied with the ammonia concentration. In the marine culture, maximum oxygen was consumed between pH 7.0 and 8.1 when the $(NH_4)_2SO_4$ concentration was 2.5 mM, and between 7.0 and 7.5 at 310 mM (Fig. 4). *Nitrosomonas*

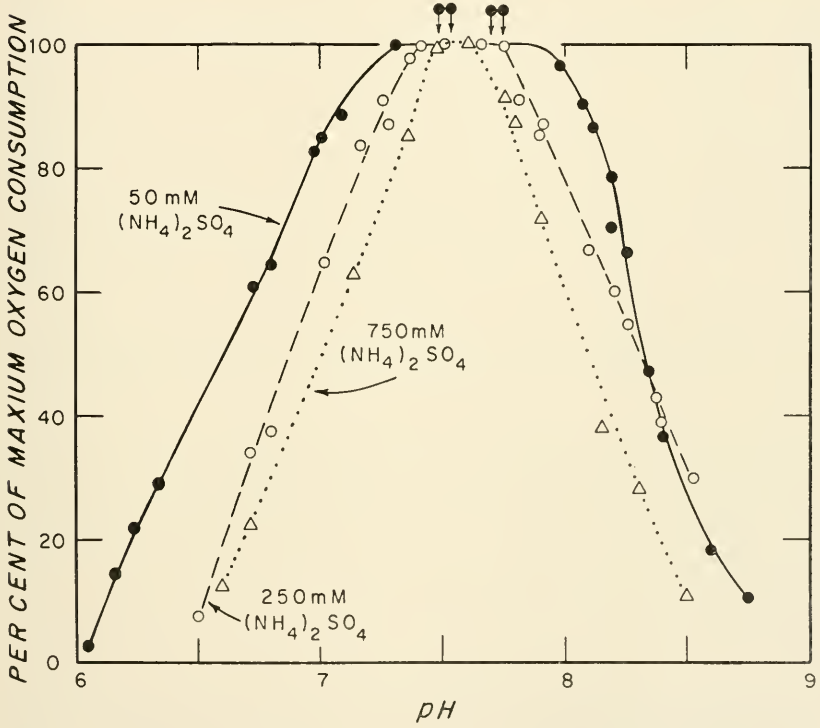

Fig. 4. The effect of hydrogen ion concentration on the respiration of ammonia-oxidizing bacteria.

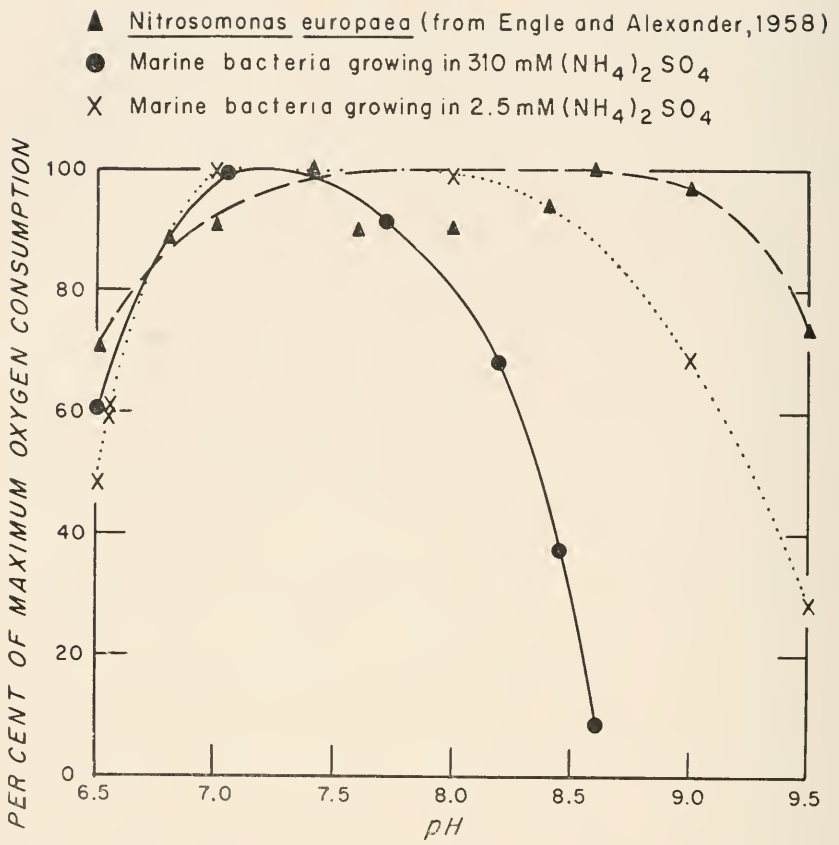

Fig. 5. The effect of hydrogen ion concentration on the respiration of *Nitrosomonas europaea.*

europaea responded similarly, having maximum oxygen uptake in a 50 mM solution between pH 7.25 and 7.9 and in a 250 mM solution between pH 7.3 and 7.75 (Fig. 5). When the ammonia concentration was increased to 750 mM, optimum respiration occurred only between pH 7.4 and 7.6.

DISCUSSION

These studies have yielded the first clues to the site of nitrification in the oceans. In waters less than 100 m deep, nitrification must occur within both the water column and sedi-

ments, since nitrifying bacteria can be cultured from both. In deep waters the distribution of nitrate and cultural studies indicate that the most probable site of nitrification is within the water column rather than the sediments as suggested by Carey (2).

The specific organisms responsible for nitrification in the oceans have been a mystery. The nitrifying bacteria cultured by Thomsen (6) were found less than 1200 m from shore and morphologically and physiologically resembled terrestrial species of *Nitrosomonas* and *Nitrobacter*. Thomsen was probably culturing terrestrial bacteria which had either adapted to the marine environment or had been recently transported into the ocean.

Since the organisms cultured from open ocean water do not resemble terrestrial nitrifying bacteria, it appears that a distinct population of oceanic nitrifying microorganisms does exist. Until these organisms are isolated in pure culture it is impossible to determine which are responsible for nitrification.

My observations of simultaneous increase of nitrites and nitrates in sea water enriched with ammonia differ from the observations of Von Brand, Rakestraw and Renn (7), who found that nitrates did not accumulate until nitrites declined. My experiments differ from theirs since I periodically added ammonia, whereas they followed the changes which occurred with time after initial enrichment with killed phytoplankton. In nature both processes could occur simultaneously.

My observations of the effect of pH on oxygen uptake by *Nitrosomonas europaea* (Fig. 5) differ from those of Engle and Alexander (3) (Fig. 4). In my experiments at very low concentrations of ammonia, oxygen consumption decreased rapidly above pH 8, whereas Engle and Alexander (3) reported that ammonia was oxidized at approximately the same rate from pH 6.8 to 9. They did observe a slight decrease in oxygen consumption followed by an increase between pH 7.6 and 8.6. Engle recently stated (personal communication) that they used a phosphate buffer below pH 8 and Tris above pH 8. In my experiments the pH was adjusted continuously. However, 0.05 M K_2HPO_4 was present in all media used. Thus the differences between my observations and those of Engle and Alexander may be accounted for by differences in technique.

SUMMARY

Nitrification and the presence of nitrifying bacteria were demonstrated in Atlantic oceanic waters collected from the continental shelf and the Sargasso Sea. These nitrifying microorganisms have been maintained in reproducible laboratory culture by periodic subculture for eighteen months. Although morphologically different from any described species of *Nitrosomonas* or *Nitrobacter,* they are physiologically similar to known strains of *Nitrosomonas europaea* and *Nitrobacter agilis.* The one distinguishing physiological difference between terrestrial and marine forms is their response to NaCl. Both terrestrial and marine forms grew for a limited period in the presence or absence of NaCl, but the terrestrial bacteria did best in the absence of NaCl and the marine forms did best in a medium with 2.5 per cent NaCl. These preliminary studies indicate that there are populations of oceanic nitrifying microorganisms within the water column.

ACKNOWLEDGMENTS

I wish to thank Dr. John Kanwisher for his generous help in using the oxygen electrode and Mr. Charles Yentsch who kindly made the measurements with the Carey Recording Spectrophotometer.

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Chapter 8

Nitrogen Fixing Organisms in the Sea*

Mary Belle Allen

Nitrogen in nature goes through a series of transformations from one chemical form into another, as summarized in Figure 1. The studies of Rakestraw and associates (12, 13) and of Watson (Chapt. 7) have demonstrated all of the steps of the nitrogen cycle, with the exception of the fixation of molecular nitrogen, in marine ecosystems. Biological fixation of nitrogen in the sea has been assumed by some (5, 8), but generally neglected by those who have been concerned with nutrient budgets in the oceans (2, 6, 4). The only replenishments of the nitrogen supply these authors have envisaged have been runoff from land and non-biological fixation during thunderstorms. The figures for over-all nitrogen balance, however, are sufficiently uncertain that the question of biological nitrogen fixation in the sea cannot be answered pro or con by reference to them.

This question can be divided into two parts: (a) do nitrogen fixing organisms occur in the sea, and, (b) if so, are they actively fixing nitrogen there? The first part of this question can be answered with an unequivocal yes. Nitrogen fixing marine microbes have been known for a number of years, since the sulfate reducing bacteria studied by Sisler and ZoBell (10) were found to carry out this reaction. However, these bacteria are strict anaerobes, whereas most of the marine environment is aerobic. A search has therefore been made for other potential biological sources of combined nitrogen in the sea. Because of the low concentration of organic matter in sea water, attention was first focused on autotrophic microorganisms, especially the blue-green algae.

Ecologists have known for a number of years (11) that in many places the upper region of the intertidal zone is dominated

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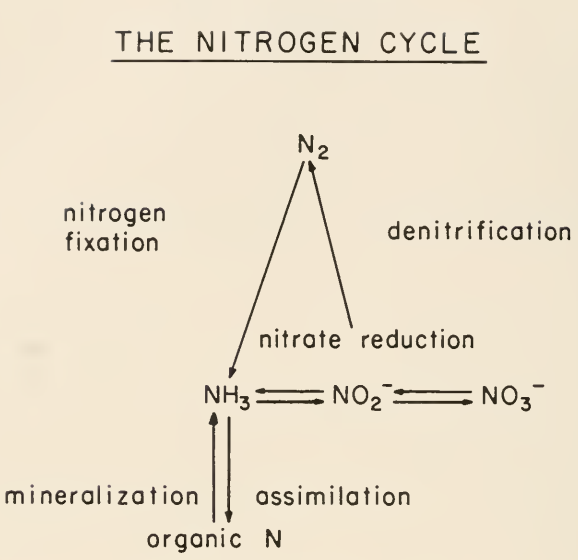

Fig. 1. The nitrogen cycle.

by blue-green algae of the genus *Calothrix*. Close examination of several intertidal zone areas has shown that *Calothrix* is also abundant on rocks at lower levels, but is obscured by the growth of macroalgae found there. *Calothrix* also appear to be fairly abundant in plankton tows made in inshore waters.

Terrestrial and fresh-water members of this genus are known to be active fixers of nitrogen. The same has been found to be true for marine isolates, as shown in Table 1. These figures were obtained with algae grown in the synthetic sea water medium given in Table 2. The appearance of a typical nitrogen fixing marine *Calothrix* is shown in Figure 2.

In the course of a routine screening of Northern California sea water samples to determine the number of nitrogen fixing organisms therein, pink yeasts of the genus *Rhodotorula* frequently developed on the plates of "nitrogen-free" media that were used. Since agar always contains some nitrogen, little attention was paid to these until similar yeasts were isolated from water samples taken in the Gulf of California at Guaymas and near Friday Harbor, Washington, and shown to grow in strictly nitrogen-free liquid media. There is at present, strong presumptive

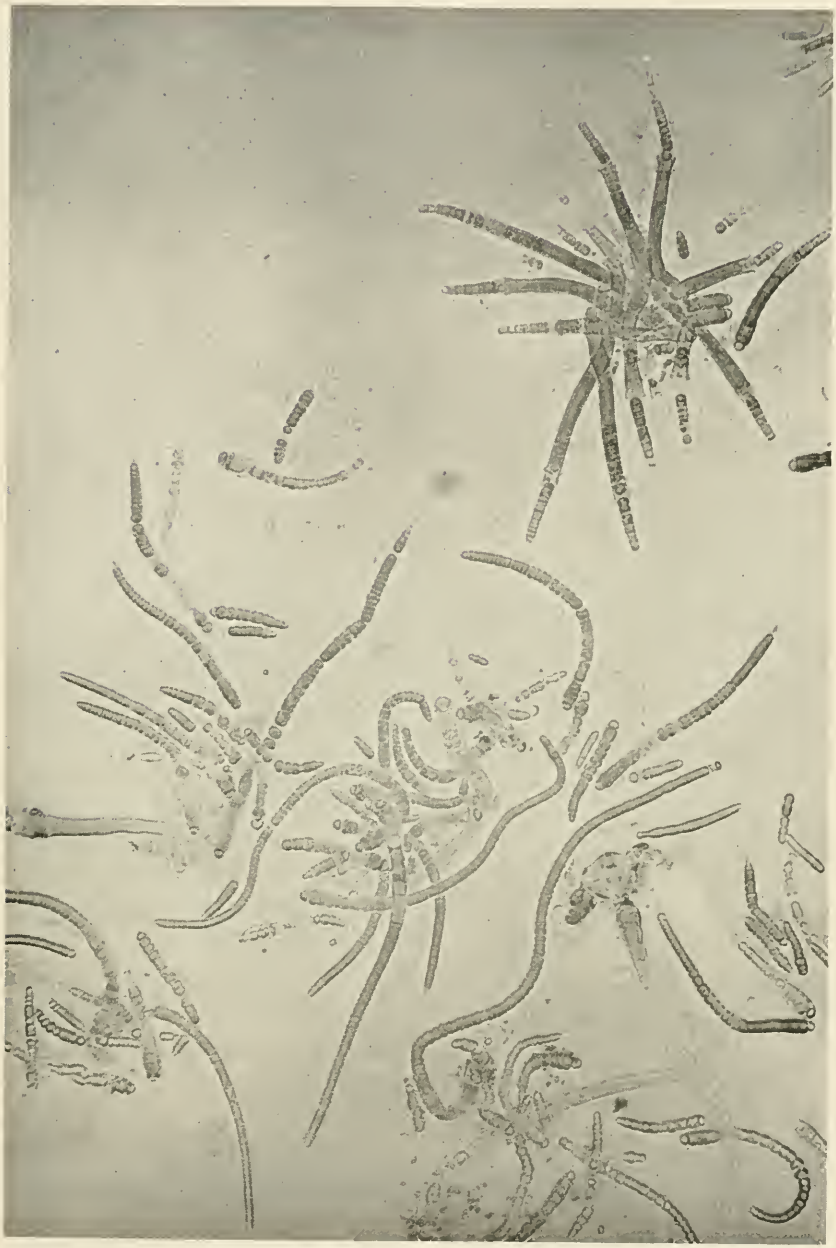

Fig. 2. Nitrogen-fixing marine *Calothrix*.

TABLE 1

NITROGEN FIXATION BY CULTURES OF MARINE BLUE-GREEN ALGAE

Culture	*Addition to Basal Medium*	*Time*	*mg N fixed/100 ml*
1	None	60 days	14.1
Calothrix crustacea	Soil extract	120 days	22.2
2	None	60 days	14.6
Calothrix	Thiamine, biotin, B_{12}	60 days	15.8
crustacea	Thiamine, biotin, B_{12}	120 days	25.2
	Soil extract	120 days	23.8
3	None	60 days	5.7
Nostoc or	Thiamine, biotin, B_{12}	60 days	4.6
Anabaena	Thiamine, biotin, B_{12}	120 days	11.0

TABLE 2

ARTIFICIAL SEA WATER MEDIUM FOR NITROGEN-FIXING ALGAE

Macroelements			*Microelements*		
NaCl	29.2	g/L	Fe	4	mg/L
$MgSO_4 : 7H_2O$	12.3	g/L	Mn	0.5	mg/L
$CaCl_2$	1.1	g/L	B	0.5	mg/L
KCl	0.745	g/L	Zn	0.05	mg/L
K_2HPO_4	0.174	g/L	Cu	0.02	mg/L
			Mo	0.01	mg/L
			V	0.01	mg/L
			Co	0.01	mg/L

evidence that these yeasts fix nitrogen. A definitive answer is currently being sought (9). A soil-dwelling *Rhodotorula* has been shown to fix molecular nitrogen (7). The appearance of these marine yeasts is shown in Figure 3.

The amount of nitrogen contributed to the oceans by these organisms is still to be determined. However, in some regions they develop to such an extent that it should be possible for them to make a major contribution to the nitrogen budget. Up to 6,000 cells of *Rhodotorula* per liter have been observed off the Florida coast (1). Blue-green algae (*Trichodesmium*) form dense blooms in the Sargasso Sea and the Indian Ocean. Near the Great Barrier

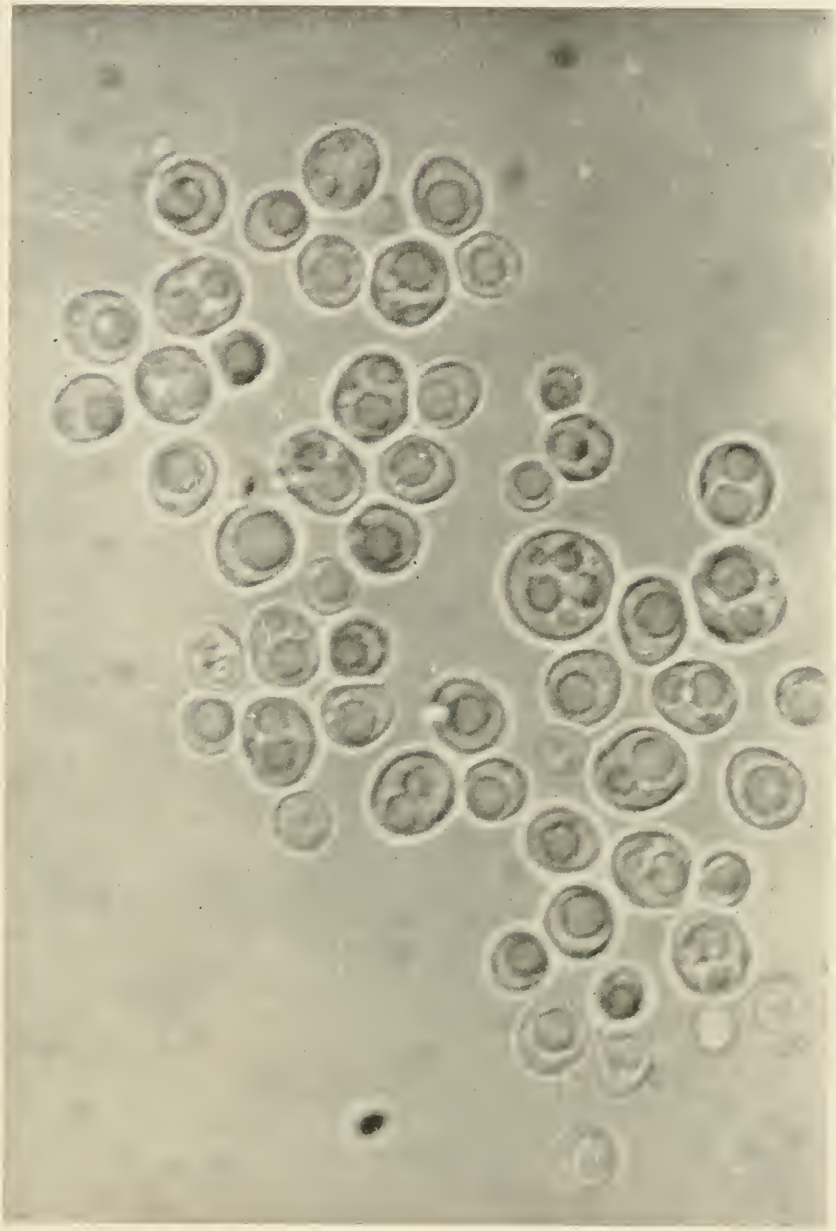

Fig. 3. Marine *Rhodotorula*.

TABLE 3

MICRONUTRIENTS REQUIRED BY NITROGEN FIXERS

	Required for Optimal Growth	*Present in Seawater*
Mo	1-100 μg/L	0.5 μg/L
Fe	4-8 mg/L	.005-.0005 mg/L
Ca	10-20 mg/L	410 mg/L
B	0.1 mg/L	4.7 mg/L

Reef of Australia these blooms may cover hundreds of square miles (14). There is reason to believe that *Trichodesmium* may fix molecular nitrogen (3), although this has not as yet been tested with pure cultures. *Calothrix* has been obtained from several samples of sea water taken up to twenty miles offshore on the California and Mexican coasts. However, routine sampling of Northern California waters (off the Farallone Islands and off Bodega Head) has indicated that they are not abundant in the phytoplankton of this region.*

It is of interest to consider the factors that may determine the growth of nitrogen fixing organisms in the sea. Among these factors are phosphate and some of the micronutrient elements. Certain micronutrients, including iron, boron, calcium, and molybdenum, have been found to be generally required by nitrogen fixing microorganisms (16). The levels found generally to be required are compared with those available in sea water in Table 3. It will be noted that Ca and B are not likely to limit growth of nitrogen fixing organisms in the sea, that Mo is on the borderline of being limiting, and that Fe appears to be in very short supply. If, however, some of the particulate iron in sea water, which is not included in these analyses, is available to the organisms, this element may not be so severely limiting.

Table 4 shows the growth of a marine *Calothrix* isolate at different concentrations of phosphate in the medium. It will be seen that appreciable growth is still obtained at phosphate concentrations as low as those in natural sea water.

* All the water samples mentioned in this paper were assayed by filtering five gallons through an HA Millipore filter (0.45 μ diam. pores), resuspending the material collected on the filter membrane in a little sea water, and plating on the medium given in Table 2. Plates were incubated at 18 - 20 C, in the light, in a moist atmosphere.

TABLE 4

GROWTH OF MARINE *Calothrix* AT DIFFERENT PHOSPHATE CONCENTRATIONS

Dry wt. *g/L*	*(PO_4)* *mM*	*grams/millimole*
0.60	1	0.60
3.70	0.33	11.2
1.84	0.1	18.4
0.72	0.033	21.8
0.44	0.01	44.0
0.16	0.0033	48.5
0.06	0.001	60

Phosphate in natural seawater is 0.003-0.00003mM.

It has thus been shown that nitrogen fixing microorganisms occur in sea water, often in considerable numbers, and that the available nutrients should permit them to grow and fix nitrogen there. Isotopic studies to determine the amount of fixation actually taking place in the ocean now appear to be called for.

ACKNOWLEDGMENTS

Thanks are due to Commander, 12th Coast Guard District, and the officers and men of the cutters *MAGNOLIA* and *WILLOW* for making possible collection of water samples at the San Francisco Lightship and the Farallone Islands; to the Friday Harbor Laboratory for hospitality; and to Dr. Joel Hedgpeth and Mr. Ward Renshaw for collection of water samples off Bodega Head. Dr. T. J. Starr and Mr. J. C. Murchio assisted with collection and processing of the Guaymas and Friday Harbor samples, and Mr. K. V. Natarajan has participated in the later laboratory experiments.

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Chapter 9

Organic Sources of Nitrogen for Marine Centric Diatoms*

Robert R. L. Guillard

INTRODUCTION

There is little information on the capacity of marine diatoms to use organic compounds as nitrogen sources. Experiments with natural populations of diatoms and bacteria led Harvey (11) to conclude that amino acids were not utilized significantly unless first deaminated by bacteria, but that uric acid and urea were. Trimethylamine, an excretory product of fishes, was not used at all.

Various marine flagellates and green algae have been examined for ability to use organic nitrogen. Some of these species inhabit environments (e.g., rock pools) where the amount of organic material is at times high. Such studies were made by Schreiber (23), Braarud and Føyn (1), Droop (4, 5), Gibor (8) and McLaughlin (17). No organic sources were suitable for all species, but the data suggest that uric acid and urea are the ones most generally utilized. Two species require amino nitrogen: the photosynthetic cryptomonad *Hemiselmis virescens* grows best on glycine (4), while the colorless phagotrophic dinoflagellate *Oxyrrhis marina* could not use glycine but grew on valine, proline, or alanine.

The growth of the attached green alga *Prasiola stipitata* (which often occurs in association with the excreta of gulls), was stimulated by 0.1 per cent glutamate or asparagine, but had no absolute requirement for an organic source of nitrogen (15).

A few fresh water green algae have been studied intensively

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and found able to assimilate organic nitrogen. They are considered important in the degradation of organic matter in contaminated waters (7).

Ryther (21) suggested that in an estuary contaminated by wastes from duck farms, the phytoplankton species that became dominant were favored by their ability to use the early products of degradation of organic nitrogenous matter, as well as by their versatility in other respects. It is not easy to assess the influence of soluble organic nitrogenous compounds in other marine environments, however the quantity is not negligible (6, 10). In coastal waters off New England the ratio total nitrogen: total phosphorous is two to twenty times higher than the ratio inorganic nitrogen: inorganic phosphorus (14); inorganic nitrogen is exhausted before phosphate, thus the capacity of an organism to use soluble organic nitrogen might be advantageous at times. The same reasoning holds for the nutrient-poor waters of the Sargasso Sea, where rapid recycling of nutrients takes place (18, 20).

The present study compares, in a rough quantitative way, growth rates of a number of species of marine algae when supplied either inorganic nitrogen or selected organic compounds. All species were compared on glycine, glutamic acid, glutamine, uric acid and urea, and were tested for their ability to grow on ammonia or nitrite.

MATERIALS AND METHODS

The method is outlined as follows. An inoculum of each species was transferred to:

(1) Sea water enriched with all nutrients except nitrogen (Table 1).

(2) Sea water enriched as in (1) but with 100 μM nitrate added.

(3) Sea water enriched as in (1) but with 100 μM of an organic compound added as nitrogen source.

(4) Enriched sea water as in (1) with both nitrate and the organic compound (this treatment was omitted in the case of glutamine).

For two organisms, flagellates ϕ and θ, 100 μM NH_4Cl was used instead of $NaNO_3$.

TABLE 1

MEDIUM 'F'

Sea Water	1 liter
$NaNO_3$	150 mg (1.765 mM)
$NaH_2PO_4 \cdot H_2O$	10 mg (72.5 μM)
$Na_2SiO_3 \cdot 9H_2O$	30 mg (10>μM)
Ferric sequestrene (1)	10 mg (1.3 mg Fe)
$CuSO_4 \cdot 5H_2O$	0.0196 mg (0.005 mg Cu)
$ZnSO_4 \cdot 7H_2O$	0.044 mg (0.01 mg Zn)
$CoCl_2 \cdot 6H_2O$	0.02 mg (0.005 mg Co)
$MnCl_2 \cdot 4H_2O$	0.360 mg (0.1 mg Mn)
$Na_2MoO_4 \cdot 2H_2O$	0.0126 mg (0.005 mg Mo)
Thiamine·HCl	0.2 mg
Biotin	1.0 μg
B_{12}	1.0 μg

[1] Ferric sequestrene (Geigy Industrial Chemicals, Saw Mill River Parkway, Ardsley, New York) is the sodium iron salt of ethylene diamine tetra acetic acid, 13 per cent iron.

At 100 μM of inorganic nitrogen, the yield of most of the algae was shown to be limited by the quantity of nitrogen under the experimental conditions.

When growth in flasks containing nitrate or ammonia was approximately maximum, yields were measured in all flasks. The yields without added nitrogen (item 1 above, amounting at most to 5 per cent of the maximum yields) were subtracted from the yields in all other treatments (items 2, 3, 4) to determine the growth due solely to the added nitrogen sources. The fraction (growth on organic source/growth on inorganic source), expressed as per cent, was taken as a measure of capacity to use the nitrogen source in question. Treatment (4) was included to detect possible inhibition by the organic compounds.

Those flasks containing organic sources in which the algae had grown comparatively little at the time yields were measured were observed for a longer time to see if growth occurred, indicating adaptation. It was also noted if the final yields approached the yields with nitrate in a reasonable time.

Culture Conditions and Methods

Stock cultures were grown in 50 ml of autoclaved medium

"F" (Table 1) in 125 ml Erlenmeyer flasks. The basic enrichment for the experiments was 'F' with nitrate omitted. Experimental flasks were washed with detergent and soaked in 30 per cent HCl. All but three species of the algae were grown under 4000 - 10,000 meter-candles of light provided by equal numbers of "cool-white," "natural," and "daylight" fluorescent bulbs at 20 - 22 C. Three species required the temperatures indicated and were provided *ca* 8000 meter-candles' illumination from "cool-white" bulbs: *Detonula confervacea*, 4 to 8 C; *Thalassiosira nordenskioldii* 8 to 12 C; *Rhizosolenia setigera*, 15 C. The buffer tris (hydroxymethyl) amino methane, (500 mg/L, pH 7.2) was added to the medium for these three species; it did not serve as a nitrogen source.

The sea water used for all experiments was collected from the Sargasso Sea and aged for several months. It contained the following (before enrichment):

NO_2 + NO_3-nitrogen	0.82 μM
ammonia nitrogen	2 μM

Methods of analysis are cited by Ketchum *et al.* (14).

Inoculum was taken either from dense stock cultures suitably diluted with sea water or from cultures prepared without added nitrogen to deplete the cells. The number of cells transferred varied from as few as 10/ml to *ca* 5000/ml. Yields of all algae except *Melosira* sp. were measured by counting cells in a hemocytometer, Palmer-Mahoney chamber, or Sedgwick-Rafter chamber, as appropriate. *Melosira* filaments were separated in a tissue homogenizer and optical densities of cultures compared in a spectrophotometer at 750 mμ. Cell numbers were assumed proportional to optical density.

Replication was provided by repeating the assay for each organism rather than by having replicate flasks in any one experiment. Thus each repetition of the experiment received inoculum from a different culture, and sometimes had a different light intensity.

Nitrate and glycine were autoclaved in the medium in some experiments but usually N-sources were sterilized separately and

added aseptically to autoclaved nitrogen-free medium. Stock solutions of glutamate, glutamine, and urea were sterilized by filtration through a membrane filter ("Millipore," 0.45 μ pore size). The entire medium containing uric acid was filter sterilized because of the low solubility of uric acid. Sterility tests of algal stock cultures, nitrogen stocks and certain experimental flasks were made with ⅔ strength nutrient broth and agar (Difco), made with both distilled water and 80 per cent sea water and with medium STP of Provasoli *et al.* (19). Three weeks of incubation at the temperatures of the algal cultures were allowed for bacterial growth.

The species of algae studied are shown in Table 2, with the locations from which they were isolated. *Coscinodiscus asteromphalus* was isolated by Miss Irma Pintner, and *Coccolithus huxleyi* (clone 92-A) by Dr. Mary Parke; the others were isolated by the author. All clones were bacteria-free except *Chaetoceros lorenzianus*. Some flagellates were included to compare with diatoms of the same habitats. Clones BT-5 and BT-6 (*Coccolithus huxleyi*) were of particular interest because they are present throughout the year in the Sargasso Sea (13).

RESULTS

Table 2 shows the vitamin requirements of the different strains and result of experiments in which growth rates were observed in media having different salinities. A critical salinity interval, when found, is given for each species. Methods are discussed elsewhere (9, 22).

Growth Without Added Nitrogen

The aged Sargasso Sea water used had about 2.8 μM/L of total inorganic nitrogen and an unknown amount of residual organic nitrogen. The increase in cell numbers without added N ranged from 0.5 to 5.3 per cent of the increase brought about by 100 μM nitrate or ammonia. The average was 2.6 per cent, which corresponds to the measured amount of inorganic nitrogen. This agrees with Harvey's observation (12) that in freshly collected sea water the amount of nitrogen that was used by two

TABLE 2

VITAMIN REQUIREMENTS, REGIONS FROM WHICH STRAINS WERE ISOLATED, AND SALINITY DATA

Species (clone)	*Isolated from*[1]	*Vitamin(s) Required*	*Salinity (‰) Permitting Growth Rate*	
			Over Half Maximal	*Negligible*
Carteria (?) sp. (BT-2)	SS	B_{12}, thiamine	36 - 20	<16
Chaetoceros sp. (BBsm)	SS	B_{12}	36 - 20	<16
Chaetoceros lorenzianus	SS	B_{12}	36 - 20	16 and below
Chaetoceros pelagicus (Ch. 4)	SS	B_{12}	36 - 24	<16
Cyclotella nana (13-1)	SS	B_{12}	36 - 20	<17.5
Coccolithus huxleyi (BT-6)	SS	thiamine	36 - 20	16 and below
Coccolithus huxleyi (?) (BT-5)	SS	B_{12}	36 - 24	16 and below
Coccolithus huxleyi (92-A)	—	thiamine	36 - 20	16 and below
Nitzschia seriata	SS	B_{12}	36 - 24	20 and below
Skeletonema (?) sp. (Men 5)	SS	B_{12}	36 - 18	9 and below
Coscinodiscus asteromphalus	VS	B_{12}	36 - 9	4 and below
Cyclotella caspia (10-5)	CSW	B_{12}	36 - 0.5	——
Cyclotella nana (7-15)	CSW	B_{12}	36 - 4	<2
Rhizosolenia setigera	VS	(2)	36 - 18	9 and below
Skeletonema costatum	LIS	B_{12}	36 - 9	4 and below
Thalassiosira nordenskioldii	VS	(2)	36 - 20	<16
Cyclotella nana (3H)	GSB	B_{12}	36 - 0.5	——
Detonula confervacea	NB	none	36 - 8	4 and below
Flagellate o	MHC	B_{12}	36 - 0.5	——
Flagellate θ	MHC	B_{12}	36 - 0.5	——
Melosira sp. (0-8)	MVO	none	36 - 8	2 and below
Thalassiosira fluviatilis	GIP	B_{12}	36 - 0.5	——

[1] SS=Sargasso Sea;
VS=Vineyard Sound, Mass.;
CSW=Continental shelf waters;
LIS=Long Island Sound;
GSB=Great South Bay, Long Island;
NB=Narragansett Bay, Rhode Island;
MHC=Milford Harbor, Conn.;
MVO=Martha's Vineyard, Oyster Pond;
GIP=Gardiner's Island Pond, Long Island;

[2] B_{12} required or markedly stimulatory.

species of algae corresponded only to the measured inorganic nitrogen.

Inorganic Sources of Nitrogen

Addition of nitrite (or nitrate) to nitrogen-free medium did not result in significant growth of flagellates ø or 0. *Skeletonema costatum, Rhizosolenia setigera,* and *Nitzschia seriata* were inhibited somewhat by nitrite; the other clones grew as well on nitrite as on nitrate. All strains grew in ammonia but the following did not grow as well on ammonia or ammonia + nitrate as on nitrate alone: *Nitzschia seriata, Coccolithus huxleyi* clones 92-A and BT-5, *Cyclotella nana* (7-15), *Cyclotella caspia, Skeletonema* sp. (Men 5), and *Coscinodiscus asteromphalus.* Inhibition by ammonia (or other compounds) was greater when the inoculum was small or nitrogen-starved and less pronounced when cultures were in dim light.

Organic Sources of Nitrogen

Studies of growth on organic sources are summarized in Table 3. Values recorded are the maximum observed for each species during the experiments.

Glutamic acid was a poor source of nitrogen except for *Coscinodiscus asteromphalus* and *Melosira* sp. (0-8) which used it as well as they did nitrate. Only three other species attained as much as a tenth the yield on nitrate. Glycine, the other alpha-amino acid, was not even as good as glutamate for most species, but *Coscinodiscus asteromphalus* reached 69 per cent of the yield on nitrate and *Skeletonema costatum* reached 18 per cent. The amide glutamine was better than glutamate for all species except *Thalassiosira nordenskioldii* (8% vs. 22%). *Coscinodiscus* and *Melosira* grew as well on glutamine as they did on nitrate or glutamate, and three other species, including two from the Sargasso Sea, reached at least 30 per cent of the inorganic nitrogen yield.

Urea was a better source than glutamine for most of the neritic and estuarine species. Only *Skeletonema* (clone Men 5) of the species isolated from the Sargasso Sea used it readily. Growth of *Chaetoceros lorenzianus* probably depended upon decomposition of the urea by bacteria.

TABLE 3

GROWTH IN MEDIUM CONTAINING ORGANIC NITROGENOUS COMPOUNDS, AS PER CENT OF GROWTH IN MEDIUM CONTAINING NITRATE

Species	*Glutamic Acid*	*Glutamine*	*Glycine*	*Urea*	*Uric Acid*
Carteria (?) sp. BT-2	0	3	3	9	5*
Chaetoceros sp. (BB sm)	0	5	3	0	3*
Chaetoceros lorenzianus	3	10	5	#	1
Chaetoceros pelagicus	0	5	2	0	0
Coccolithus huxleyi (BT-6)	5	9*	3	0	0
Coccolithus huxleyi (?) (BT-5)	0	0**	0**	0	3
Coccolithus huxleyi (92-A)	3	30	1	0	18
Cyclotella nana (13-1)	4	13*	4	3	2
Nitzschia seriata	12	41	0	10	5
Skeletonema (?) sp. (Men 5)	1	14	1	90	22*
Coscinodiscus asteromphalus	120	103	69	18	12
Cyclotella caspia (10-5)	10	18	1**	70	40*
Cyclotella nana (7-15)	0	17	0	133	39
Rhizosolenia setigera	0	16	6	22	0
Skeletonema costatum	0	4	18	60*	23
Thalassiosira nordenskioldii	22	8	1	22	25
Cyclotella nana (3H)	1	5	7	60*	70*
Detonula confervacea	3	8	6	13	14
Flagellate o+	1	9	4	14*	82*
Flagellate θ+	0	30	0	73	37
Melosira sp. (0-8)	85	100	5	50	60
Thalassiosira fluviatilis	0	3	3	10	15

* Cultures continued to grow significantly.

** Cultures attained a significant yield later.

\# Variable growth, due to bacterial action on the urea.

+ NH_4Cl used instead of $NaNO_3$.

On uric acid, only two of the presumably oceanic algae—*Coccolithus huxleyi* (clone 92-A) and *Skeletonema* (clone Men 5)—attained a yield over 5 per cent of that on nitrate. On the other hand, all the coastal and estuarine species except *Rhizosolenia* reached yields of at least 12 per cent and six of the twelve species grew at least as well on uric acid as on urea.

Toxicity and Other Effects

The following were somewhat inhibitory in the presence of nitrate: glycine to *Coccolithus huxleyi*, uric acid to *Cyclotella caspia* and clone BT-6 of *C. Huxleyi;* urea to *Chaetoceros pelagicus* and *Skeletonema costatum*. Growth of clone BT-5 of *C. huxleyi* was delayed (again in the presence of nitrate) by all organic sources for about two days, but final yields were about the same as in nitrate alone. The green alga BT-2 (*Carteria?*) also showed a delay but finally grew excellently on uric acid.

In a few experiments the inoculum died on transfer to an organic N source alone (*i.e.*, without nitrate); in some cases when the source was known from other experiments to be utilizable. This occurred more frequently when the inoculum was small, severely depleted, or put into strong light. Some species may only survive and grow at low light intensity if given a N-source that is assimilated slowly. This would be significant ecologically, but has not been investigated.

Urea and uric acid brought about morphological changes in a few species. *Cyclotella caspia* cultures grown on uric acid were much darker in color than those supplied nitrate. The amount of chlorophyll *a* per cell was much larger in the uric acid grown cells than in nitrate grown cells, though the cell volumes were only slightly larger. In one experiment the values were 71×10^{-8} μg/cell in uric acid and only 3.6×10^{-8} μg/cell in nitrate. This effect on chlorophyll *a* content was not noticeable with other species, but no measurements were made. McLachlan and Yentsch (16) noted that peptones increased chlorophyll production by *Dunaliella tertiolecta* (see this reference for methods).

DISCUSSION

Harvey's conclusion (11) that the simple amino acids do not serve directly as a source of nitrogen for marine algae is confirmed, with the notable exception that the bottom-dwelling diatoms *Coscinodiscus asteromphalus* and *Melosira* (0-8) used glutamate and glutamine as readily as inorganic nitrogen in these experiments. Possibly these species have heterotrophic leanings. Bruchmayer-Berkenbusch (3) reported that amino acids influenced auxospore formation in *Melosira nummuloides*.

The species isolated from estuarine or neritic environments generally used either urea or uric acid or both significantly, again in general agreement with Harvey's findings. However, of the nine clones isolated from the Sargasso Sea, only one, *Skeletonema* (?) (clone Men 5) did so; this clone, it should be noted, was able to grow at somewhat lower salinities than the others isolated from the Sargasso Sea. The two Sargasso clones of *Coccolithus huxleyi* (BT-5 and BT-6) were unable to use urea or uric acid, while clone 92-A (from the algal collection at Plymouth, England) was able to use uric acid about as well as *Skeletonema* (clone Men 5). *C. huxleyi* is in fact common in polluted Scandinavian waters (2). Inability to use urea and uric acid may be widespread in oceanic populations. For such algae, the best nitrogen source of those studied is glutamine.

The organisms able to use uric acid best came from polluted estuarine waters. There is evidence suggesting that the extent to which such organisms dominate the plankton can at times be associated with their capacity to grow on uric acid. *Cyclotella nana* (clone 3H) dominated the waters in Great South Bay from which it was isolated (9) and grew rapidly on uric acid. *Cyclotella caspia* also used this source well and was common in Great South Bay. Two other organisms from Great South Bay were studied—*Thalassiosira fluviatilis* and a small green flagellate (not otherwise mentioned in this paper, but similar to BT-2). These algae were almost always present in the plankton, but never in large numbers; both used uric acid more slowly than *Cyclotella nana* (3H). Great South Bay has a rich and varied flora of diatoms and flagellates. Phosphorus is present in excess in its waters, and nitrogen becomes available in water of low salinity at the heads of small creeks and streams. It is interesting that those planktonic organisms that have been observed to dominate its waters have not only been euryhaline, but able to use nitrogen in organic combination relatively rapidly.

SUMMARY

A survey of growth of marine planktonic algae with nitrate, nitrite, ammonia, urea, uric acid, glycine, glutamate and glutamine as nitrogen sources (100 μM) included 15 clones of centric

diatoms, one pennate diatom, two estuarine flagellates, an oceanic green flagellate, and three coccolithophorides.

The two estuarine flagellates used only ammonia among the inorganic N-sources; the other algae used nitrate and nitrite as well. While most organic sources allowed some growth above that in the N-free medium, the alpha amino acids were generally poor, except that the bottom-dwelling diatoms *Melosira* and *Coscinodiscus* grew as well on glutamate as on nitrate. Glutamine was a somewhat better source in general. Urea and uric acid were consistently better for most estuarine and neritic clones, but not for the oceanic ones.

There was no evidence that naturally occurring organic nitrogen in the sea water employed was used by any algae.

Evidence suggests that direct utilization of uric acid may be significant in some environments.

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Chapter 10

Limited Heterotrophy of Some Photosynthetic Dinoflagellates*

L. Provasoli and J. J. A. McLaughlin

STRAINS USED

Gyrodinium californicum Bursa. A unialgal culture kindly given us by Dr. B. Sweeney was purified by repeated micropipette washings. She had cultured it from waters in Sorrento Slough during bloom condition (10,000 cells/ml); in the sea off Scripps pier only a relatively few were present. The Slough, situated at the mouth of a small river near La Jolla, California, is dry most of the year and divided from the sea by a sandbar; at high tides the sea overflows the bar. In previous publications this organism was referred to as *Gyrodinium* sp. and was later identified by A. Bursa (10, 13, and Bursa, in preparation).

Exuviaella cassubica, Wolosz., *Peridinium chattoni* Biecheler, and *P. balticum,* Lemm. were abundant in a brackish pond at Sippewisset, Cape Cod, Mass. *Potamogeton pectinata* and *Ruppia maritima* were also growing in the pond which had a salinity of 0.63 per cent. The dinoflagellates were purified in 1955 by micropipette washings. *Gymnodinium splendens* Lebour, and *Gyrodinium resplendens* Hulburt, were purified from waters off Mamaroneck, Long Island Sound in 1954. *G. resplendens* was first washed six times, then grown in enriched seawater containing 200 units/ml of penicillin and 1 unit/ml each of chloramphenicol, neomycin, and polymixin B; after fifteen days growth in antibiotics, the culture was bacteria-free (purified by Mrs. Susan Egloff). *G. splendens* was purified by micropipette washings.

Gyrodinium uncatenum Hulburt, kindly given us by Dr.

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Richard E. Norris who purified it, was abundant in a pond in Berkeley Aquatic Park, California. *Amphidinium rhynchocephalum* Anassimowa, from a sample of water of the inland Salton Sea, and *A. carterii** from a unialgal culture kindly supplied by Dr. Robert Guillard (isolated from Great Pond, Falmouth Mass.) were both purified in 1955 by transfer and growth for fifteen days in a medium containing 600 units/ml of penicillin followed by a few micropipette washings.

All the strains of dinoflagellates, except *A. carterii* and *Exuviella cassubica*, were lost because of repeated failures of the refrigerator-incubators. Knowledge of their nutrition is therefore sketchy. Cultures were grown in screw-capped tubes, incubated at 15 to 20 C under 200 to 400 foot-candles of "cool white" fluorescent light.

GROWTH IN ARTIFICIAL MEDIA

Construction of reproducible, non-precipitating marine media was approached in two convergent ways: *a*) by studying the nutrition of single marine species in the hope of finding common requirements; *b*) by finding ways to simplify seawater-like solutions, to avoid precipitation during autoclaving, and to buffer the artificial media.

G. californicum, as the first marine organism obtained by us in bacteria-free culture, was a major test organism in devising good media (15, pp. 402-10). An early medium for *G. californicum* (13) shows how clumsy were the first attempts (Table 1). When we began with marine organisms it was known that *Gymnodinium splendens* grew in aged seawater enriched with N, P, and soil extract (17). Soil extract for freshwater algae could be replaced by B-vitamins and trace metals (13). Soil extract was similarly replaced for *G. californicum*. But, to avoid precipitates, we employed a high concentration (20 mg%) of ethylenediamine tetraacetic acid (EDTA). Such concentrations of EDTA created difficulties. In calcium-rich media (and in solutions similar to seawater) EDTA forms the dicalcium salt which is poorly soluble; also the excess EDTA had to be compensated

* Formerly designated as *A. klebsii*.

for by raising manyfold the concentrations of trace metals. Small variations in pH resulted in deficiencies or toxicities of available trace metals. These difficulties were intensified when the level of chelators and trace metals was high. When *G. californicum*, *E. cassubica* and the cryptomonad *Rhodomonas lens* (14) proved rather flexible in respect to salinities and variations in monovalent/divalent ratios. These obstacles were partly circumvented by lowering the salinity and calcium concentrations.

During the search for a non-toxic pH buffer we found that the three *Gyrodinium* are sensitive to triethanolamine (inhibitory at 30-100 mg%; toxic above). Tris (hydromethyl) amino-methane (TRIS) is not inhibitory for the same species up to 600 mg%. A concentration of 100 mg% is adequate for most marine algae, although Droop (2) found that TRIS is inhibitory for *Oxyrrhis marina*. The use of diluted over-chelated trace metal mixtures and of a pH buffer (TRIS) resulted finally in non-precipitating media such as ASP_2, ASP_2NTA, ASP_6 (15). These media were designed to be — and indeed are — all-purpose media: they permit growth of diatoms, cryptomonads, chrysomonads, and dinoflagellates, and have been recently employed for blue-green algae (19), chlorophytes (8) and red algae (4, 5, 6). This versatility was achieved by arbitrarily selecting levels of nutrients well within the zone needed or tolerated by several species of marine algae and flagellates. Although these media were derived from media like DC, MGC, AC, (Table 1) which were arrived at experimentally with a single organism (respectively *Amphora perpusilla*, *G. splendens*, *Amphidinium carteri*), they differ by being more complete, e.g., containing silica (which is needed only by diatoms and some chrysomonads) and several additional B vitamins even though so far only B_{12}, thiamine or biotin have been shown to be needed by marine algae. None of these media is *the* best for any one species, e.g., ASP_1, ASP_2, ASP_2NTA, $ASP_{12}NTA$, and DV (modified from DC) supported growth of the three *Gyrodinium*, *G. splendens*, and of the two *Amphidinium* and suitably diluted (to salinities of 0.6-1.0%), of *P. balticum* and *P. chattoni*.

TABLE 1

MARINE MEDIA (mg %)

	Gyro. californicum	*Gymno. splendens*	*Amphi. carteri*	*Amphora perpusilla*
		(MGC)	(AC)	(DC modified = DV)
$NaNO_3$		100.0	10.0	50.0
KNO_3	10.0			
KH_2PO_4	2.0			
K_2HPO_4			1.0	3.0
Monoethyl PO_4		5.0		
$MgSO_4.7H_2O$	30.0	0.6gm	0.5gm	0.5gm
$MgCl_2.6H_2O$	0.3gm			
NaCl	2.4gm	2.4gm	1.8gm	1.8gm
Ca (as Cl)	5.0	10.0	10.0	10.0
KCl	30.0	60.0	80.0	60.0
EDTA	20.0	3.0	3.0	3.0
Fe (as Cl)	0.3	0.03	0.08	0.04
Fe (as SS) (1)		0.2 (1)		
Zn (as Cl)	0.4	0.015	0.015	0.015
Mn (as Cl)	1.0	0.12	0.12	0.12
Mo (as Na salt)	0.05			
Co (as Cl)	0.003	0.003	0.003	0.003
Cu (as Cl)	0.0003		0.0012 (2)	0.0012 (2)
S 1 Metals	0.5 ml./100			
Boron	0.2	0.6	0.6	0.6
Na H glutamate	2.0			
DL-Lysine	1.0			
DL-*Leucine*	0.2			
Nitrilotriacetic acid		10.0		
Tris		100.0	100.0	100.0
B_{12}	0.01 μgm	0.01 μgm	0.003 μgm	0.3 μgm
Thiamine HCl			0.05	
Biotin			0.05 μgm	
Vitamins 8a (4)				0.1 ml./100
Na_2CO_3			3.0	
pH	7.5	7.8-8.0	7.8	7.5-7.8

(1) Sulphosalicylic acid used to dissolve $FeSO_4(NH_4)_2SO_4$

(2) Omitted from later media

(3) S 1 Metals: 1 ml. of the mixture contains the following metals: Sr (as Cl) 1.3 mg., Al (as Cl) 0.05 mg., Rb (as Cl) 0.02 mg., Li (as Cl) 0.02 mg., I (as KI) 0.005 mg., Br (as NaBr) 6.5 mg.

(4) See Provasoli *et al.* (15).

These stock media save time in screening newly-collected organisms. One or more of these media will generally support growth and so serve to establish clonal bacteria-free cultures.

SALINITY TOLERANCE

Peridinium balticum and *P. chattoni* are stenohaline brackish organisms. While motile for a long time at seawater concentration, they divide only in more dilute media. The optimal concentration is one-third to one-fourth seawater (0.8-1.2% salinity), the upper limit being 2.0 per cent (14). *P. balticum* was thought to be euryhaline (1) because it was found in ponds ranging from brackish to seawater. The seeming discrepancy may be explained by our observation that it stays motile at 0.05-3.5 per cent salinity. ASP_1 medium diluted to one-third or other media diluted to 1 per cent salinity are adequate media. *Exuviaella cassubica*, obtained from the same brackish pool, is on the contrary euryhaline; it grows in salinities from 0.08 to 3.5 per cent. Growth is better in media of 1-2 per cent salinity (i.e. half-strength ASP_2 or ASP_1). The other species of dinoflagellates have an optimal salinity between 2.0-2.5 per cent, the limits ranging from 1.5 to 4.0 per cent.

AUXOTROPHY, PHOTOAUTOTROPHY, AND HETEROTROPHIC ABILITIES

The aforementioned dinoflagellates, like many other photosynthetic algae, need one or more vitamins. All need B_{12}; *Amphidinium carteri* and *A. rhynchocephalum* also require biotin and thiamine.

Specificity toward various cobalamins differs slightly among the various species. All utilize the dichlorobenzimidazole and benzimidazole analogues, and Factor III (5-hydroxybenzimidazole); they do not utilize pseudo B_{12} and Factor B(B_{12} minus nucleotide). The three *Gyrodinium* do not utilize Factor A (2-methyladenine) nor Factor II (2-methylhypoxanthine) which are utilized by the two *Amphidinium;* Factor A is also utilized by *P. balticum* and *E. cassubica*. The specificity of *G. splendens* and *P. chattoni* was not determined.

The activity of the utilizable cobalamins measured as final

growth is substantially similar to the one of B_{12} (Table 2). On the contrary, some analogs are more active than B_{12}, for other auxotrophs, i.e., pseudo vitamin B_{12} for *Euglena gracilis* (3), and Factor III for *Goniotrichum elegans* (5). Aside from the minute heterotrophy represented by the need for growth factors, all the species tested are good photoautotrophs. Nitrate is an excellent N source (optimal range 5-30 mg%). Ammonium sulfate is toxic for *G. californicum* at 0.5 mg%, for *E. cassubica*, *P. chattoni*, and *P. balticum* at 1 mg%, and for *A. carteri* and *A. rhynchocephalum* at 10 mg% (lowest concentrations tried). Unfortunately these concentrations far exceed those in seawater. Quite likely at very low concentrations, ammonia is a good N source even in the alkaline range; ammonia in the neutral or acid range is non-toxic and utilized by most – probably all – algae.

TABLE 2

GROWTH RESPONSE TO DIFFERENT B_{12} ANALOGUES (CELLS PER ML.)

B_{12} Analogues	*Amphidinium carteri*	*A. rhyncho-cephalum*	*Gyrodinium californicum*	*G. uncatenum*	*G. resplendens*
10 mμg%	(1)	(1)	(2)	(2)	(2)
No addition	6,000	6,900	1,000	2,000	500
B_{12}	240,000	341,000	190,000	260,000	190,000
Pseudo B_{12}	5,900	10,000	1,000	1,000	700
Factor A	151,000	181,000	1,000	1,000	800
Factor B	7,000	7,000	1,000	40,000	2,000
Factor III	270,000	373,000	140,000	280,000	220,000
Factor H	71,000	159,000			
Benzimidazole analogue	336,000	210,000	110,000	230,000	140,000
Dichlobenz-imidazole analogue			90,000	270,000	260,000

(1) Forty days growth in ASP_2 (with biotin and thiamine added), no carbon sources.

(2) Two months growth in ASP_2 (without vitamins) enriched with 10 mg% each of glycine, asparagine, arginine and glutamic acid.

It is of ecological interest to know whether even photoautotrophs have heterotrophic abilities. The dissolved organic matter

present in seawater (0.2-20 mg C/L), if utilized by the algae, might have ecological meaning.

A few amino acids were tried as N sources. Arginine and asparagine are utilized by the three *Gyrodinium* and the two *Amphidinium;* glycine by the two *Amphidinium*, *G. californicum*, and *G. splendens;* glutamic acid and alanine by the two *Amphidinium*, and *G. splendens;* the *Amphidinium* also utilize methionine. Growth in amino acids is not better than in nitrate. Asparagine, alanine, glycine, and glutamate, tried as C sources in a medium containing nitrate, were not used by *E. cassubica*, *P. balticum*, and *P. chattoni*, either alone or in combination (1-10 mg%). The three *Gyrodinium* behave similarly when each amino acid was added singly (10-50 mg%); when the four amino acids were added as a mixture (10 mg% each) growth was increased 2 to 3X. A similar increase in growth above the controls was obtained by adding soil extract. Complete amino acid mixtures, peptones, yeast and other hydrolysates did not aid growth and often inhibited at low levels (30-100 mg%) — a presumable indication of limited heterotrophic abilities. The *Gyrodinium* and *Amphidinium* species share with most algae, utilization of glycerophosphate, adenylic, guanylic and cytidylic acids as P sources.

DISCUSSION

Even the scant nutritional data available offer some ecological guide posts. These dinoflagellates, aside from their minute heterotrophic need for vitamins, are, as noted, good photoautotrophs. The three *Gyrodinium* reach a total growth of 30-60,000 cells/ml in four to five weeks in mineral media enriched with vitamins (ASP_2); in the same medium the *Amphidinium* grow to 1-1.5 million cells/ml. That heterotrophic abilities are limited is shown by these observations:

a) Amino acids utilized as N sources do not elicit more growth than nitrates.

b) Addition of carbon sources increases growth of the *Gyrodinium* modestly (2 to 3 times).

c) Peptones or various hydrolysates do not enhance growth.

This limited heterotrophy and the ability of most algae to

utilize some organic P compounds shows that complete mineralization is not necessarily needed for their nutrition. Even incomplete mineralization permits a more rapid cycling of the N and P sources; this is particularly important for phosphorus which is often limiting, or close to limiting.

Because of the need for media permitting isolation and cultivation of the greatest variety possible of marine algae, emphasis has been to find metabolites and concentrations good for all. The successes so far may encourage the conclusion that algae have much the same requirements. This is only partly true. The ability of all to utilize some organic N and P compounds and to require only three vitamins should be viewed only as a widening of the number of metabolites (i.e., ecological factors) for which the algae compete.

Species do bloom and succeed one another — an obvious sign of diversity in requirements and tolerances for various physical and chemical factors. Biotic and antibiotic interactions as well as the nutritional factors may here be important. Competition experiments in common media or differential enrichments of natural waters containing their living flora are needed. Thomas (18) obtained in the same sample of water early growth of diatoms followed by late growth of a *Gymnodinium* when he added a complete enrichment (N, P, Si, trace elements, soil extract); the diatoms failed to grow when Si or the trace metals were omitted, but *Gymnodinium* grew. Johnston (7) enriched many samples of seawater with N, P, Si, and chelated trace metals; this enrichment favored the growth of spring diatoms in all samples while the dinoflagellates, even when abundant in the natural inoculum, failed to grow. Is this to be interpreted only as an antibiotic effect? Silica favors the growth of diatoms; do the diatoms then excrete substances inhibiting the dinoflagellates? Although the most evident factor may be an antibiosis, the diatoms may be favored, besides silica, by a differential tolerance and need for the other constituents of the enrichment. Needed now are detailed comparisons of the requirements and tolerances of the organisms.

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Chapter 11

Nutritional Characteristics of Some Chrysomonads

I. J. Pintner and L. Provasoli

INTRODUCTION

Diatoms and dinoflagellates, because of their conspicuous blooms, were considered the most important components of the phytoplankton. However, the chrysomonads, often neglected because they are too small to be caught by fine-mesh nets or too fragile to withstand the usual fixatives, appear to be as important. The μ-flagellates seem to constitute a good part of the primary producers in the sea around England (8). Rodhe (11) employing fractionated C_{14} filtration, demonstrated that in Swedish Lakes, the net-plankton algae (retained by the finest net) represent in most cases, only 10 to 20 per cent of the total primary production; the nannoplankton (less than 70μ) constituting the bulk. A similar situation apparently exists in the warm seas (Mediterranean, Atlantic, Indian Oceans) where flagellates, coccolithophorids and blue-green algae seem the predominant crop (3). Bernard and Lecal (3) often found phytoplankton maxima well below the photic zone; *Coccolithus fragilis* in the Mediterranean grows in the non-photic zone as well as in the photic zone — an implication that this photosynthetic organism has a functional heterotrophy. The freshwater chrysomonads *Ochromonas malhamensis* and *O. danica* (1, 6) are photoautotrophs with a very well developed heterotrophic ability, but little if anything is known of the nutritional versatility of the marine species; *Prymnesium parvum*, which is a brackish organism (9) is endowed with some heterotrophy. As a preliminary to studying nannoplankton chrysomo-

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nads, we have established bacteria-free cultures, by treatment with antibiotics and micropipette washings of the following chrysomonads kindly given to us by Dr. Mary Parke of the Plymouth Laboratory, who had isolated them in unialgal culture: *Coccolithus huxleyi* 92a, *Pavlova gyrans* 93, *Ochrosphaera neapolitana* 162, *Syracosphaera* sp. 181, and *Hymenomonas* sp. 156. All grow autotrophically in chemically defined media: *Pavlova, Syracosphaera* and *Hymenomonas* prefer DV (10); and *Ochrosphaera* and *Coccolithus* grow best in $ASP_{12}NTA$, a medium originally designed for high-salinity algae by Provasoli (7).

NUTRITIONAL REQUIREMENTS

Salinity Tolerance

Different salinities were achieved by keeping constant the enrichment (N, P, trace metals, vitamins, and pH buffer) and varying (in the same proportion as in the above media) the concentrations of Na, Ca, K, and Mg. *Coccolithus huxleyi* and *Ochrosphaera* are stenohalines of oceanic type; on the contrary *Syracosphaera, Hymenomonas* and especially *Pavlova* are wide-range euryhalines (Table I). While *Pavlova* and *Syracosphaera* do not show a well defined salinity optimum, *Hymenomonas* definitely prefers the 12 to 32 per mil range. The *in vitro* data for *C. huxleyi* correspond rather closely with the field observations of Braarud (4, 5): *C. huxleyi* was found in the sea and polluted waters of the Oslofjord at salinities of 18‰ or above. *Pavlova,* the most euryhaline, lives in the brackish dilute Baltic Sea.

Vitamin Requirements

The 5 species need vitamins. With the accumulation of data a chrysomonad pattern for vitamins is emerging. The thiamine requirement is predominant over B_{12}; biotin, an uncommon requirement for other algal groups, is needed by some freshwater chrysomonads. As for the marine species, *Stichochrysis immobilis* does not need vitamins, *Hymenomonas carterae* requires only B_{12}; *Pleurochrysis scherffelii, Coccolithus huxleyi, Ochrosphaera neapolitana, Syracosphaera* 181, and *Hymenomonas* 156 require only thiamine; *Hymenomonas elongata, Isochrysis galbana,*

TABLE 1

SALINITY TOLERANCE (OPTICAL DENSITY AFTER 1 MONTH GROWTH)

Salinity ‰	*Pavlova gyrans*	*Syracosphaera* 181	*Hymenomonas* 156	*Coccolithus huxleyi* 92a	*Ochrosphaera neapolitana* 162
52		0.46	0.31	0.01	0.19
42		0.24	0.72		
32	0.46	0.35	1.8	0.21	0.23
21	0.46	0.28	2.0	0.23	0.20
19				0.19 (0.21)*	0.02
17			1.9	0.14 (0.10)*	
16				0.0 (0.03)*	
12	0.45	0.30	1.84		
10			1.07		
8	0.76	0.27	0.38		
6.4		0.18	0.15		
4.2	0.62	0.09	0.03		
3.2	0.12				
2.7	0.07				

* Values for *C. huxleyi* isolated by R. Guillard from Sargasso Sea.

Microglena arenicola, Monochrysis lutheri, Prymnesium parvum, and *Pavlova gyrans* require B_{12} and thiamine.

Trace Metals

The requirement for trace metals seems low for all. At least in the first transfer in media containing no trace metals, growth is as good as with addition of various trace metal-chelate mixtures for *Coccolithus* and *Ochrosphaera*. For the other species the addition of metal-chelated mixtures is slightly stimulatory; however, a similar effect is obtained for *Hymenomonas* by simple addition of a chelator (no added trace metals). These results do not prove that Cu, Co, Zn, Mn, Fe are not needed, rather that the need is low and is satisfied by the impurities brought in by the "chemically" pure salts of the medium. In the absence of metal chelators (as in "no trace metals") probably a part of the heavy metals, perhaps significant, is in the form of a fine precipitate. If so, unexacting *Coccolithus* and *Ochrosphaera* may be able to utilize precipitates; and the effect of the addition of a

chelator alone or of chelated trace-metal mixtures (which are over-chelated) for *Hymenomonas* may denote a preference for the solubized form. Obviously, reliable data on trace metal requirements and concentrations can only be had by virtual elimination of them (2).

HETEROTROPHIC ABILITIES

Phosphorus and Nitrogen

As for most of the algae tested so far, the aforementioned chrysomonads utilize besides inorganic phosphate, glycerophosphoric, adenylic, guanylic, and cytidylic acids.

Single amino acids and amino acid mixtures and single amines were tested in concentrations ranging from 1-10 mg %; for the utilized N- sources additional concentrations (up to 50-70 mg %) were also tried. Amines were tested because *Gyrodinium cohnii* utilized betaine and because many marine invertebrates and bacteria produce amines and it is not unlikely that amines would be present in seawater. *Hymenomonas* and *Syracosphaera* utilized the widest range of organic nitrogen. *Hymenomonas* utilized best glutamate, aspartate, alanine, arginine, and lysine; leucine, methionine, tyrosine, ornithine, citrulline, canavanine, and γ-aminobutyric acid are also utilized but less well. *Syracosphaera* utilized best aspartate, tryptophan, arginine, and lysine; alanine, glutamate, canavanine, ornithine, citrulline and γ-aminobutyric acid are less utilized. *Ochrosphaera* utilized only arginine, taurine, ornithine, citrulline, and agmatine. *C. huxleyi* utilized a mixture of histidine, arginine and lysine; each one of the components is less utilized than a combination of the three amino acids. *Pavlova* does not utilize any of the organic sources tried. Urea was tried but at very low levels (0.01-1 mg %); only *Syracosphaera* seemed to utilize urea as well as nitrate; *Hymenomonas* and *Pavlova* showed some utilization at the highest concentration tried (1 mg %).

Nitrate was the best inorganic N- source; maximum growth was obtained in the 10-50 mg % range. Only *Hymenomonas* utilized better the amino N of glutamate, aspartate and alanine than the N of nitrate (based on ratio optical densities: weight of N). The utilization of the organic N sources for the other

organisms was lower than nitrate, either as N or weight of compounds.

The five species differed in tolerance toward $(NH_4)_2SO_4$: *C. huxleyi* did not utilize it in the range 0.01-1 mg %, the latter concentration being toxic. The other chrysomonads utilize ammonia (the best concentration is 10 mg %) and only *Pavlova* and *Hymenomonas* can withstand 20 mg %. It is interesting that these species are resistant to ammonia in the alkaline range since *Prymnesium parvum* is so sensitive to it that ammonia salts are employed to prevent poisonous outbreaks of *Prymnesium* in the dilute brackish fish ponds in Israel. This toxicity declines sharply with salinity (20 mg % $(NH_4)_2SO_4$ is inhibitory at 16‰ and toxic at 8‰) and ammonia is a good N source for *Prymnesium* in the neutral or acid range (9).

Carbon Sources

The following carbon sources were tried at 10-50 mg % (the stimulatory ones were tried at higher concentrations): acetic, fumaric, aspartic, succinic, lactic, glutamic, pyruvic (aseptic addition), and malic acids; alanine, asparagine, glycine,

TABLE 2

Growth Response to Carbon Sources in Light (Optical Density; 1 Month Growth)

	Hymenomonas sp. 156	*Pavlova gyrans* 93	*Syracosphaera* sp. 181
No addition	0.30	0.51	0.11
Na lactate 50 mg %		0.46	0.47
Na lactate 100 mg %	1.00-1.32	0.36	0.95
Na lactate 200 mg %	1.90-2.40		
Na lactate 400 mg %	1.70		
Na pyruvate 50 mg %	0.98		0.15
Na pyruvate 100 mg %	1.56		
Na pyruvate 200 mg %	1.64		
Na acetate·$3H_2O$ 50 mg %	0.32	1.56	0.39
Glycerol 50 mg %	0.22	0.90	0.25
Glycerol 100 mg %		1.16	0.54
Glucose 50 mg %	0.42	0.40	0.27
Glycine 50-200 mg %	0.6		0.16

sucrose, glucose, glycerol, propylene glycol, monacetin, diacetin, and N-acetyl glutamate. A large growth response was elicited by the utilized carbon sources for *Hymenomonas* and *Pavlova*, much less by *Syracosphaera* and *Ochrosphaera* (Table 2). Growth of *C. huxleyi* was stimulated, but only slightly, by acetate, glutamate, pyruvate, acetyl glutamate, sucrose and propylene glycol. Lactate and pyruvate were good C sources for *Hymenomonas*; acetate and to a less extent glycerol for *Pavlova;* lactate and to a lesser degree glycerol for *Syracosphaera;* propylene glycol and to a lesser extent glycine for *Ochrosphaera*. Leucine is stimulatory for *Hymenomonas*: malic, sucrose and acetyl-glutamate for *Ochrosphaera*. Lactate in high concentration elicits a six to eight fold growth increase of *Hymenomonas* in light but it does not substitute for photosynthesis: no growth was obtained in darkness. No attempts at growth in darkness have yet been done with the other heterotrophic species.

DISCUSSION AND CONCLUSIONS

In exploring the heterotrophic abilities of a few chrysomonads and to elicit significant differences, we used concentration levels far above any likely to be found even in coastal polluted waters. The data therefore serve only to determine the versatility of the organisms; one might then make better guesses as to their behavior in nature. For instance, it would be logical to postulate that most of the oceanic forms like *Coccolithus huxleyi* would show stenohalinity and that littoral organisms would in general be more euryhaline. From this postulate, and from the apparent higher content in organics of littoral waters, one might be tempted to see whether the euryhaline forms (presumably littoral) are more heterotrophic than the stenohalines, preferring a relatively higher salinity. Indeed the euryhaline *Hymenomonas* and *Syracosphaera* can utilize the widest range of organic N and C sources and the stenohaline *Coccolithus* has very poor heterotrophic abilities. But the euryhaline *Pavlova*, though responsive to some organic sources of *Ochrosphaera* utilizes a few organic nitrogen sources and is stimucarbon, cannot utilize any organic N sources, and the stenohaline lated by several C sources. With such a limited sampling one

must not succumb to the temptation to describe *Pavlova* and *Ochrosphaera* as physiologically transitional forms.

Heterotrophic abilities need not be considered the sole cause of the possible colonization or preference of some organisms for organic-rich waters. Many organic substances are good trace-metal solubilizers. Their beneficial action could be in making available iron and manganese which are present mostly as particulates in seawater. Thus a non-heterotrophic photolithotrophic organism incapable of utilizing particulate trace metals may benefit. The colonization of the polluted waters of the Norwegian fjords by *Coccolithus huxleyi*, which is a photolithotroph practically devoid of heterotrophic abilities and with an apparent low requirement for trace metals, cannot be ascribed to either effect of organic solutes. Perhaps it grows there and in the open sea because it resists pollution and can compete favorably in both environments with other species, either because the other species may be more sensitive to pollution or because other variables, not considered in the present work like temperature, light intensity, etc., may give *Coccolithus* the edge.

While heterotrophy, vitamins, and trace-metal requirements can be a primary cause of predominance of some organisms over others, they should not be considered all-encompassing. Other intrinsic physiological abilities, including division rates of the species and their versatility or response to other variables, and their ability to produce or withstand the possible actions of antibiotics of other species—all have to be explored to explain predominance and succession of species. We should examine all possible factors; or, to say it fashionably, let us view ecology synoptically and holocoenotically, with due evaluation of relevant interacting parameters.

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Chapter 12

Axenic Cultivation of a Pelagic Diatom

Giorgio Soli

Attempts to cultivate pelagic diatoms axenically in a defined medium were started because bacteria-free organisms appeared necessary for carrying out studies of the effect of marine phytoplankton on the propagation of sound waves in the sea, with particular attention to absorption and scattering phenomena.

Many difficulties beset the cultivation of pelagic diatoms, in a defined medium free of bacteria. The reasons for these difficulties seem to be rather complex.

Bacteria may exercise a beneficial action on the diatoms, by slowly buffering the medium, by influencing heavy metal solubility, or by lowering the oxidation-reduction potential of the medium during the early phases of diatom growth (1). However powerful buffers like TRIS (2-amino-2-(hydroxymethyl)-1, 3- propanediol), chelating agents and substances which lower the oxidation-reduction potential, do not appear to substitute for bacterial action. It is possible that the bacteria affect diatom growth by furnishing active metabolites and by changing the physical-chemical environment in early stages.

Antibiotics are usually employed for obtaining axenic cultures of diatoms and dinoflagellates. These microorganisms appear to be very sensitive to doses of antibiotics which are effective in inhibiting completely bacterial growth. Therefore, the selection of the right antibacterial agent, as well as the exposure time, should be points of particular attention. The culture medium should also contain all those vitamins and growth factors which the bacteria are presumably furnishing.

This line of thought was followed in the attempt to obtain sterile cultures of pelagic diatoms. During the course of this work, the possibility of a syntrophic relationship between the bacteria

and the diatoms was considered.

The following complex defined medium supported good growth in bacterized cultures, of *Chaetoceros dydimus, Asterionella japonica* and a species of *Coscinodiscus* (Table 1).

TABLE 1

MEDIUM FOR GROWTH OF DIATOMS

NaCl	20 gr	Adenine	100 mcg
$MgCl_2$	4.5 gr	Uracil	100 mcg
Na_2SO_4	3.5 gr	Xanthine	100 mcg
$CaCl_2$	1.0 gr		
KCl	600 mg	Thiamine	30 mcg
$NaHCO_3$	40 mg	Choline	10 mcg
KBr	60 mg	Nicotinic acid	5 mcg
Na_2SiO_3	20 mg	P-amino benzoic ac.	2 mcg
		Pyridoxine	1 mcg
KNO_3	200 mg	Riboflavin	1 mcg
K_2HPO_4	35 mg	Biotin	1 mcg
K_2glycerophosphate	1 mg	Folic acid	0.1 mcg
		B_{12}	0.1 mcg
		Ca pantothenate	300 mcg
H_3BO_3	6 mg	Inositol	500 mcg
$FeCl_3$	2 mg	L-ascorbic acid	200 mcg
NaF	1 mg	Citric acid	20 mcg
$LiNO_3$	200 mcg		
$MnCl_2$	100 mcg	Aspartic acid	25 mcg
$ZnSO_4$	50 mcg	Beta-alanine	15 mcg
KI	50 mcg	Glutamic acid	15 mcg
Na_2MoO_4	10 mcg	Glycine	15 mcg
$CoCl_2$	10 mcg	l-leucine	10 mcg
$CuSO_4$	5 mcg	Histidine	10 mcg
		Serine	10 mcg
EDTA	10 mg	Tryptophane	5 mcg
TRIS	500 mg	Cystine	5 mcg
Re-distill. H_2O	1000 ml	Methionine	3 mcg

While realizing that a great number of the organic substances included in the medium may not be required, it was thought that a complex medium offered better chances of success, and could be later dissected. The concentrations of the various growth factors proved adequate, and non-toxic, if not required.

This medium, without TRIS, was sterilized at first by filtration through an UF glass filter, then, after addition of TRIS at

a pH of 7.8, was autoclaved. The loss of some of the heat-labile substances apparently did not disturb diatom growth.

Soon it became clear that the bacteria, while presumably exercising a beneficial action, were also responsible for cessation of growth and death of the diatoms. When bacterized cultures of *Chaetoceros dydimus* were transferred in fresh medium, the diatom developed abundantly, but when growth was completed, the bacteria multiplied rapidly, and destroyed the diatom cells.

Several bacteria were isolated from cultures of *Chaetoceros dydimus, Asterionella japonica* and *Coscinodiscus.* They were all Gram-negative rods, strongly saccharolytic, tentatively indentified as belonging to the family *Pseudomonadaceae* and *Achromobacteraceae.* Since these bacteria could grow in the synthetic medium to which 0.1 per cent glucose was added, it was concluded that the bacteria were thriving, very likely, at the expense of the sugars produced by the diatoms during photosynthesis. The diatom medium without added glucose and without diatoms did not support bacterial growth, when inoculated with the bacteria.

Antibiotics were used at the beginning in the attempt to eliminate the bacteria. To select the most effective antibiotics, the bacteria, plated on sea water peptone agar, were tested with Difco antibiotic sensitivity disks (Table 2).

Polymyxin B and Neomycin were selected as being the most effective and the least toxic against the diatoms. Medium with fifty μgm/ml of both antibiotics was effective in keeping the bacterial count down. However, at this concentration, resistant mutants developed, which made impossible their elimination without damage to the diatoms.

By using an alcoholic solution of iodine (2-3 drops of 0.1 per cent solution in 10 ml of diatom suspension for 1-2 minutes) and by subsequent centrifuging and washing in sterile medium, it was finally possible to eliminate the bacteria and grow the diatom *Chaetoceros dydimus* in pure culture.

The exposure time seemed to be very critical: an exposure to the iodine solution of five minutes kills the diatoms by irreversibly bleaching the cells.

Sterility of diatom cultures was tested by inoculating sea

TABLE 2

EFFECT OF ANTIBIOTICS ON BACTERIA ISOLATED FROM DIATOMS

	Isolate No. 1 Chaetoceros	*Isolate No. 2 Chaetoceros*	*Isolate No. 1 Asterionella*	*Isolate No. 2 Asterionella*	*Isolate No. 1 Coscinodiscus*
Polymyxin B	+ RM	+	+ RM	+ RM	+
Neomycin	+ RM	—	+ RM	+	—
Viomycin	—	—	—	—	—
Streptomycin	+ RM	—	+ RM	+ RM	—
Penicillin	—	+	—	—	+ RM
Terramycin	—	—	—	—	—
Aureomycin	+ RM	—	—	—	—
Tetracycline	—	—	—	—	—

(Difco antibiotic sensitivity disks of medium strength)
+ inhibition — no inhibition RM=insurgence of resistant mutants

water peptone agar and the diatom medium (added with 0.1 per cent glucose) with samples of diatom cultures.

Chaetoceros dydimus in bacteria-free cultures seems to have better pigmentation, longer chain length and a shorter generation time.

A minimum generation time of 11 hours was recorded, the culture reaching a concentration above 3×10^5 cells/ml in ten to eleven days, when incubated at 19 C, under twelve hours of illumination a day, at 200 foot-candles (Figure 1). Average size of single cells is 14 x 20 microns.

Growth in bacteria-free cultures continues up to thirteen to fourteen days, under these experimental conditions, at which time auxospores start to appear. This compared with a growth period of approximately eight days for bacterized cultures, at which time growth usually declines, as shown in Figure 1.

The medium is now being dissected and more detailed studies of the association between diatoms and bacteria are planned, since very little is known of the interaction between these microorganisms in the sea.

Diatom bloom, termination of growth and succession of forms in the natural environment, may be phenomena in which bacteria play an important role.

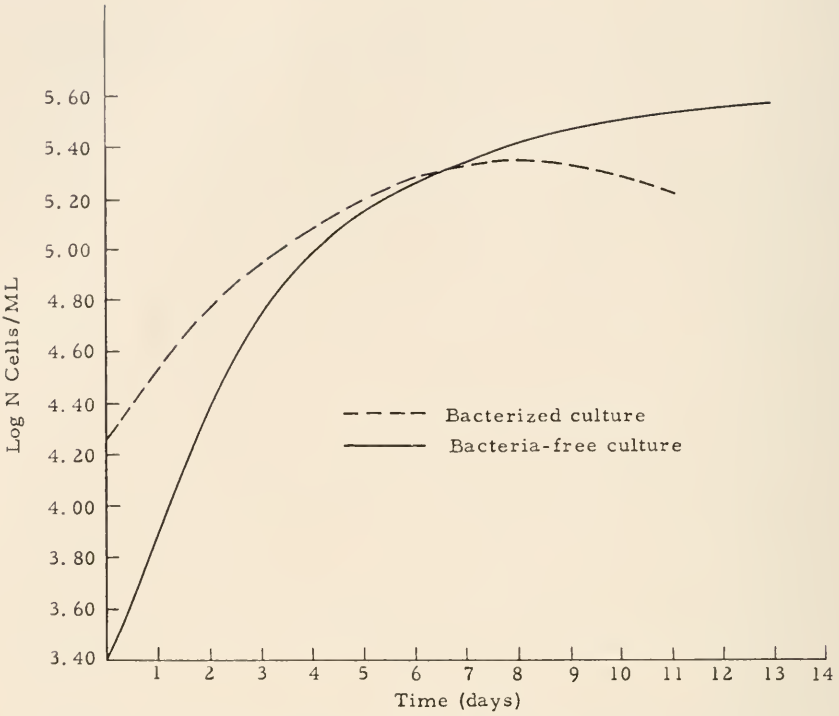

Fig. 1. Growth of *Chaetoceros dydimus* in Synthetic Medium.

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Chapter 13

Fission Rate of Plankton Algae of the Black Sea in Cultures

L. A. Lanskaya

The present material is the result of the observations of many years, done, at first, under the leadership of Prof. Morozova-Vodjanitskaja. It describes the division rate in culture, of species of plankton algae blooming in the Black Sea. These data can serve as initial material for judging the rate of renewal and dying off of phytoplankton in the sea, the potential productivity of each of the examined species, and also grazing by consumers.

The algae were grown on filtered sea water enriched with the components of the "Erdschreiber" and a modified Allen-Nelson solution. The cultures were grown in a box under natural light (about 1000 lux in winter to 3800 lux in summer). The temperature of water in the experimental vessels with algae was usually higher than in the sea: 1-2 C in summer and autumn, and 5-6 C in winter and spring-time.

Unialgal cultures were obtained by picking cells or chains directly from a plankton-net sample. Mixed cultures were obtained by inoculation of a certain volume of water taken with a bathometer.

In most cases the division rate of planktonic algae was studied in cultures reared from several cells. The experiments lasted usually five to seven days, as the overgrowth of the culture markedly influences the division rate of cells.

The division rate of the algae at different periods of a year was determined two to three times a month. The diurnal rhythm of the division rate of cells was also determined. Every observation was carried out with a fresh culture, even though many seasonally occurring algae grew well in cultures for five to six months, and year-long forms can be grown for several years.

The experimental investigations were accompanied by continuous observations on the quantitative changes of plankton algae in the sea by direct count of cells.

The two main groups of plantonic algae of the Black Sea, the diatoms and the dinoflagellates (in general more than 50 species) were studied from 1952 to 1960, with special attention to the species: *Chaetoceros socials, Chaetoceros curvisetus,*

TABLE 1

MAXIMUM DIVISION RATE OF PLANKTON ALGAE OF THE BLACK SEA IN CULTURES

Species	*Month of Maximum Development*	*Time Between Division of Cells (in Hours)*	*Mean Monthly Temperature (C°)*
Diatomeae			
Skeletonema costatum (Grev) Cl.	IV	3	17
Chaetoceros socialis f. *vernalis* Pr.-Lavr.	V	8	19
Chaetoceros socialis f. *autumnalis* Pr.-Lavr.	IX	10	21
Chaetoceros curvisetus Cl.	IV, V	14	17–19
Leptocylindrus danicus Cl.	VIII	11	25
Cerataulina bergonii Perag.	IX	12	21
Thalassionema nitzschioides Grun.	V, IX	19	19–21
Coscinodiscus granii Gough	VI	18	24
Coscinodiscus janischii A.S.	X	30	18
Rhizosolenia alata Bright	V	20	19
Rhizosolenia calcar avis Schultze	VIII, IX	28	21–26
Ditylum brightwellii (West) Grun	III	24	13
Dinoflagellatae			
Exuviaella cordata Ostenf	VIII	15	26
Prorocentrum micans Ehr.	VII, VIII	12	26
Peridinium steinii Jorg.	VII	19	26
Peridinium triquetrum (Ehr.) Lebour	IX	10	17
Peridinium trochoideum (Stein) Lemn.	XI	16	24
Gymnodinium wulfii Schiller	VI, VII	14	23–26
Glenodinium lenticula (Bergh) Schiller	VII	24	26
Ceratium furca (Ehr.) Dujardin	VII	48	26
Volvocineae			
Chlamdyomonas minima Schiller	VII	12	26
Carteria willei Schiller	VI	6	24

Skeletonema costatum, Leptocylindrus danicus, Cerataulina Bergonii, Thalassionema nitzschioides, Prorocentrum micans, Exuviaella cordata, Peridinium trochoideum, Peridinium triquetum, Gymnodinium wulfii, and others. Monocultures of the pigmented small flagellates, *Chlamydomonas minima,* and *Carteria willei* were the first grown from the Black Sea.

The division rates differ for the various species of plankton algae (Table I). Among diatoms during mass blooms *Skeletonema costatum* has the highest division rate (3 hours) and *Coscinodiscus janischii* the lowest (30 hours). Among dinoflagellates, *Peridinium triquetrum* was notable for the greatest division rate (10 hours), and *Ceratium furca* for the lowest (48 hours). *Carteria willei* had the greatest division rate (6 hours) among the small flagellates.

The division rate in cultures of the same species of algae varies markedly during the year. For *Skeletonema costatum* it varied from three hours (maximum) in April-May to 56 hours (minimum) in November; for *Chaetoceros socialis* from 8 to 84 hours; (maximum in May, minimum in February); for *Leptocylindrus danicus* from 11 to 65 hours; (maximum in August-September, minimum in November-December); for *Prorocentrum micans* from 12 to 127 hours (maximum in July-August, minimum in January); for *Peridinium triquetrum* from 12 to 50 (maximum in April-May, minimum in July); for *Exuviaella cordata*—from 15 to 90 (maximum in August, minimum in December); for *Chlamydomonas minima* from 12 to 120 hours (maximum in July, minimum in December).

The division rate of the predominant species slows down markedly as their blooms decrease. Therefore the average monthly division rate has lower values than the minimum ones (Table 2).

As a rule, the neritic seasonal forms, giving in the sea a short burst of bloom, have the highest division rate. Such species, in spite of the high content of nutrients in experimental cultures often die off, form auxospores or resting stages after reaching maximum division rate.

The highest division rate of cells in cultures is usually observed in the period of the observed numerical maximum of

TABLE 2

AVERAGE DIVISION RATE OF PLANKTON ALGAE OF THE BLACK SEA IN CULTURES

Species *Months*	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Diatomeae												
Skeletonema costatum (Grev) Cl.	28	22	17	12	10	14	—	—	24	24	30	32
Chaetoceros socialis Laud.	—	53	32	17	13	14	22	16	18	21	24	32
Chaetoceros curvisetus Cl.	41	28	23	20	22	25	—	—	32	29	37	66
Leptocylindrus danicus Cl.	39	—	—	—	—	20	23	22	19	22	35	39
Cerataulina bergonii Perag.	89	38	30	26	27	29	34	27	23	27	31	37
Thalassionema nitzschioides Grun.	65	46	35	28	23	—	—	35	25	28	40	44
Coscinodiscus granii Goulgh.	94	87	74	48	—	34	32	26	34	44	50	71
Rhizosolenia alata Bright	65	—	99	45	47	42	—	72	83	111	115	89
Rhizosolenia calcar avis Schultze.	42	60	70	79	—	—	38	33	34	38	46	47
Ditylum brightwellii (West) Grun.	37	35	31	27	25	38	—	—	—	42	44	48
Dinoflagellatae												
Exuviaella cordata Ostenf.	58	49	42	40	32	24	22	22	24	30	35	46
Prorocentrum micans Ehr.	80	66	59	42	31	24	18	25	29	37	40	60
Peridinium triquetrum (Ehr) Lebour.	—	—	28	16	17	31	40	—	—	—	—	—
Peridinium steinii Jorg.	—	—	—	48	33	24	26	30	35	54	—	—
Peridinium trochoideum (Stein) Lemm	62	48	39	38	27	24	30	36	40	46	55	60
Gymnodinium wulfii Schiller	48	48	40	38	30	22	24	30	35	40	42	54
Glenodinium lenticula (Berdh) Schiller	—	—	—	48	35	26	28	29	35	48	--	—
Volvocineae												
Chlamydomonas minima Schiller	48	42	—	—	—	24	16	18	26	30	32	44
Carteria willei Schiller	—	—	—	—	18	12	16	21	24	26	30	48

the same species in the sea. The period of a high division rate for many species in cultures lasts always somewhat longer than the period of mass development in the sea.

However, in some years their maxima do not always coincide in time. This is apparently due to some biotic relationships between the different species of planktonic algae. The observations

carried out with mixed cultures showed that some algae (*Skeletonema costatum, Chaetoceros socialis* forma *vernalis*) in the period of their intensive multiplication inhibit the development of *Cerataulina bergonii, Thalassionema nitzchioides, Chaetoceros curvisetus, Rhizosolenia alata* and others, while in monocultures the latter divide with a comparatively high rate. A quick disappearance of some algae in the sea is not always connected with the decrease of their potential of multiplication because in cultures they do not show any decrease in fission rate during the same period. It is then probable that this is due to some limiting factors. Decrease in growth may also depend on the abundance of consumers of algae in the sea besides the changes in content of nutrients and the biotic relationships. Zooplankton organisms energetically consuming plankton algae, in fact, decrease the initial value for the population of the latter. Maybe this is one of the main reasons of the lower quantitative values of some species of dinoflagellates and small flagellates in the open part of the Black Sea. In spite of the fact that in cultures the division rate of diatoms and dinoflagellates is nearly equal, the quantity of *Prorocentrum micans, Exuviaella cordata, Peridinium triquetrum, Gymnodinium wulfii* and others in the sea does not exceed 300 thousand cells per liter while diatoms often reach 20 million cells per liter. This may be an indirect confirmation of intensive grazing of dinoflagellates.

The experimental data show that the fission rate of diatoms and dinoflagellates is diurnal: it is usually higher during daylight than at darkness. The minimum fission rate was observed in summer from midnight till 4 o'clock in the morning and till 7-8 o'clock at winter time, i.e., before daybreak.

Since the algae were grown in unialgal cultures, but not free of bacteria, it was interesting to investigate the influence of bacteria on algae. The number of bacteria was determined by counts on a peptone-meat infusion-agar. Actively-dividing algal cultures depressed bacterial development; the number of bacteria sharply decreased. In weak cultures with slow algal division, especially after the cells begin to die off, the number of bacteria greatly increased.

Not all the species of planktonic algae equally depressed

bacterial growth. Bacterial growth was inhibited in cultures of the diatoms *Nitzchia closterium, Thalassionema nitzchioides, Cerataulina bergonii,* and of the dinoflagellates *Prorocentrum micans, Gymnodinium wulfii.* In the cultures of the diatoms *Leptocylindrus danicus, Skeletonema costatum, Chaetoceros socialis, Ch. curvisetus, Ch. lauderii* this inhibition was not so clear. In the cultures of *Coscinodiscus* sp. a parallel development of algae and bacteria was observed. Bacteria which grew on agar plates were representative of saprophytes widespread in the Black Sea: *Pseudobacterium subluteum, Chromobacterium rhenii, Bacillus idosus* and some others. There was no observed correlation between defined species of algae and bacteria.

CONCLUSION

The algae with a marked seasonal prevalence of development have a higher fission rate and a shorter period of intensive multiplication. Seasonal forms have periods of rest, while the year-long forms have a period of rest which is less distinct. However, the year-long forms of phytoplankton have maximal development at different periods of a year (spring, summer or autumn). The observed biological cycle is apparently the result of physiological changes in the cell itself. For the Black Sea the development of many forms of phytoplankton is throughout the year with distinct spring, summer and autumn maxima. From the average monthly fission rate of the plankton algae (Table 2), it is possible to conclude that besides the diatoms, the dinoflagellates and small flagellates play a great role in the productivity of the Black Sea.

Correlation between the number of cells of algae and that of bacteria may change during the year according to the systematic composition, and especially to the physiological condition of the phytoplankton.

Chapter 14

Some Nutritional Relationships Among Microbes of Sea Sediments and Waters*

Paul R. Burkholder*

In the fluid marine environment, important growth-regulating substances, such as water-soluble vitamins and various antibiotic substances, are synthesized, excreted, and used in diverse ways by the natural populations of algae and bacteria. The present discussion is concerned chiefly with certain coactions among small autotrophic and heterotrophic plants that live in the sediments and waters of shallow seas.

It is now recognized that auxotrophy, the requirement for exogenous supplies of special metabolites in very small amounts, predominates in algal groups with well-developed tendencies toward saprophytic and holozoic modes of nutrition, and is less frequently distributed in groups with strong vegetal tendencies (8). Diatom blooms of northern seas (4) and inhabitants of marine muds (5) commonly require such B vitamins as thiamine, biotin, and cobalamines. Recent studies in our laboratories (12) have shown that among marine littoral Cyanophyta more than 50 per cent of isolates growing in pure culture exhibit requirements for cobalamins. The auxotrophic habit appears to be widely distributed among the different phyla of autotrophic and heterotrophic microorganisms (10, 7, 6) that live on land as well as in the waters of the earth.

In order to appraise the significance of B vitamin auxotrophy in marine bacteria, we have isolated, in dilution pour plates of rich media, more than 6,000 cultures from waters and

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sediments obtained from tropical, temperate, and sub-arctic regions. The composition of media suitable for isolation, testing, and maintenance of many marine bacteria is given in Table 1. For the purpose of determining special requirements for vitamins, amino acids, purines, pyrimidines, etc., chemically defined media were prepared by adding suitable supplements to artificial sea water. In some instances vitamin-free casein hydrolysates supplemented with cystine and tryptophane were used to satisfy nitrogen needs, or when indicated, each of the desired amino acids was used in the proportion present in casein. Requirements were determined by testing growth of each organism in a series of media having single omissions of compounds from the complete medium. For certain studies of B vitamin relationships in gel substrates, addition of 1.5 per cent Difco purified agar to the liquid media proved satisfactory.

The general growth requirements of 1748 aerobic, heterotrophic bacteria are presented in Table 2, for the purpose of indicating the characteristics of populations commonly occurring in marine muds. Analysis of the vitamin requirements of these cultures has provided data on the statistical occurrence of vitamin deficiency in this whole group of auxotrophic bacteria. The data, summarized in Table 3, show that biotin and thiamine are most frequently required, cobalamin and nicotinic acid stand next, while pantothenate and riboflavin requirements occur infrequently. The occurrence of vitamin deficiency may be a single or multiple requirement in various cultures. Some need as many as four B vitamins. Upon further inspection, it is found that the specificity of response to different forms of the vitamin B_{12} group or the intermediates of thiamine, or different structures related to biotin or nicotinic acid, may vary to a considerable degree among the different isolates. Thus all B_{12} requiring isolates appear to respond to cyanocobalamin, but some are unable to utilize pseudovitamin B_{12}, or factors A and B. Numerous thiamine-deficient strains show ability to grow in response to either the pyrimidine or thiazole moieties; Some biotin requirers can use desthiobiotin, and so on.

Our data, along with results of many other investigators, indicate widespread requirement for exogenous sources of B vita-

TABLE 1

Composition of Various Media Used for the Isolation, Maintenance, and Testing of Aerobic, Heterotrophic Bacteria from the Sea. The pH is Adjusted to 7.2

Ingredients	*Artificial Sea Water*	*A Isolation and Maintenance*	*B*** Vitamin Tests*	*C Minus Amino Acids*
Distilled water	To 1 l			
NaCl	23.476 gm			
Na_2SO_4	3.917 gm			
$NaHCO_3$	0.192 gm			
KCl	0.664 gm			
KBr	0.096 gm			
H_3BO_3	0.026 gm			
$MgCl_2 \cdot 6H_2O$	10.610 gm			
$SrCl_2 \cdot 6H_2O$	0.040 gm			
$CaCl_2 \cdot 2H_2O$	1.469 gm			
Trypticase		2 gm		
Soytone		2 gm		
Yeast extract		2 gm		
Marine mud extract*		100 ml		
Natural sea water		900 ml		
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$			7 mg	7 mg
Difco agar**		15 gm		
Artificial sea water			To 1 l	To 1 l
Casamino acids (-vitamins)			5 gm	
Dextrose			2 gm	2 gm
Na succinate			2 gm	2 gm
Cystine			100 mg	
Tryptophane			100 mg	
K_2HPO_4			50 mg	50 mg
Adenine			5 mg	5 mg
Cystosine			5 mg	5 mg
Guanine			5 mg	5 mg
Thymine			5 mg	5 mg
Uracil			5 mg	5 mg
Xanthine			5 mg	5 mg
Biotin			1 μgm	1 μgm
Cobalamine		1 μgm	1 μgm	1 μgm
Nicotinic acid			500 μgm	500 μgm
Pantothenate			800 μgm	800 μgm
Riboflavin			500 μgm	500 μgm
Thiamine			800 μgm	800 μgm
NH_4NO_3				0.5 gm

* Mud extract is prepared by autoclaving 1 kilogram of wet marine mud together with 1 liter of sea water for 20 minutes and filtering. Preserve under toluene and store in a refrigerator.

** Difco purified agar, 15.0 gm per liter, was used in making media for vitamin tests in plates.

*** Vitamins are omitted from this medium in appropriate fashion, depending upon the experimental design.

TABLE 2

General Growth Requirements of 1748 Cultures Isolated From Sea Sediments and Waters

	Media Requirements				
Source of Cultures	*I Minerals and Carbon Source*	*II I Plus Vitamins*	*III I Plus Amino Acids*	*IV II Plus III*	*V Isolation Medium A*
Long Island Sound, near Milford, Conn.	85	24	66	77	98
Gulf of Mexico, near Mobile, Ala.	161	17	54	29	8
Region of Woods Hole, Mass.	176	32	84	138	179
Region of Gaspe, Quebec	85	9	138	120	188
Totals	507	82	342	364	473
Per Cent	29	5	20	21	25

TABLE 3

Number of Bacterial Cultures Requiring each Vitamin for Growth in a Chemically Defined Medium

Vitamin	*Number of Cultures*
Biotin	505
Thiamine	501
Cobalamine	153
Nicotinic acid	97
Pantothenate	15
Riboflavin	1

mins by both autotrophic and heterotrophic marine microorganisms. Groups of organisms showing saprophytic and holozoic tendencies are known to exhibit pronounced auxotrophic characteristics, and marine bacteria conform to this general rule. The blue-green algae living in marine muds also exhibit marked requirements for vitamin B_{12} compounds, as might have been expected in view of their close phylogenetic relationship with bacteria. Recognizing the common requirements among planktonic, benthic, and littoral marine microorganisms for vitamins of the B

group, it is important to inquire into the origins and availability of these substances in the marine environment.

PRODUCTION AND OCCURRENCE OF B VITAMINS IN MARINE ORGANISMS

Earlier studies have indicated the widespread occurrence of B vitamins in marine muds (2), algae, and ocean waters (8, 13). The concept that some of the B vitamins arise by biosynthesis in the marine environment has been supported by various investigations. As a basis for studies of vitamin exchanges among marine microorganisms, further determinations have been made for production of the four B vitamins required most frequently by auxotrophic marine algae and bacteria.

For our present studies of vitamin production, two methods were employed: crude enrichment cultures, and pure cultures of bacteria in a chemically defined medium. Following a suitable period of growth, the samples of broth cultures were prepared for assays in accordance with simple modifications of standard methods (1). Microbiological tests for the B vitamins were performed in poured plates seeded with suspensions of vitamin-requiring bacteria selected out of our screening program. The basal assay medium is indicated in Table 1. Supplements of appropriate vitamins were added to the medium as required, so that growth of the assay organisms would indicate the amount of a vitamin in standard solutions or in crude bacterial culture broths applied in one-half inch paper discs to the surface of the seeded agar test plates. Calculations of vitamin potency of unknowns could be made by reference to standard curves obtained in these plate assays.

Growth and vitamin production of marine bacteria in crude cultures of enriched marine muds were studied in flasks containing sea water, phosphate, and organic sources of carbon and nitrogen. The numbers of bacteria were determined at the beginning and after three days, by the dilution method, and vitamins were assayed with the standard agar plate technique. As the numbers of bacteria increased, so also did the amounts of the four important B vitamins.

Production of vitamins was determined in pure cultures of

1,054 isolates grown in medium B. It was found that physiologically significant amounts of biotin and thiamine were produced by about half of the cultures, while nicotinic acid and B_{12} were synthesized in appreciable amounts by somewhat fewer isolates. Vitamin production by auxotrophic cultures having certain specific vitamin requirements was also studied with the usual procedures. Results obtained in studies of nine different groups of cultures, each with its own pattern of vitamin requirements, indicated that some cultures produced no detectable amounts of the four vitamins, while others formed one or more of the B vitamins in about the same amounts as in cultures having no special requirements (Table 4).

TABLE 4

PRODUCTION OF B VITAMINS BY SELECTED GROUPS OF GROWTH-FACTOR-REQUIRING BACTERIA. AVERAGE PRODUCTION OF VITAMINS IS INDICATED FOR THE VARIOUS NUMBERS OF CULTURES IN EACH GROUP BY SHOWING THE RADIAL ZONES IN MM. OF GROWTH PROMOTION AT THE EDGE OF THE PAPER DISCS LAID ON SEEDED AGAR PLATES CONTAINING TEST BACTERIA. —=REQUIREMENT, 0=NO REQUIREMENT.

Number of Cultures	*Pattern of Requirements*				*Relative Productivity*			
	Biotin	*Thiamine*	*Nicotinic Acid*	B_{12}	*Biotin*	*Thiamine*	*Nicotinic Acid*	B_{12}
29	—	0	0	0	—	26=0		28=0
					—	3=2	29=9.5	1=6
7	0	—	0	0	1=0	—		7=0
					6=8	—	7=10.3	
2	0	0	—	0	1=0	2=0	—	2=0
					1=2		—	
3	0	0	0	—		2=0		—
					3=10.7	1=4	3=10.3	—
3	—	0	0	—	—	3=0		—
					—		3=10.7	—
5	0	—	0	—	2=0	—		—
					3=10.3	—	5=12.3	—
1	0	—	—	0		—	—	1=0
					1=13	—	—	
1	—	—	—	0	—	—	—	1=0
					—	—	—	
1	—	—	0	—	—	—		—
					—	—	1=12	—

Production of B vitamins by selected marine bacteria was studied at intervals over a period of twenty-four hours for the purpose of determining whether these substances are excreted during the period of growth. Isolates P9 and P85 from Puerto Rico, and B739 from Long Island Sound, were inoculated into a series of flasks, each containing 10 ml of medium B (see Table 1). Growth was allowed to take place at 30 C on a rotary shaker. At different time intervals, the optical density of the growing cultures was determined at wavelength 600mμ, and samples of the culture broths were taken for vitamin assays. An aliquot was centrifuged to remove the bacteria, and the supernatant was collected and heated to 100 C for three minutes. Another portion of the whole culture was also heated in the same way, to kill the cells and liberate bound vitamins. Assays on the two portions gave indications of the total and excreted B vitamins present at different times during the growth period. The experiments were repeated on different days, with similar results. Typical data obtained for cultures P85 and B739 are shown in Figures 1 and 2. Antibiotic production and synthesis of five B vitamins by P9 are presented in Figure 3.

It became apparent from the experimental data that appreciable amounts of B vitamins are synthesized by some cultures of marine bacteria, concurrent with the rise in the growth curve. The vitamins biotin, riboflavin, nicotinic acid, and pantothenate are produced and excreted into the medium during the period of rapid growth. Other vitamins, such as B_{12} and sometimes thiamine, are produced and retained within the cells, but can be released by denaturation of the cell constituents through heating at boiling temperature for a brief period (3-5 min.). The beers of culture P9 were inhibitory to the vitamin B_{12} assay organism, making this test impossible. The chief difference in the vitamin data obtained with culture P9 and the others, is that thiamine was excreted into the medium along with the other vitamins.

Production of B vitamins during growth of pure cultures of green algae, blue-green algae, and diatoms, isolated from marine muds, has also been studied. Simple demonstrations of vitamin excretion may be obtained by placing portions of an algal culture, growing in vitamin-free ASP-2 agar medium (9), on the surface

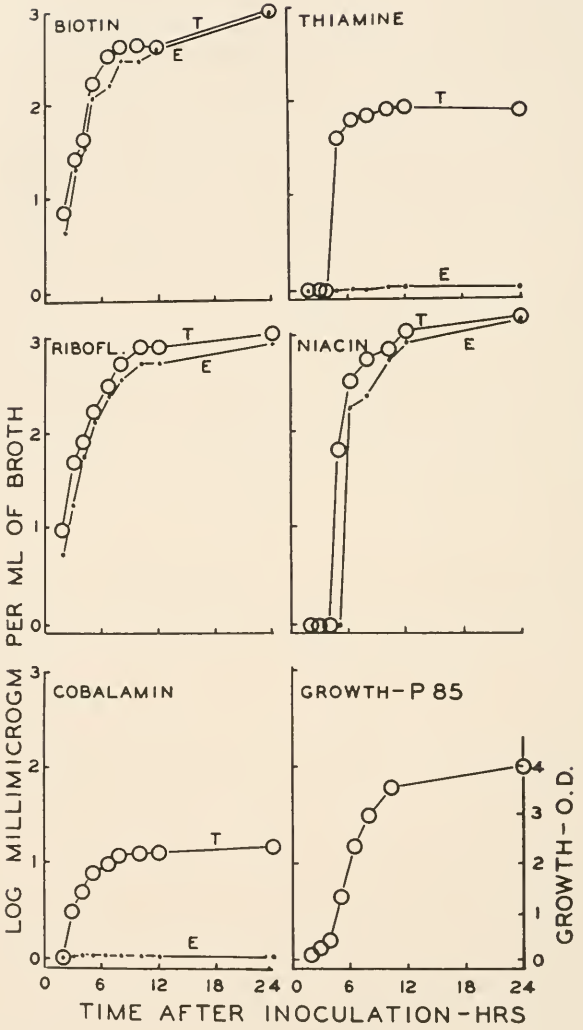

Fig. 1. Production and excretion of B vitamins by culture P85 obtained from the sea near La Parguera, Puerto Rico. Note that thiamine and cobalamins are not excreted during growth, but can be released from the cells by heating.

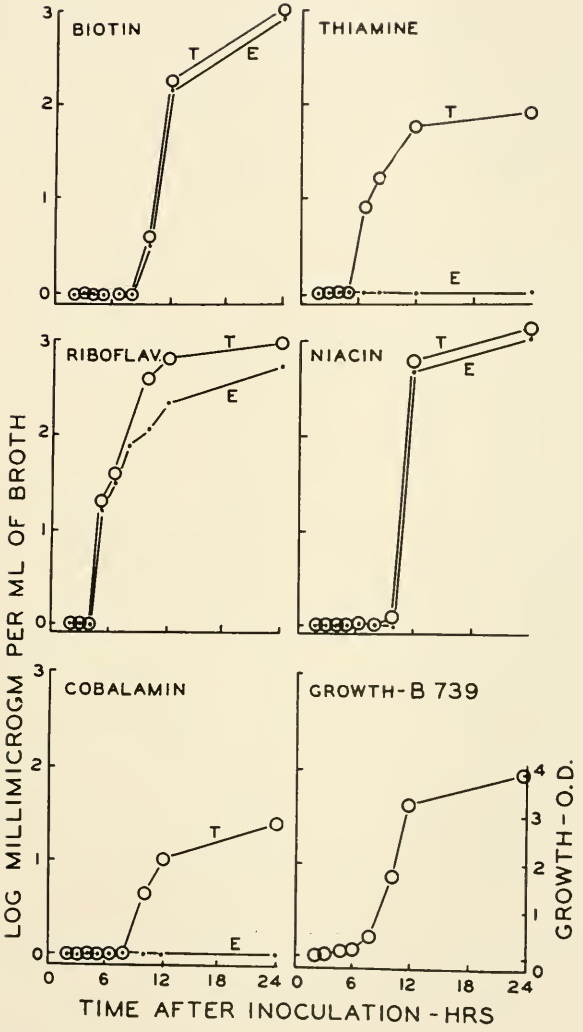

Fig. 2. Production and excretion of B vitamins by culture B739 obtained from Long Island Sound.

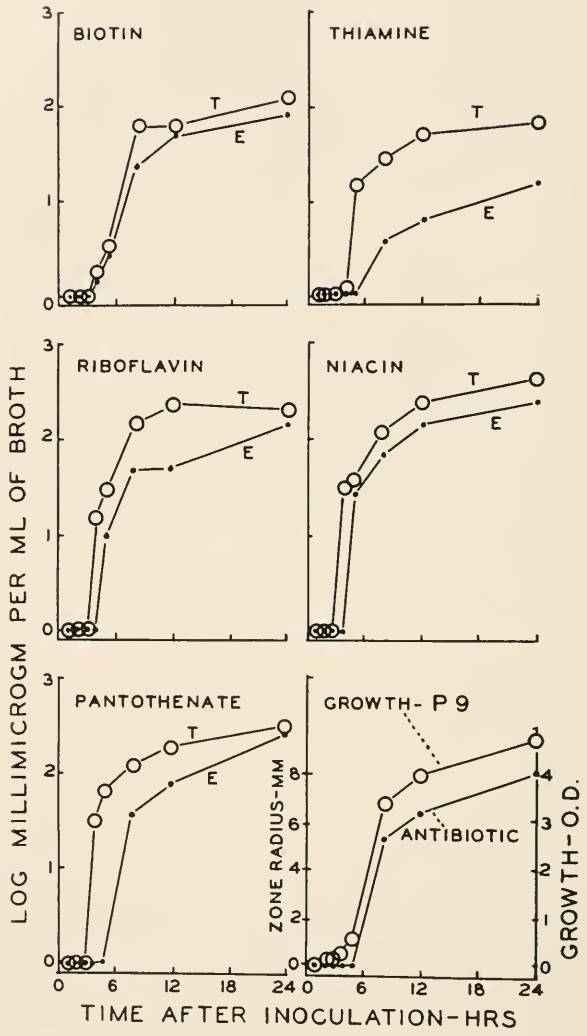

Fig. 3. Production of an antibiotic substance and B vitamins by culture P9, a marine *Serratia* obtained from Puerto Rico. Note that this bacterium excretes thiamine, along with other B vitamins.

of test plates of vitamin-requiring bacteria. Zones of growth promotion indicate that a supply of required vitamin produced by the algae has diffused into the seeded agar plate, where stimulation of the vitamin-deficient bacteria occurs.

Although it is beyond the scope of this discussion to present all of our observations concerning various effects of marine algae upon growth of bacteria, it can be stated that macro-algae, as well as planktonic dinoflagellates and diatoms, produce special substances with both inhibiting and promoting properties for bacteria. The antibacterial action of red algae and certain dinoflagellates has already been emphasized (3). Some typical results that have been obtained in recent tests of the effects of extracts from tropical and temperate blooms of phytoplankton on seeded plates of four laboratory strains and ten isolates of marine bacteria are presented in Table 5. The *Gonyaulax* (identity not certain) "red tide" was collected in Puerto Rico during January, 1961 and the plankton bloom of *Skeletonema* was obtained from Long Island Sound in February, 1961. When these crude plankton materials, and other marine algae, were placed upon seeded Difco yeast beef agar plates, inhibition zones occurred around the algal material and striking circular zones of heavier growth were formed beyond. In specially prepared agar plates lacking single vitamins and seeded with marine bacteria that require these vitamins, pronounced zones of inhibition frequently occurred, and usually marked stimulation of growth was observed in the form of halos around the points where the algae were applied (Fig. 4). Such results may be explained by the diffusion of antibacterial substances outward to cause zones of inhibition, and further diffusion of B vitamins beyond these zones of inhibition to produce halos of growth in vitamin-deficient agar. It can also be demonstrated that algae supply complete sources of carbon and nitrogenous materials for growth of marine bacteria.

The production of a diatom bloom in Long Island Sound occurs regularly every February, as a result of the increased phosphate, nitrate, vitamin B_{12}, and sunlight at this time of the year (13). So pronounced is the bloom that the above listed factors which contributed to it, except sunlight, are removed from the water by the first of March. Since vitamins of the B_{12} group

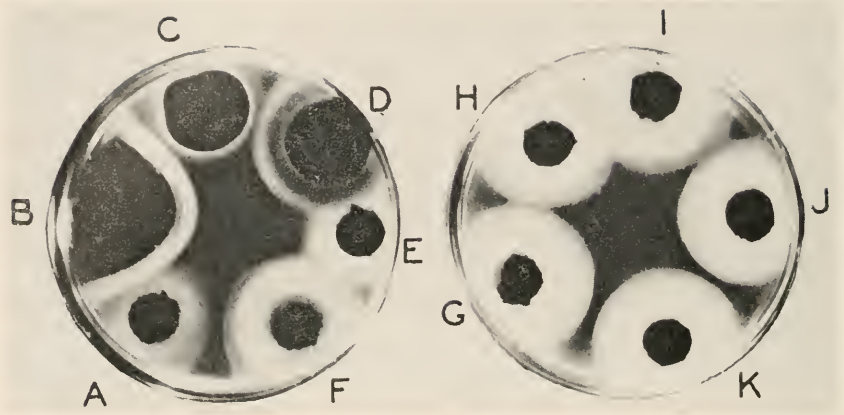

Fig. 4. Response of a marine thiamine-deficient bacterium (B1866) to various marine algae applied on seeded plates containing medium B without vitamin B_{12}. A, *Sargassum lendigerum;* B, *Gonyaulax tamarensis;* C, *Zonaria zonalis;* D, *Wrangelia argus;* E, *Acanthophora spicifera;* F, *Laurencia obtusa;* G, *Penicillus capitatus;* H, *Ceramium nitens;* I, *Halimeda opuntia;* J, *Caulerpa racemosa;* K, *Coelothrix irregularis.* Note the dark zones of inhibition around applications B, C, and D, and the light areas of growth stimulation by thiamine supplied by the algae.

disappear from solution in the waters of Long Island Sound concurrently with the development of extensive blooms of the cobalamin auxotroph, *Skeletonema costatum,* one may inquire what is the fate of these substances? Similar blooms of dinoflagellates often occur in tropical waters. The organisms that are dominant in these blooms belong to groups which have pronounced auxotrophic tendencies, but their absorption and use of vitamins is not well understood.

Microbiological assays of lyophilized powders of *Skeletonema* and *Gonyaulax* (?) blooms were performed with various auxotrophic marine isolates, and with two strains of *E. coli,* 113-13 sensitive to cobalamins, and 26-18 sensitive to methionine. Both blooms showed appreciable vitamin B_{12} activity and other vitamins, along with antibiotic properties, exhibited on suitable assay agar plates seeded with indicating bacteria. Apparently blooms can absorb certain vitamins, as well as synthesize others and store them as a vast reservoir, available for predators and direct utilization by those auxotrophic microbes that can tolerate the antibiotic properties.

It is of considerable interest to know what use can be made of this vast amount of organic matter assembled in the cells of diatoms possessing antibacterial activity. Accordingly, hot aqueous extracts of the diatoms were prepared in sea water and a series of media were made so as to contain different amounts of extract in the range from 1.4 to 22.0 grams per liter. Bacteria obtained from Long Island Sound and from Puerto Rico were

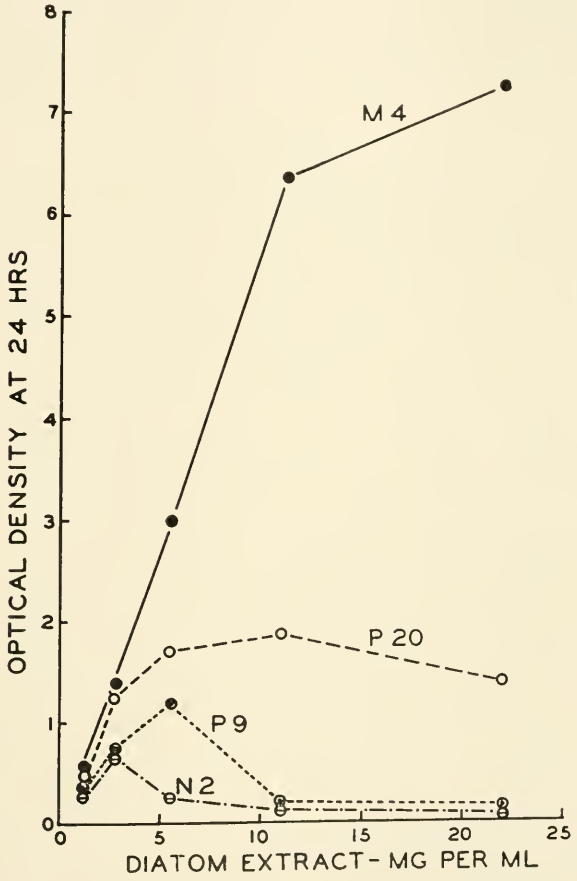

Fig. 5. Growth responses (after 24 hr.) of marine bacteria cultivated in sea water containing different concentrations of an aqueous extract prepared from a bloom of the diatom, *Skeletonema costatum,* taken from Long Island Sound, February, 1961. Note that bacterium M4, isolated from the plankton bloom, grows very well, whereas other isolates appear to be inhibited by antibiotic activity of the diatoms (compare Table 4).

inoculated into these media and allowed to grow at 30 C on a shaker for twenty-four hours. The calculated optical density of bacterial growth was determined. Typical results obtained for several bacteria are presented in Figure 5. Excellent growth of isolate M4, obtained from the water where the bloom was collected, is shown by the steadily rising growth in proportion to the concentration of diatomaceous extract supplied in the medium. Other bacteria, obtained from remote geographic locations, were unable to take advantage of the organic substrate beyond the lowest concentrations, presumably because of the inimical effects of the antibacterial substance present in the diatoms.

TABLE 5

GROWTH PROMOTION AND INHIBITION OF BACTERIA BY PHYTOPLANKTON (CHIEFLY *Skeletonema costatum*) FROM LONG ISLAND SOUND AND BY *Gonyaulax tamarensis* FROM LA PARGUERA, PUERTO RICO. RADIAL ZONES OF INHIBITION (INSIDE) AND LARGER RADII OF PROMOTION ARE RECORDED IN MM. —=NO. RECORD.

Laboratory Strains	*Medium*	*Inhibition, mm*		*Promotion, mm*	
		Skeletonema	*Gonyaulax*	*Skeletonema*	*Gonyaulax*
S. aureus	YBA*	−13	−13	+17	+17
E. coli	YBA	− 3	− 7	+ 6	+11
B. subtilis	YBA	− 8	−14	+19	+18
M. smegmatis	YBA	− 7	−11	+12	+15
Marine Isolates					
B 491	Medium A**	− 3	−10	+15	0
Cap 20	Medium A	0	—	+10	—
Cap 34	Medium A	− 4	−10	+12	—
B 1866	B-B_1***	− 5	−11	+20	+19
B 746	B-Biotin	− 5	− 3	+10	+12
C 259	B-Panto.	− 7	−10	+16	+16
N 35	B-Nicotinic Acid	− 7	−12	+10	+16
MY 36	B-B_2	− 3	—	+13	—
WH 310	B-Panto.	− 7	−13	+15	—
N2	B-B_{12}	− 6	−15	+23	—

* YBA signifiies Difco yeast beef agar.
** Formula for Medium A is given in Table 1.
*** Formula for Medium B is given in Table 1.

It has been found, also, that many bacteria isolated from the marine environment produce antibiotic substances that are very active against marine bacteria and algae, as well as against

common "laboratory" strains. Certainly antibiosis must have considerable ecological significance in the oceans (3, 11).

EXCHANGES OF VITAMINS AMONG GROWING MICROORGANISMS

In order to show that actual vitamin exchanges take place simultaneously between different kinds of microorganisms, special experiments have been devised with cultures possessing recognizable characteristics. Several examples will be given to illustrate the principle of metabolite exchange in the phenomenon of syntrophism.

For the purpose of demonstrating the contribution of vitamin B_{12} by bacteria to the blue-green alga *Synechocystis*, seeded plates of the latter were prepared, with ASP-2 medium and 1.0 per cent purified agar, poured into sterile plastic petri dishes. In some experiments, this medium was supplemented with Difco vitamin-free Casamino acids 0.5 per cent, dextrose 0.2 per cent, and sodium succinate 0.2 per cent, in order to encourage more rapid growth of bacteria. Bacterial cultures, known to produce vitamin B_{12}, were impregnated in paper discs and placed on the algal-seeded plates, or the bacteria were inoculated directly on the surface of the agar seeded with B_{12}-requiring *Synechocystis*. After several days of incubation in an atmosphere of air and 1% CO_2 at 28 C in a light room, zones of the blue-green algae growing in ASP-2 medium became evident around the pads containing bacteria that produce vitamin B_{12}. In the other plates containing organic additions, where the inoculated bacteria grew and produced B_{12}, there developed excellent growth of the blue-green algae intermingled with the feeder bacteria.

Effects of excreted vitamins have been demonstrated between a bacterium that produces large amounts of many B vitamins and other bacteria requiring riboflavin and nicotinic acid (Fig. 6). Also the simultaneous exchange of riboflavin for pantothenate has been observed between cultures My36 and N43, growing in an agar plate lacking these required vitamins. If simultaneous import and export of vitamins did not take place in a vitaminless environment, no growth of such deficient cultures could occur.

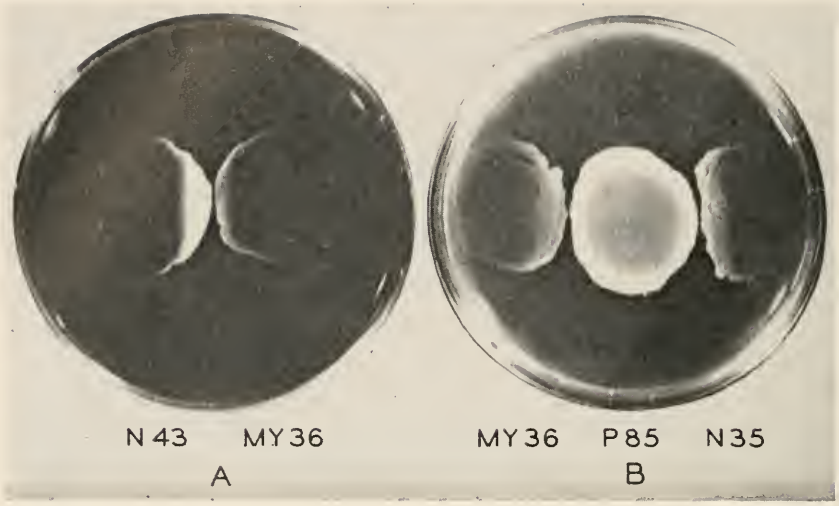

Fig. 6. Exchange of certain B vitamins synthesized by matched auxotrophs allows growth of bacteria, within the areas of mutual influence, in agar media where the required vitamins had been omitted. A, culture My36 B_2^- Panto$^+$exchanges with culture N43 B_2^+ Panto$^-$, with resulting mutual benefit. B, culture P85 Nicotinic acid$^+B_2^+$ contributes these vitamins to cultures N35 Nicotinic acid$^-$ and My36 B_2^-.

Other methods of demonstrating exchange of B vitamins have been used with liquid media. Complementary types of vitamin-deficient bacteria are inoculated singly and as mixtures, into suitable media lacking the required vitamins. Thus, culture 238 requiring thiamine and culture 507 requiring biotin were alone unable to grow well in a medium lacking both of these vitamins; in mixed culture, however, good growth was observed. Two vitamin B_1-deficient cultures served as an example for exchange of intermediates in syntrophic mixed culture. Culture 276 required pyrimidine, and culture 776 required thiazole. Alone, neither culture was able to grow well in a medium lacking vitamins; together they apparently exchanged the thiazole produced by culture 276 and the pyrimidine formed by culture 776, and both grew well in a medium that was initially without thiamine.

CONCLUSIONS

1. Among the auxotrophic algae and bacteria of shallow

sea waters and sediments, requirements for biotin, thiamine, cobalamine, and nicotinic acid occur commonly, while needs for exogenous pantothenate, riboflavin, and other vitamins are found infrequently.

2. Production of various B vitamins is demonstrable in cultures of many algae and bacteria isolated from the sea. Certain vitamins are excreted along with other metabolites during growth of some algae and bacteria while others, such as B_1 and B_{12}, are often more tightly bound within the cells. While the presence of such vitamin substances is readily observed in crude collections of phytoplankton and marine macrophytes, one may not easily conclude that all of these substances are synthesized in organisms where they are found in nature.

3. The direct availability of vitamins and other metabolites formed by primary producers in the sea may often be regulated by selective inhibitory action of "antibiotic" substances produced by plankton on a vast scale or by various algae and bacteria in more localized habitats.

4. Products of organisms commonly are utilized in sequence by others, in accordance with the general principles of metabiosis, and it has been demonstrated, also, that simultaneous exchanges of metabolites can occur among living microorganisms in marine communities. Our observations indicate that complex relationships in mixed populations often involve delicate adjustments of syntrophism, antibiosis, and other types of coactions.

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Part 2
Geomicrobiological Activities of Marine Microorganisms

Chapter 15

Problems of Thermophilic Life in Polar Regions

R. H. McBee

The search for thermophilic bacteria in polar regions appears to be a foolish venture. It would be interesting to know what train of thought led to the first examination of materials from polar regions for thermophiles. As far as I have been able to determine, the first such examinations were of soils from Antarctica and were made by Mme. Tsiklinsky in 1905 (9). Her results were negative. Egorova (2), however, found aerobic spore-forming bacteria capable of growing at 80 C in soil samples from barren islands in the Kara Sea and mud from the bottom of the Arctic Ocean at 79° N. latitude. He apparently did not enumerate the thermophilic bacteria in these materials nor did he classify the bacteria found. He did not present any explanation as to how or why thermophilic bacteria were present in this environment.

Ocean bottom mud from off the California coast was examined for thermophilic bacteria by Bartholomew and Rittenberg (1). Although this is not a polar region, the *in situ* temperatures were below 10 C. Thermophilic counts of 600-800 bacteria per gm were found in the top twelve inches of the mud. Four cultures which grew well at 60 C but not at 37 C were isolated. They were all large gram-positive spore-forming rods but they were not classified. No attempt was made to explain their presence or significance.

McBee and McBee (6), attempted to examine the polar thermophile problem on a quantitative basis and to determine what factors were associated with their occurrence. This study was conducted at Point Barrow, Alaska (71° 20′ N. latitude). A wide variety of soil and water samples were examined for aerobic thermophilic bacteria. Of 59 such specimens, thermophilic bacteria, one or more per gm were found in 38. Thirty-four

of the samples were also examined for mesophilic bacteria by a 35 C plate count with tryptone-glucose-extract agar and for coliform bacteria with desoxycholate lactose agar. Only two samples had fewer than one mesophilic organism per gm whereas twenty were negative for coliforms. Of these 34 samples, thermophiles were present in twenty-one in numbers ranging from one to 2,200 per gm. The soil samples showing the greatest numbers of thermophilic bacteria were from areas where there had been either old or recent Eskimo camps.

Fifteen pure cultures of thermophilic aerobic bacteria were obtained from the Point Barrow specimens. Classification of these, using the tests recommended by Smith *et al.* (8), resulted in ten being identified as *Bacillus stearothermophilus*, three as *Bacillus coagulans*, and two as *Bacillus licheniformis* (5). No new species were found.

During the course of a brief biological survey in the Ross Sea area of Antarctica in January and February of 1957 a large number of random bacterial cultures were made in an effort to determine the distribution of these organisms in Antarctica. Some of these results have been published (4), whereas others have not. Among the materials examined for mesophilic and thermophilic bacteria were:

1. Large intestine contents and feces of two Adelie penguins, one South Polar skua and two Weddell seals.
2. Mud from an ocean bottom core taken in Kainan Bay at a depth of about 400 fathoms.
3. Two samples of sandy soil from near Pram Point.
4. Old horse manure from under the eaves of Scott's hut on Hut Point. This material dates from 1911-1912.
5. Two mixtures of moss and lichens from near Williams Naval Air Facility and Pram Point.
6. Three algal encrustations of rocks found in small fresh water ponds near Williams Naval Air Facility.

All of these specimens contained mesophilic bacteria, although only anaerobic bacteria were obtained from the intestinal contents of the birds and seals. No molds or thermophilic bacteria were found in any of these materials. All of the cultures for thermophilic bacteria were incubated aerobically except that

glucose agar shake tubes were also prepared from the bird and seal specimens.

This complete failure to find thermophilic bacteria in specimens from Antarctica is puzzling since they have surely been introduced many times during the last 50 to 60 years. It is true that there are no large accumulations of decomposing organic material in this part of Antarctica, but man along with dogs and horses have been in this area repeatedly for half a century. The absence of thermophilic bacteria from the horse manure is especially interesting since this type material has repeatedly been used as a source of thermophilic cellulose fermenting bacteria. It may be that only anaerobic thermophilic bacteria were present and that these were undetected because only aerobic conditions were used on this culture. One would hardly expect that all aerobic spore-forming thermophilic bacteria would have died during the 45 years of exposure of Antarctic conditions since more than 1,000 coliform bacteria per gm were still present in the sample examined.

The relative abundance of aerobic thermophilic bacteria in arctic regions and their absence or relative scarcity in antarctic regions where they have been sought has led me to the following speculations.

1. Arctic regions are in general not as inhospitable as is Antarctica. There is an abundant growth of grasses, lichens, mosses and flowering plants in arctic areas free of ice and snow during the summer. Antarctica, on the other hand, is devoid of grasses and flowering plants except on parts of the Palmer Peninsula which extends northward to approximately 63° S latitude. Even the antarctic mosses and lichens generally barely hold their own against cold, dryness and a scouring wind. There is no accumulation of plant remains to help with the formation of a true soil which could support a large microbial population.

2. Arctic animals are quite numerous and range in size from the lemming to the musk ox and caribou. Both herbivores and carnivores are included. They supply variety to the organic material and also continually reinoculate the soil with mesophilic, (and possibly thermophilic bacteria) from their digestive tracts. The effect of the lemmings and the other animals on the arctic

soil population of microorganisms has not been assayed, but it is probably considerable during the years of large populations. Antarctic land animals are restricted to birds and seals, both of which are primarily carnivorous and dependent upon the ocean for their food. They contribute very little towards the building of a population of soil microorganisms except in such isolated places as penguin rookeries. The microbiology of these has not been studied.

3. Arctic regions along major rivers and the ocean continually receive soil, organic materials and microorganisms from continental land masses which have relatively warm summer periods. This inoculation is both air- and water-borne. The vast oceans surrounding Antarctica drastically reduce such inoculations by both air and water.

4. Finally there is a native human population in most arctic regions. This is entirely lacking in Antarctica where up until the last few years man has been only a rare summer visitor. Man tends to accumulate organic litter, which, along with the increased temperature of his habitation, would give small areas with high microbial populations.

It is possible that not all of the observations concerning thermophilic bacteria in polar regions can be explained by these generalizations, but several of them may be.

1. The Kara Sea receives the water from the Tobol, Irtysh, Ob, Angara and Yenisei Rivers which drain a tremendous area east of the Ural mountains extending from about 60° to 114° E longitude and from about 46° to 73° N latitude. Water-borne silt from this area is probably the source of the thermophilic bacteria found by Egorova in the bottom mud and the island soil.

2. The thermophilic bacteria found in the Point Barrow, Alaska region by McBee and McBee (6) were nearly all in or near Eskimo habitation. One sample from near the dog stakes of a recent Eskimo camp had nearly 200 aerobic thermophiles per gram as well as anaerobic thermophilic bacteria. Similar soil from a few yards away showed no aerobic thermophilic bacteria in a one gram sample even when lemming feces were included. Abandoned Eskimo houses and the area surrounding them such

as Nuwuk Village at the tip of Point Barrow were littered with decaying organic material. Even though the village had been abandoned for nearly 35 years this litter showed a high thermophile count. Grassy or mossy tundra type soil, on the other hand, showed a relatively high count of mesophilic bacteria but few thermophiles.

The conclusion that we reached was that a high content of organic material other than dead plants was necessary for the growth or survival of thermophilic bacteria in polar regions.

One cannot ignore the possibility of thermophilic bacteria actually being normal polar soil microorganisms. It has been demonstrated (3) that thermophilic bacteria can grow at reduced temperatures in the soil. Whether they can actually multiply below the freezing point as do mesophiles (7) needs to be determined.

One important point to remember when speaking of thermophilic bacteria is that many of them are not obligately thermophilic but grow very well at temperatures below 30 C.

Before we overemphasize the importance of polar thermophilic bacteria we need to re-examine the soils and digestive tracts of animals in the torrid and temperate zones for a variety of types of thermophilic bacteria other than the aerobic sporeformers. After learning what conditions are conducive to the growth and survival of thermophilic bacteria in warmer climates I believe we can better understand why we find them or do not find them in polar regions.

There is a tendency to regard the microbiology of polar regions as being unique. I believe, however, that when we study similar environments in other regions we will find the outcome of many of our polar studies to be predictable.

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Chapter 16

Possible Influence of the Earth's Magnetic Field on Geomicrobiological Processes in the Hydrosphere*

Frederick D. Sisler* and Frank E. Senftle

INTRODUCTION

It is well recognized and obvious that in marine environments, physical, chemical and biological forces work in combination and are responsible for much of the diagenesis and geology of sedimentary deposits. However, the precise nature of the interrelationships and the total impact of the more obscure forces on the overall geological processes taking place are not so obvious. In this paper, attention is focussed on the admittedly obscure relation between the earth's magnetic field and geomicrobiological processes in the oceans and the superficial sediments.

Figure 1 shows our earth as a ball covered largely with water, most of which can be considered as a dilute electrolyte containing charge carrier. The principle charge carriers in ocean water consist of simple ions, complex molecules and minute microorganisms. These carriers are transported by ocean currents and, if their path happens to be at right angles to the earth's magnetic field, a force will be exerted on them perpendicular to the direction of flow. This force will cause a given particle to trace out a curved path. If the flow of current is continuous, as it is in ocean currents, one might expect electric potential differences to be set up between different parts of the ocean.

As far back as 1832, Faraday considered the possibility of potential differences in a moving fluid electrolyte and actually performed some crude experiments in the tideway under Water-

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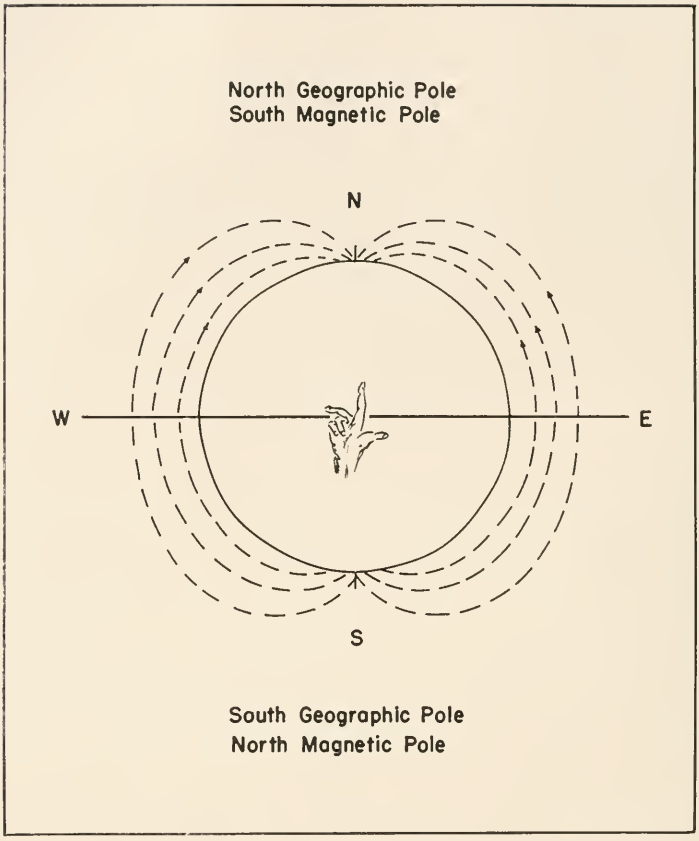

Fig. 1. The earth's magnetic field.

loo Bridge in London (3). Chapman and Bartels (3) describe a number of subsequent experiments performed by the British Admiralty showing the effects of the vertical component of the earth's field in producing potential differences. More recently Bauer and Norton (2) have postulated a similar situation to exist in some rivers. Fossil shells recovered from one side of a river bank during a field study were found to be badly decalcified. Shells found in the opposite bank in the same formation were well preserved. They concluded that a physical-chemical process was taking place involving induced electrical currents resulting from the conducting river water flowing across the earth's mag-

netic field. They also speculated that the oxidation-reduction potentials believed to have existed would have an influence on the growth of the biota occurring on opposite sides of a flowing river. In all of the above experiments only the ability of the vertical component of the earth's field has been considered as a potential producing mechanism. What are the effects of the horizontal component and what can be learned of the charge carriers by considering the associated processes? As an example and for simplicity only those oceanic regions adjacent to the equator will be considered. Here the verticle component of the earth's magnetic field is essentially zero and the horizontal component is maximum.

THEORETICAL CONSIDERATIONS

When a charged particle of mass m and charge e crosses a magnetic field of H gauss at right angles and with a velocity v, it will trace out a path such that the impressed force due to the magnetic field will be equal to the centrifugal force. Thus*

$$Hev/c = mv^2/r \qquad (1)$$

and

$$r = vc/H \cdot \frac{1/e}{m} \text{ (cm)} \qquad (2)$$

where r is the radius of curvature of the path and c is the velocity of light. The direction of the curved path will be determined by Fleming's right hand rule (see Fig. 1).

In the northern hemisphere of the equatorial zone, the vertical component of the earth's field is zero, the horizontal component is maximum, and the ocean current is from west to east. According to the right hand rule, negative charge carriers will trace out curved paths such as to move them toward the bottom of the ocean. As the operation is continuous, one might expect a relative concentration of negative charge in the vicinity of the ocean bottom. At the same time there would be a concentration of positive charge near the surface of the ocean. The net result is a sustained potential difference between the surface and the bottom of the ocean. In the southern hemisphere a similar situa-

* This theoretical equation has not been corrected for viscosity effects which are important for particles larger than 1μ.

tion will exist except as the ocean current is in the opposite direction, the polarity of the charge will be reversed, and the negative charge will be observed near the surface. Thus along the surface of the ocean just north and south of the equator a significant potential difference should occur and give rise to electron flow from south to north. Likewise near the ocean bottom along the equator there should be a similar region of opposite polarity which will give rise to an electron flow in the opposite direction.

In the polar region the horizontal component of the earth's field is essentially zero, but the vertical component is maximum. The polar ocean currents, however, are not as well defined as they are in the equatorial zone, and one cannot anticipate the result with any degree of confidence. It will suffice to say that the resultant force will be such as to cause a lateral rather than a vertical potential gradient such as discussed by Chapman and Bartels (3). In any region between the polar and equatorial latitudes, the resultant force will reflect both components of the earth's magnetic field.

The above hypothesis, while physically sound in principle, must be investigated in terms of the known parameters. The horizontal component of the earth's field will be considered to be nominally at 0.5 gauss. The velocity of surface ocean currents in the middle latitudes range from zero to about 4 km per hour (9). The other important parameter is the ratio of total charge to mass of the carrier.

In considering equation (1) it is obvious that the radius of curvature of the path of the charge carrier will be largely dependent on the ratio e/m. The carriers range in size from simple ions to macromolecules and particles up to the size of microorganisms (1 μ). For small ionic carriers, e/m is large and the radius of curvature is small, say of the order of one meter. Excluding local convection currents, etc., such charge carriers will have a relatively constant velocity and hence will simply circulate in circles as they move along with the current. For larger carriers the e/m ratio is smaller and the radius of curvature is larger. The vertical velocity gradient, however, is steep and thus while the radius of curvature of the path for high currents is large

at first, it decreases rapidly with the lower velocities at depth. A charged bacterial particle will thus start out on a broad circular path toward the bottom, but as it goes down it will execute an ever tightening circular path. It will then be essentially trapped at some depth where the velocity approaches zero. In relatively shallow water the velocity may be low enough such as to coincide closely with the bottom, whereas in the ocean deeps it may be considerably above the ocean floor. In either case there will be a tendency to cause a separation of charge between the surface and the ocean bottom which will be maximum in the equatorial region. For the purposes of this analysis, it is thus expedient to consider only those charge carriers whose radii of curvature is commensurate with coastal and ocean depths between, i.e. 0.05 to 3 km.

Figure 2 shows the radius of curvature vs the velocity for various values of the total charge to mass ratio. It can be seen that for normal ocean currents of 0.01 to 4 km/hr, the charge to mass ratio which will be important to this process will vary between about 10^5 and 10^9. Figure 3 shows the mass vs the charge for the charge to mass ratios in this range. If the carrier possesses a unit charge, the possible mass range for normal ocean currents will be 5×10^{-15} to 10^{-19} grams respectively. This mass range is in the colloidal particle size. It is therefore possible to essentially rule out the migration of singly charged inorganic metallic ions in sea water as the cause of a potential difference such as being considered here.

One now asks what are the possibilities of magnetic influence on multiple-charged particles of larger mass such as large colloids or microorganisms? Although not much is known about the electron charge on the surface of marine microorganisms, an order of magnitude calculation is instructive. Bacteria and other microorganisms generally carry a negative charge. The adsorbed excess negative ions on the surface of the organism give rise to a strongly attracted positive ion layer adjacent to the surface of the particle. Successive layers adjacent to this socalled "electric double layer" become increasingly less positive as they grade out into the bulk fluid medium. An electric potential, the zeta potential, is de-

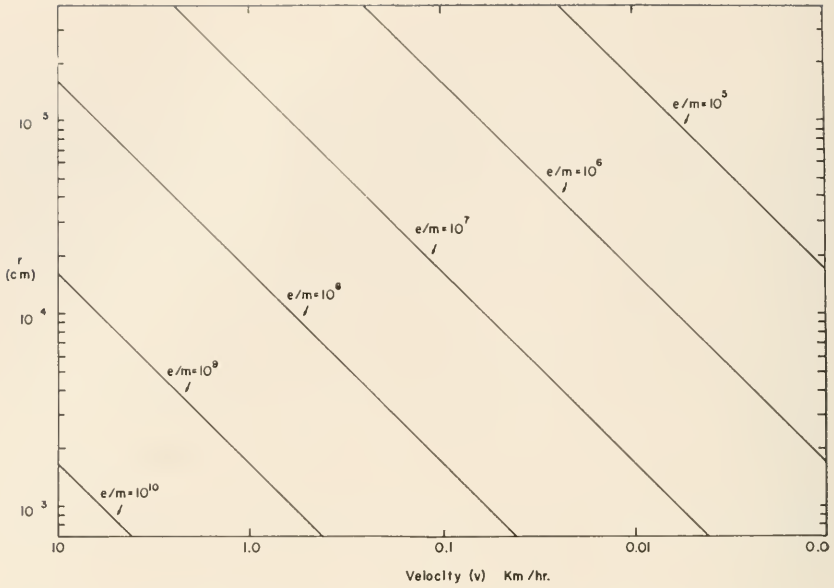

Fig. 2. The radius of curvature (r) for various particles of e/m moving at velocity of currents (v).

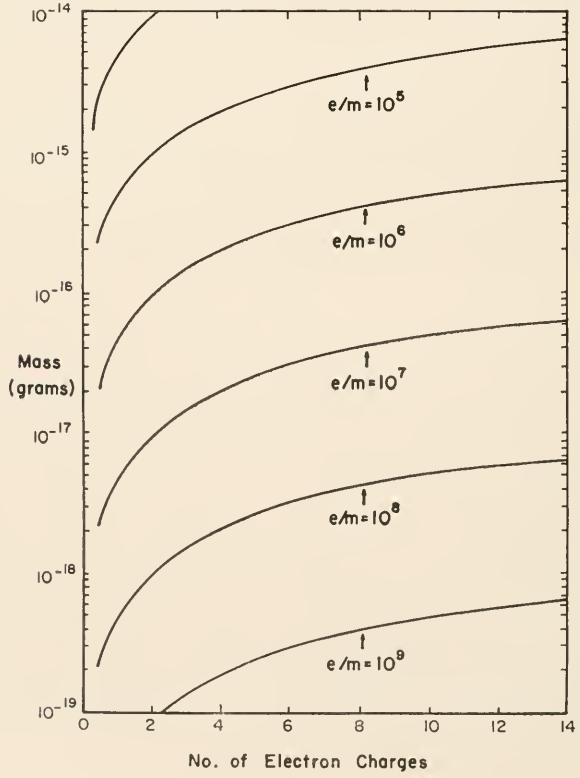

Fig. 3. Mass versus the charge values for e/m ratios in the range 10^5 to 10^9.

veloped across the diffuse part of the double layer. Moyer (5, 6) has shown that the surface charge per unit area, σ, is given by

$$\sigma = \zeta D/4\pi\lambda$$

where D is the dielectric constant of the medium and λ is the thickness of the diffuse part of the double layer. Using this type of calculation, Abramson (1) has shown that typhoid bacilli carry about 3×10^5 electron charges. Accepting this as a rough figure although it may be a little high for some bacteria, and the fact that bacteria of this type have a mass of about 10^{-12} grams, then the charge to mass ratio is approximately 1.5×10^8.

From Figure 2 it can be seen that a charge to mass ratio of this magnitude falls in the shaded area for current velocities of from 0.3 to 4 km/hr, i.e. between these currents a charge carrier of bacterial size can be deflected to the ocean bottom for depths of from 50 m to 3 km respectively. Average equatorial ocean currents, however, are closer to 0.3 km/hr and thus correspond to a radius of curvature of about 50 m. Depending on the surface current and the current gradient the radius of curvature will decrease with depth for a given e/m ratio.

For the e/m ratio being considered here, the radius of curvature can be shown to approach a radius of about one meter when the current velocity drops to about 0.006 km/hr. For an average surface current of 0.3 km/hr, a velocity of 0.006 km/hr is not unreasonable for depths of 50 to 100 meters. When the radius of curvature and velocity drop to a value of this order of magnitude, the bacterial charge carrier will be trapped. The net result will be a zone of enriched bacteria in the ocean which will vary somewhat in depth depending on the current and the e/m ratio. Of course, convection and other local currents will interfere with this hypothetical picture to some extent, but over broad regions it is reasonable to assume that a zone of bacterial enrichment can be produced by this process.

It is proposed that the above effect is a contributing factor leading to the known distribution of bacteria in the sea, and is at least in part the reason for a charge distribution in ocean water. At an average depth of about 50 m there is known to be a distinct peak in the vertical distribution of marine bacteria. This high bac-

terial zone, which coincides with the pelagic zone, varies somewhat in depth and at night generally rises to the surface as can be observed by the fluorescent light emission of many of the plankton. If the electromagnetic force on the bacteria varies with the surface current and current gradient, one would expect the bacteria zone to vary; and also at night when the surface wind and current velocity are generally low, the zone would be expected to approach close to the surface. Heretofore, the fact that the bacterial zone coincides closely with the pelagic zone has been explained as due to the attachment of the bacteria to planktonic organisms. It appears that the electromagnetic force on these particles is large enough to bring about a similar distribution pattern.

The very large bacterial enrichment in the bottom muds especially near shore can also be explained by this mechanism. Under conditions of high wind velocity and thus higher surface currents, the radius of curvature of particles can increase greatly. The enriched zone of bacteria will then be depressed and from Figure 2 it can be seen that it is entirely feasible that the zone may come in contact with the bottom. The organisms will then be trapped and as the bottom silts present a large surface area for attachment, they will ultimately be localized on the ocean bottom. The extremely large bacterial population in the top few centimeters of bottom muds is well known and appears adequately explained by this hypothesis.

It appears therefore that masses of colloidal size carrying single or multiple charges up to masses of the size of marine bacteria carrying thousands of electron charges have the appropriate charge to mass ratio to account for a surface to bottom charge separation in the ocean of equatorial regions and the known vertical bacterial distribution in the sea. It is apparent that once such carriers reach the vicinity of the ocean floor they are essentially trapped in this region. If local currents carry them up from the bottom they soon are caught in the prevailing bottom currents which, being at right angles to the horizontal component of the earth's magnetic field, will return them to the bottom. It may well be that the iron and manganese which may be precipitated by bacteria (4) near the ocean bottom are directly connected

with the not infrequent intersection of the enriched bacterial zone with the bottom muds as the result of high ocean currents produced by storms.

EXPERIMENTAL WORK

Only a limited amount of experimental work can be done in the laboratory to check the above hypothesis and a preliminary experiment was performed as a feasibility test. A cell was constructed of clear plastic as shown in Figure 4. Water was circulated in a closed circuit through the tubes "a b." The side arms "c" and "d" were plugged with agar containing a universal pH

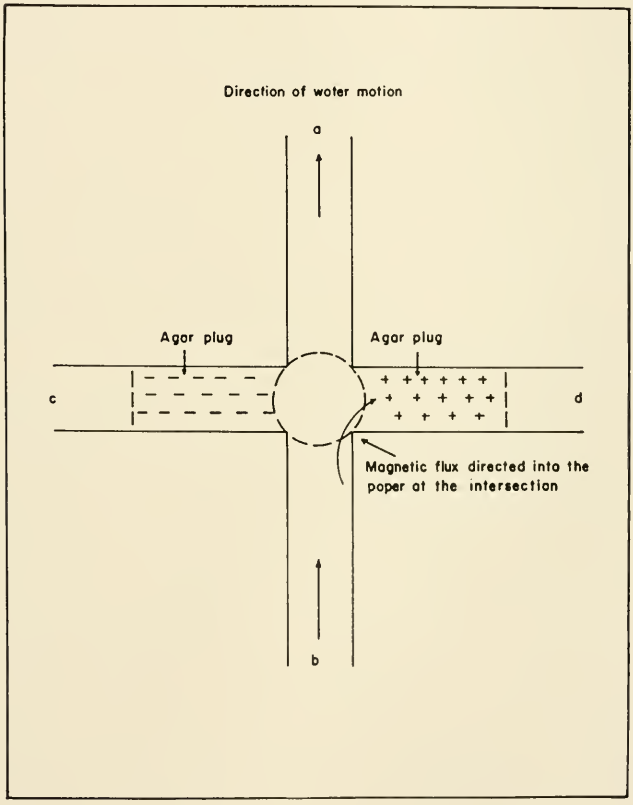

Fig. 4. Experimental cell showing effects of magnetic field perpendicular to the center as indicated by ion trap side arms.

indicator. Two permanent Alnico bar magnets were mounted perpendicular to the plane, and on either side of the cell at the junction of the tubes "ab" and "cd." These provided a field of about 800 oersteds at the junction. After pumping water through this cell for several hours the indicators showed one of the side arms to be basic while the other was acidic. The probes of a pH meter were then inserted into the agar and a reading taken.

In the first experiment, tap water containing some bacteria, cells and colloidal material was circulated for four hours. The initial water had a pH of 6.2_2 while after the experiment the pH in the agar of the side arms of the cell was found to be 5.8_2 and 6.6_2 respectively. Repetition of the experiment with raw sea water containing bacteria and colloidal material, but at a slightly lower flow rate and for six hours, showed similar changes but at a smaller magnitude as compared to the tap water. Using a slightly modified glass cell the experiment was repeated, using sea water which had been previously filtered through a millipore filter to remove all particulate matter. The results in this latter experiment even after circulation for 24 hours were reduced to only about 0.1 of a pH unit between the two side arms of the cell. It thus appears that filtration removed essentially all of the large charge carriers. The carriers must therefore consist of very large colloids or charge carrying bacteria.

If one assumes that the ratio of e/m is approximately 10^8, the flow rate 20 liters/hour, a tube diameter of 0.5 inches and a magnetic field of 800 oersteds, it can be shown from equation (1) that the radius of curvature will be approximately 0.4 centimeters. The measured radius of curvature from the geometry of the cell was a little greater than 0.5 centimeter. It appears that bacteria or possibly large colloids are causing the observed change in pH.

The experimental data tends to support the hypothesis that the vertical motion of bacteria in the sea is influenced by the ocean currents in the horizontal component of the earth's magnetic field. However, the data so far are much too fragmentary and need to be supported by further experimental work both in the laboratory and in the field. A more sophisticated laboratory experiment should be performed similar to the one described above and in which radioactive labelled bacteria are used. The

change in potential with depth in the ocean and correlation with surface velocity would also shed much light on the significance of the proposed electromagnetic mechanism.

EFFECT OF THE EARTH'S MAGNETIC FIELD ON MICROBIOLOGICAL PROCESSES IN OCEAN BOTTOM SEDIMENTS

The above hypothesis not only explains the effect of the ocean currents and the earth's magnetic field on the vertical motion and distribution of microorganisms, but may also be used to explain other anomalous effects on the physiological processes of sessile organisms in bottom sediments. It is generally recognized that microorganisms in marine sediments perform numerous oxidation-reduction reactions involving the transfer of electrons. Certain microbiological processes in the sea bottom appear to contribute to the production of electric currents. The existence of telluric currents within the earth's crust including the sea floor is well recognized. Although telluric currents are considered to originate from geophysical forces, e.g., differential solar heating of the earth's crust, some local telluric currents appear to result from a much more complicated series of circumstances and, to involve biological processes. In this latter instance, difference in oxidation-reduction potentials between adjacent sedimentary deposits would be expected to influence the direction and magnitude of these local currents. As microbiological activity has a profound influence on the oxidation-reduction conditions within the sea floor, one may reasonably inquire about the impact of an induced emf resulting from the movement of sea water electrolytes across the earth's magnetic field, upon biologically induced electrochemical phenomena.

Field investigations and laboratory tests by one of the authors reveal that, under certain conditions, a substantial fraction of the electric currents in the sea bottom can be accounted for by biological processes. Because of the difficulties encountered to date in measuring and mapping electric fields in the sea, the picture is as yet incomplete as to precisely what fraction and under what set of conditions the biologically induced electric currents

supplement the geophysical telluric currents. This is clear, however, that where there is a large difference in oxidation-reduction potential between the sea floor and surface waters of the sea, biological forces are actively contributing to the phenomenon.

Sediment samples collected from regions of high potential difference between the sea floor and the overlying water, such as usually observed in anaerobic basins of the marine environment, contain large numbers of bacteria and other microorganisms, and invariably a large population of sulfate-reducing bacteria. When such sediments are placed in a laboratory cell designed to measure the emf and electric current flow between the contained sediment and a sample of sea water collected in the same region as the sediment, the observed emf and current usually correlates well with similar measurements taken *in situ* (7). Where correlation is poor, a closer inspection of the field data, which includes oceanographic observations of water currents and tidal oscillations, strongly suggest that the induced emf resulting from water movement across the magnetic lines of force of the earth either mask or neutralize the biologically induced electric currents expected to occur in the region.

Using specially designed probes to measure both emf and electric currents on the sea floor and between the sea floor and overlying water, fluctuations in voltage and current have been observed during continuous recordings at an anchored station (8). It is believed, although not conclusively proven at this time, that such fluctuations, in some instances appearing as a low frequency oscillating current, are caused by the above mentioned interrelation between water movement and the earth's magnetic field. It is also believed, but not proven, that magnetically induced variations in emf and electric currents affect the physiology of the microflora in the sediments. Isolated microorganisms, when placed in an electrolytic cell, display changes in physiological processes and in orientation when the electric current in the cell is reversed by an outside power source. The authors are presently conducting laboratory tests to assess both direct and indirect effects of magnetic fields on the functions of microorganisms.

ACKNOWLEDGMENT

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Chapter 17

The Role of Microbes in Genesis and Weathering of Sulfur Deposits*

S. I. Kusnetzov

About 90 per cent of sulfur deposits of the earth are of sedimentary origin. Very often their genesis is connected with the activity of microbes of the sulfur cycle in nature under marine conditions. Epigenetic and syngenetic genesis of sulfur deposits take place also under recent conditions. We have studied their microbiology in the USSR (6a).

SYNGENETIC GENESIS (Fig. 1)

The sediments of Balkasch sea and of the sea of Tambukan contain quartz sand, Calcite and Dolomite with elementary sulfur up to 0.25 per cent (Table 1) which originates mostly from desulfurication.

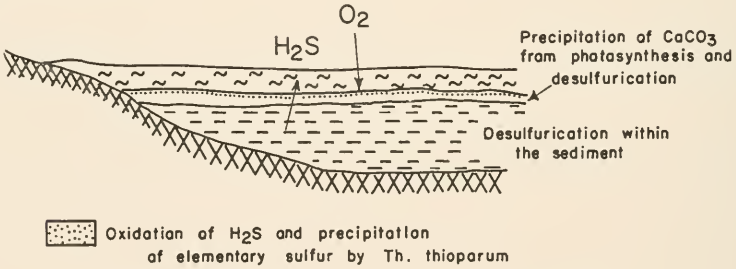

Fig. 1. Sulfur beds of syngenetic origin (scheme).

The sulfates are transformed to carbonates which are settled together with sulfur and other sedimentary materials. Desulfurizing bacteria are very common in saltwater lakes, especially in the surface layers of the mud (Table 2).

Experiments with sulfur isotopes have shown that in Solenoje

* Translated from German into English and presented by W. Schwartz.

TABLE 1

SULFUR, CARBONATES AND QUARTZ SAND IN THE SEDIMENTS OF BALKASCH SEA (PER CENT OF DRY WEIGHT)

	Elementary Sulfur	*SiO_2*	*CaO*	*MgO*	*CO_2*
Sandy mud	0.05	53.99	10.51	2.58	18.04
	0.25	38.17	18.62	3.85	16.02
Gray mud	0.25	25.71	27.70	3.56	23.40
	0.21	27.50	25.81	3.55	21.48
Light colored	0.12	20.96	29.42	4.98	26.23
mud	0.11	14.41	26.95	11.61	31.52

TABLE 2

VERTICAL DISTRIBUTION OF DESULFURIZING BACTERIA/ML IN THE SEDIMENTS OF SOME SALT LAKES.

Depths of Sediment (m)	*Umreschewo Lake (Eutrophic)*	*Mogilnoje Lake (Eutrophic)*	*Maibalyk Lake (Mesotrophic)*	*Repnoje Lake*	*Balkasch Lake Containing Epsomite ($MgSO_4 7 H_2O$)*
0	1,000	300	500	100,000	500
0.25					700
0.50	1,000	100	30		500
1.0		10	60	100	
2.0			100	100	
3.0				1,000	
4.8				100	
6.0				10	
7.8				1	

lake 19 mg H_2S/L are produced daily (4). Ivanov (3) has found high numbers of *Thiobacterium*[1] and of purple-sulfur microbes in the sulfur containing sediments of Sernoje lake. About 30 per cent of the sulfides in the lake water are oxidized to sulfur by *Thiobact. thioparum*. Purple-sulfur-microbes oxidize sulfur to sulfate. In the littoral or Belvod lake, only the sediment contains H_2S in quantities of 150-500 mg/L and rH_2 becomes low only at

[1] *Thiobacterium Lehm.* et *Neum.*, nov comb. of Krassilnikow (7) corresponds to *Thiobacillus Beij.* of Bergey's Manual.

the interface between water and mud. *Thiobact. thioparum* has only been found in the zone where sulfur may be precipitated (6).

EPIGENETIC ORIGIN (Fig. 2)

Shor-su (Usbekian SSR) is an example of an epigenetic sulfur bed, the H_2S of which originates from an oilfield in the neighborhood (12). The microbiological conditions have been studied by Ivanov (3). Desulfurizers live in the saltwater of the oilfield; they produce 0.2 mg/L H_2S daily. The rH_2-value of the oilwater is about eight to ten. Within the open anticline, groundwater and H_2S-containing oilwater are transported by gas-pressure of the oilfield. Near the surface, the ascending water has contact with oxygen-containing surfacewater. Here, at a rH_2 of twelve to sixteen, *Thiobact. thioparum* lives in great numbers. About 50 per cent of H_2S and sulfides, dissolved in water, are oxidized to sulfur, and the part of oxidation by *Thiobact. thioparum* is about 20 per cent; the rest is chemical oxidation. During diagenesis, colloidal and fine-crystallized sulfur are transformed to coarse-crystallized sulfur:

Formation of Ca sulfide During Desulfurication	$CaSO_4 \cdot 2H_2O \rightarrow CaS$
Solution of Colloidal and Fine-crystallized Sulfur	$2\ CaS + 2H_2O \rightarrow Ca(OH)_2 + Ca(HS)_2$
	$Ca(HS)_2 + 4\ S \rightarrow CaS_5 + H_2S$
Accumulation of Coarse-crystallized Sulfur and of Calcium Carbonate	$CaS_5 + CO_2 + H_2O \rightarrow CaCO_3 + 4\ S + H_2S$

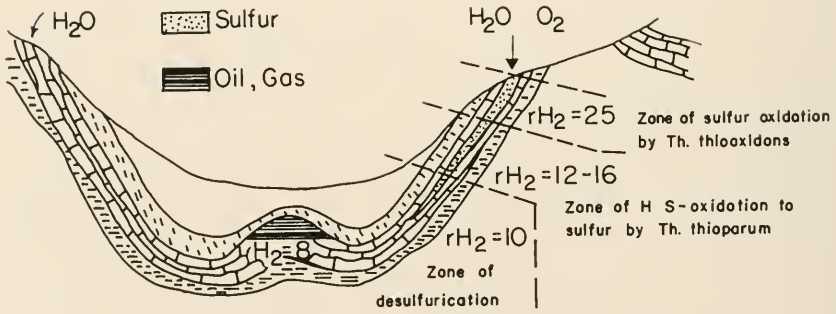

Fig. 2. Epigenetic sulfur beds of Shor-su (scheme).

Weathering in sulfur beds and mines of sulfidic ores begins at the outcrop. The sulfur beds of Gaurdagh, District of Chardzhuj, Turkmenian SSR (Fig. 3) are situated in a territory of arid climatic conditions with about 120 mm of precipitation yearly during the summer months. Weathering and oxidation happen mostly during this time of the year. The pH value of the water is about one. The limestone shows heavy weathering. Even from great distances, the light color of the weathered limestone is remarkable (Fig. 1). The microbiological weathering is caused by *Thiobact. thiooxidans* present at numbers of 10^3 to 10^5 cells/g limestone. The uppermost layers of the sulfur bed of Shor-su point out the same weathering.

Microbiological weathering is stopped by dryness, by absence of surface-water, by high levels of groundwater which is poor of oxygen, or by H_2S. Karawaiko (5) also found, in the sulfur beds of Rosdol Ukranian SSR, that surface contact, weathering and occurrence of *Thiobact. thiooxidans* coincide (Table 3).

American scientists (1, 8, 11) have first found the relations between the weathering (oxidation) of Pyrite, Marcasite and other sulfidic ores and the activity of *Thiobact. ferrooxidans* and

TABLE 3

Distribution of *Thiobact. thiooxidans* in Fresh and Weathered Sulfur Containing Limestone (pH of Water About 1).

Sample	*pH of Sample*	*Number of Thiobacterium (uer Gm. Limestone)*
(1) weathered sulfur containing limestone	7.64	10^2
(2) sulfur containing limestone with Alunite	6.90	10^3
(3) loamy sediment below the zone of (1)	5.81	10^4
(4) fresh sulfur contraining limestone	7.50	0

Thiobact. thiooxidans. In the USSR Ljalikowa (9) has found that *Thiobact. ferrooxidans* has its redox-optimum between rH_2 22 and 30. This species is spread all over the earth in coal seams with iron sulfides and in mines of sulfidic ores. The oxidation is a combined biochemical and chemical process with the forma-

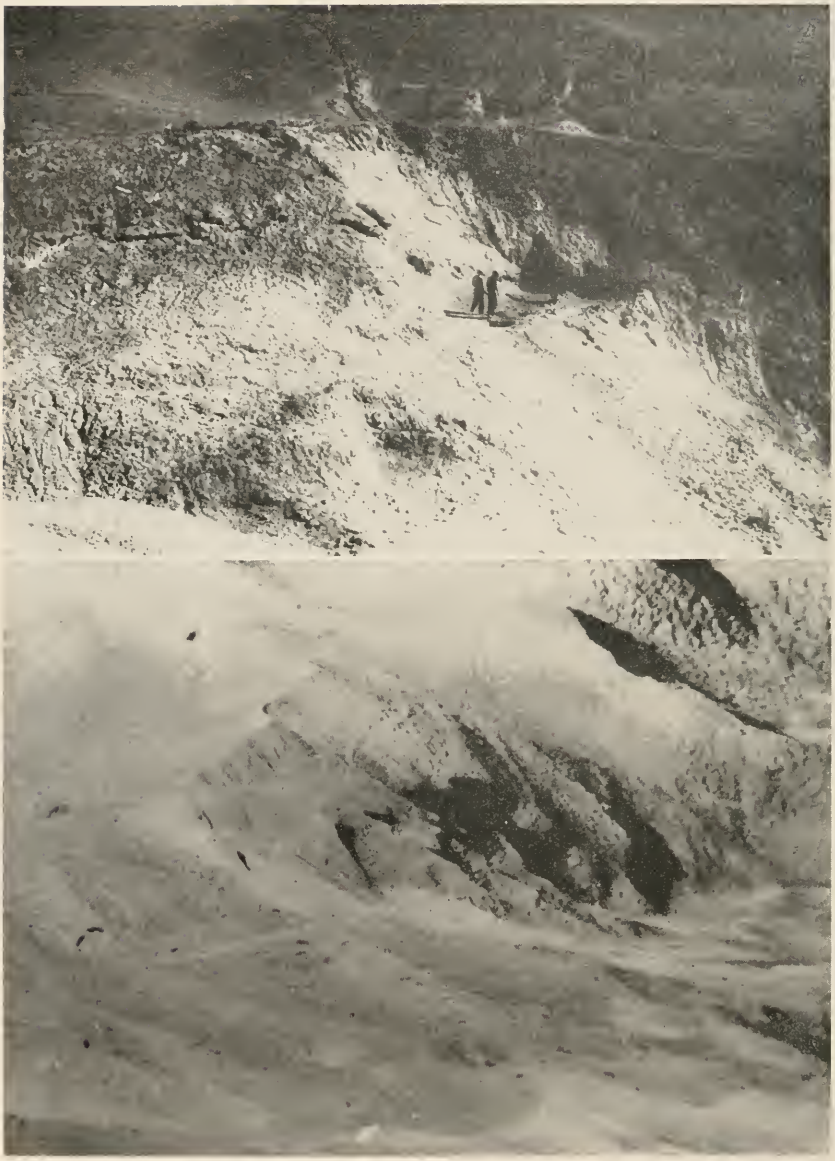

Fig. 3. Sulfur beds of Gaurdagh, weathering of limestone.

tion of H_2SO_4 and a great number of sulfate minerals which are found in the weathering zone of mines (12). Researches have been made in the mines of Akhtala near Alaverdi, Armenian SSR, (10) and in some mines of hydrothermal origin in the Ural district of Dektarskoje and Kzasnogvardejskoje. The dropping water of the galleries has a pH between 1.7 and 4.1 with about 10^5 *ferrooxidans* cells/ml. The water which is pumped up from the mine has still a higher bacterial content. On the average, the water contains 1.025 g Cu/L as $CuSO_4$ and 1.026 g/L H_2SO_4. Fresh ore which has not yet contact with oxygen and water is free of *Thiobacterium*.

SUMMARY

The genesis of sedimentary sulfur beds and the weathering of elementary sulfur and of sulfidic ores is by combined biochemical and chemical processes. Microbes of the sulfur cycle, especially desulfurizers and species of the genus *Thiobacterium* (*thiobacillus*) are active also under recent conditions in the syngenetic and epigenetic genesis of sulfur beds and in weathering processes of sulfur beds and sulfidic ores of various origin. Examples from USSR are discussed.

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Chapter 18

Behaviour of a Suspension of Microbes, Migrating Through Sediments Under Marine and Limnic Conditions

M. Wagner and W. Schwartz

It is of interest to learn how microbial cells will behave in groundwater moving in sediments and sedimentary rocks, or in surface water moving in a vertical direction from the surface of the earth into the underground. Different events may happen: Active or passive migration of the cells with/without multiplication may occur within the streaming water and the substream sediment; cells may grow through the sedimentary material, form surface films, ("Aufwuchs") attach to the surface of particles of the sediment, or be retained, adsorbed, or eluted within the sediment.

The microbes used in our experiments were:

Micrococcus sp., strain B/lc (0.6 x 1.0 μ),[1] isolated from drilling-mud of an oil-well.

Nocardia sp., strain beach sand I (1.0 x 2.4 μ, threads up to 12.0 μ, coccoidal cells 0.8 μ diameter), isolated from beach sand.

Serratia marcescens, (1.0 x 1.5-2.2, average 1.8 μ), culture collection.

Strain H/8b, (1.0 x 1.5-2, 2 μ), rods forming threads, no flagella and spores, isolated in 1949 from oil of an oil-well.

Bacillus mycoides, (1.5 x 4-5, average 4.6 μ) culture collection.

We have tested the vertical migration of the cells in model experiments with suspensions of bacteria in seawater (3.5%) and in fresh water through an 80 cm-column (Fig. 1) of two types of sandy sediments (Table 1).

[1] (In brackets, size of the cells from broth agar cultures, 3 days old.)

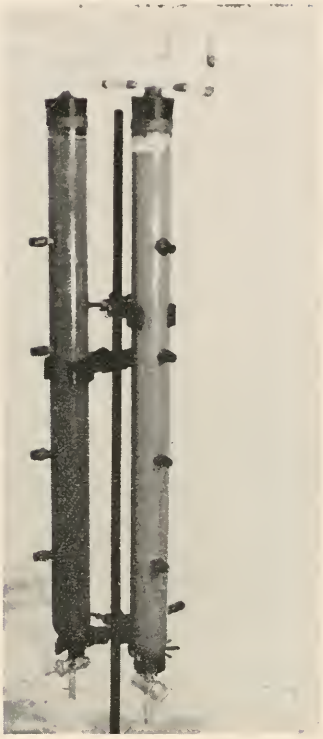

Fig. 1. Glass-columns filled with sand for model experiments. The inside of the glass tubes were covered with a thin water-repelling film of paraffin before filling with sand. Lateral openings allow samples to be taken from different levels. Both ends of the tube are closed and airproof.

During 48 h, a suspension of 10^5-10^6 cells /ml of one species in seawater and fresh water respectively passed through the columns at a speed of 2 ml/min. Thereafter, the columns were washed with sterile water (seawater and fresh water respectively) at 1 ml/min during eight days. The number of living cells in the dropping water was determined by plate culture on nutrient agar, at intervals, smaller at the beginning of the experiment and greater later on. The first cells appeared within the first minutes. Transportation through the sediment was influenced by shape and size of the cells, by the type of the sediment and by marine/limnic conditions.

The influence of physical factors,—size of grains of the sedi-

TABLE 1

Characteristics of the Sediments

	Sea Sand from the Beach of the Baltic Sea	*Tertiary Quartz Sand from Hohenbocken*
Mineralogical composition	Alluvial sand with 96% quartz, 2% feldspar, 1% hornblende, 0.5% garnet, 0.2% mica	Chiefly quartz
Particle size (sieve-set DIN 1171)	1.0-0.2 mm fine sand; rounded grains, 90% between 0.3 & 0.6 mm.	Edged grains 0.2-0.1 mm. ("Schluff")
Pore volume	36 Vol. %	41 Vol. %
Adhering water (water capacity)	11.7 Vol. %	22.3 Vol. %
Permeability for water	23.3 Vol. %	18.7 Vol. %
Water-content	0.4 Vol. %	0.4 Vol. %

ment, size of cells,—was in general discernible (Table 2) but not sufficient to understand all the phenomena. Besides, there was the influence of the medium (seawater, fresh water), and there were specific unknown qualities of single species of microbes, e.g., in the behaviour of *Serratia marcescens*, which need further investigation.

Micrococcus B/lc had the highest degree of transmigration with 91 and 97 per cent cells/ml of the suspension recovered after eight days under limnic conditions and 65 and 96 per cent under marine conditions (Table 2); during the second period of the experiment, (washing out with sterile water) the number of cells/ml of dropping water were in general the highest. Also with *Nocardia*, the transmigration was high even though the rods were big. The coccoidal cells migrated the fastest. The big rods of *B. mycoides* were restrained nearly completely and only a small number of cells appeared in the dropping water after thirty minutes in fresh water and seasand.

Transmigration was lower in seawater than in fresh water. This may be due to an influence of the salt content of the water upon the sediment and upon the surface conditions of the bacterial cells. The difference between fresh water and seawater

TABLE 2

HIGHEST DEGREE OF TRANSMIGRATION AFTER 8 DAYS OF SOME SPECIES OF MICROBES THROUGH A COLUMN OF SAND (NUMBER OF LIVING CELLS/ML. AS % OF CELL CONTENT/ML. OF THE MIGRATING SUSPENSION)

	Marine Conditions		*Limnic Conditions*	
	Sea Sand (1.0-0.2 mm)	*Quartz Sand (0.2-0.1 mm)*	*Sea Sand (1.0-0.2 mm)*	*Quartz Sand (0.2-0.1 mm)*
(1) *Micrococcus* B/lc (0.6 x 1.0 μ)	96	65	97	91
(2) *Nocardia spec.* (1.0 x 2.4 μ) (0.8 μ)	71	36	87	62
(3) *S. marcescens* (1.0 x 1.8 μ)	0.7	6.8	82	72
(4) Strain H/8b (1.0 x 1.9 μ)	17	4.6	53	5.3
(5) *B. mycoides* (1.5 x 4.6 μ)	0	0	3.5	0

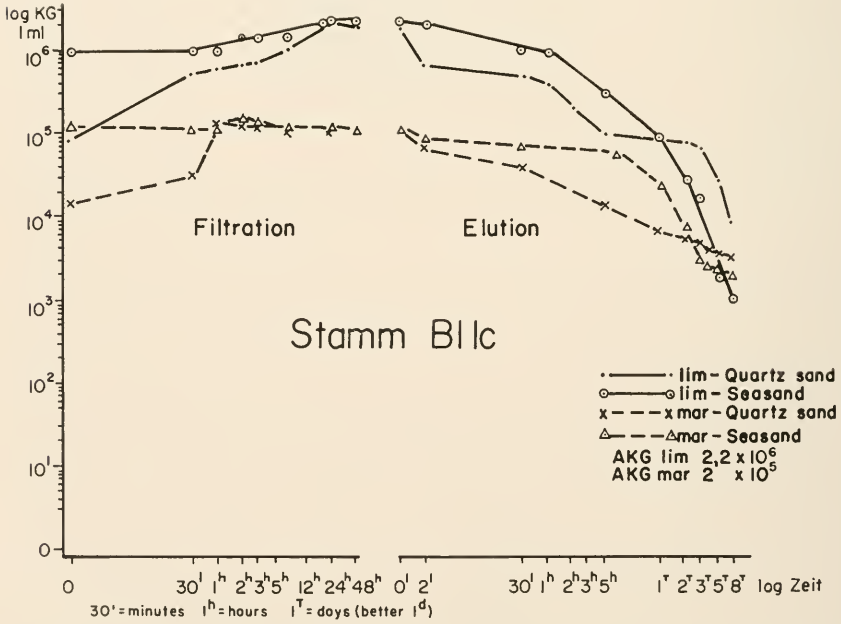

Fig. 2. Micrococcus. B/lc. Behaviour during filtration and elution under marine and limnic conditions. Abscisse: log time. Ordinate: log living cells/ml water.

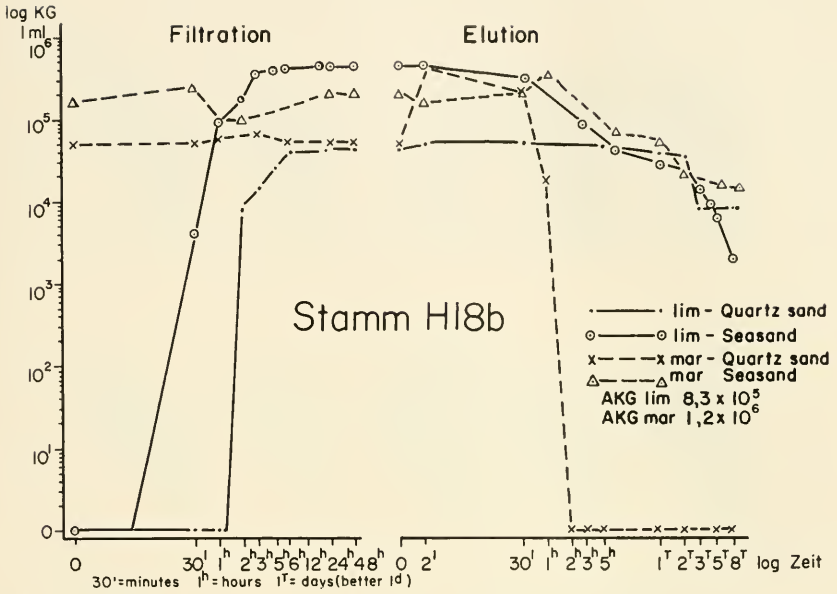

Fig. 3. Strain H/8b. Behaviour during filtration and elution under marine and limnic conditions. Abscisse: log time. Ordinate: log living cells/ml water.

(limnic/marine conditions) was low with the small cells of *Micrococcus* B/lc. It was high with *Serratia* for both sediments, with *Nocardia* for quartzsand and with strain H/8b for seasand.

Only *Serratia* had, under marine conditions, a higher transmigration-per cent in quartzsand. A comparatively high number of cells was retained in seasand, though this sediment was more coarse.

The cells of *Serratia* and of strain H/8b have nearly the same size but the transmigration-values are quite different in seasand/marine and quartzsand/limnic. The behaviour of *Micrococcus* B/lc and of the rods of strain H/8b may be typical for small cocci and for rods of medium size (Figs. 2, 3).

Within the sand-column, most of the cells of *Serratia*, strain H/8b and *B. mycoides* were retained in the upper 15 cm, especially under marine conditions where the transmigration was lower. The small cells of *Micrococcus* and the cells of *Nocardia* were distributed nearly through the column by 48 hours. During the second period of the experiment the number of cells was lowered,

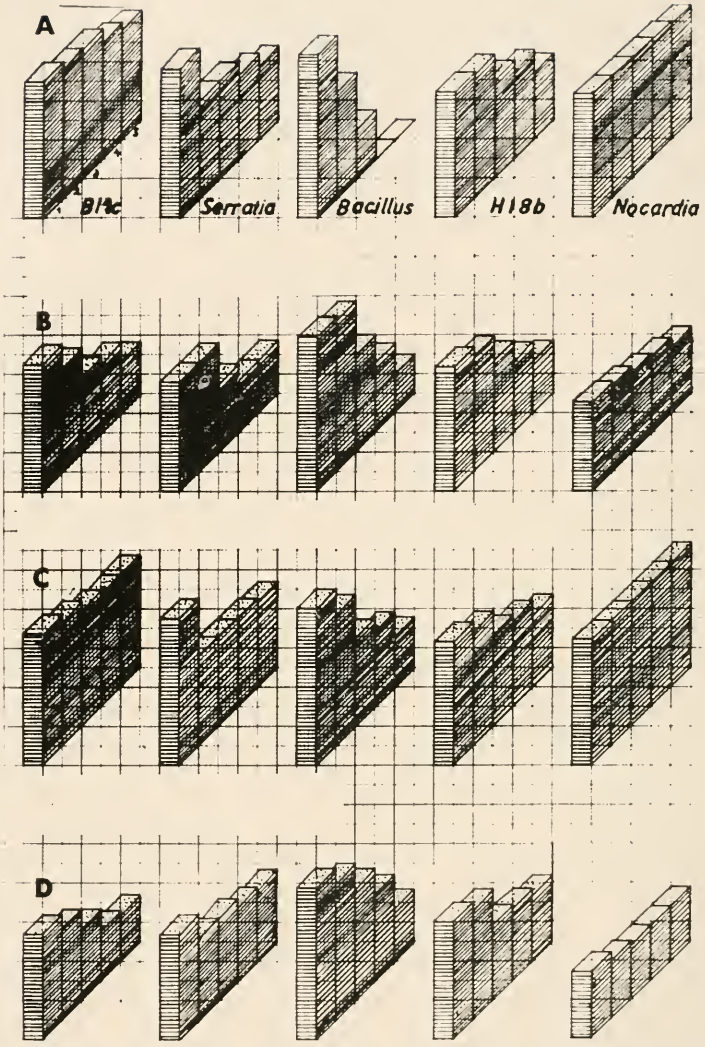

Fig. 4. Distribution of the cells within the sand column after 48 hours transmigration-time and eight days elution-time. Every 5 mm of height corresponds to cell content of $10^1/cm^3$ sand. 1 = highest, 5 = lowest sector of the columns.

Quartzsand (a) after 48 hours transmigration-time
(b) after 8 days elution-time
Seasand (c) after 48 hours transmigration-time
(d) after 8 days elution-time

but, in general, the distribution within the column was not changed (Fig. 4).

We have tried to make a balance between (a) the total number of the cells in 5,760 ml of suspension which passed through the column; (b) the total number of cells which transmigrated; (c) the total number of cells washed out from the column during the second period of the experiments, and (d) the total number of cells retained within the column at the

TABLE 3

TOTAL BALANCE OF NUMBER OF CELLS IN 5,760 ML OF SUSPENSION WHICH PASSED THROUGH EACH COLUMN (A) TOTAL NUMBER OF CELLS IN THE SUSPENSION; (B) CELLS FILTERED THROUGH THE COLUMNS; (C) CELLS WASHED OUT FROM THE COLUMNS; (D) CELLS RETAINED WITHIN THE COLUMNS.

(B), (C) AND (D) IN PER CENT OF TOTAL NUMBER IN THE SUSPENSION

	Sea Sand/Marine			*Quartz Sand/Marine*		
	(b)	(c)	(d)	(b)	(c)	(d)
Micrococcus B/1c (a) = 1.15×10^9	69.1%	8.3%	22%	61.2%	4.4%	35%
Nocardia (a) = 5.1×10^9	33.0	4.0	62	32.0	1.5	66
Serratia (a) = 2.6×10^{10}	0.5	0.01	98	3.4	1.7	96
Strain H/8b (a) = 6.0×10^9	13.4	3.4	87	3.3	0.1	96
B. mycoides (a) = 1.8×10^8	0	1.3	98	0	0	100
	Sea Sand/Limnic			*Quartz Sand/Limnic*		
	(b)	(c)	(d)	(b)	(c)	(d)
Micrococcus B/1c (a) = 1.3×10^{10}	93.5%	3.0%	5%	83.5%	4.8%	16%
Nocardia (a) = 2.3×10^9	70.1	0.1	27	48.0	0.2	61
Serratia (a) = 6.9×10^9	85.5	5.4	11	67.1	4.3	29
Strain H/8b (a) = 4.8×10^9	48.2	2.9	50	4.4	4.2	92
B. mycoides (a) = 9.8×10^8	0.1	0.05	100	0	0	100

end of the experiment[2] (Table 3). During the term of the experiment the total number of cells remained nearly constant. After eight days of washing out with sterile water, the columns contained still a remarkable number of cells. The relation to the size of the cells and of the grains in the sediments is recognizable here also. *Serratia* differs as to the low number of cells which are retained under limnic conditions, compared with the high numbers of strain H/8b.

SUMMARY

Model experiments with five species of bacteria and two types of sandy sediments have shown that transportation of cells through the sediment, washing out, and distribution in different layers of the sedimentary column were in general influenced by the size of the cells and the size of the grains of the sediment. Under marine conditions (suspensions of cells in seawater) a higher number of cells were retained than under limnic conditions. Besides the physical laws which regulate retention and elution, specific factors bound to single species of bacteria were effective. After eight days of washing out, the sediments still contained changing numbers of living bacteria.

[2] (b), (c) and (d) in per cent of (a). Calculation of (b) and (c) from cell contents and quantities of dropping water. The estimation of (d) is only approximate from the cell contents of five samples of 300 cm^3 of sand apiece (total volume of sand in a column 1,500 cm^3).

Chapter 19

Redox-Conditions and Precipitation of Iron and Copper in Sulphureta

R. Suckow and W. Schwartz

The development of a sulphuretum in nature is connected with changes of redox-conditions in the mud and the supernatant water. The term "Sulphuretum" was introduced by Bass-Becking (1). First of all, the ecological conditions are characterized by the presence of H_2S, originating from desulfurication, putrefaction, or vulcanism. The biotope is modified by temperature, pH, salinity, light, nutritional materials, etc. Sulphureta occur in deep water or low water, or even within the tidal range; at different temperatures, even under thermophilic conditions; at pH up to 9-10 or more; in fresh water, brackish water, seawater and concentrated brines. In general, conditions are anaerobic but without sharp limits and there are in a sulphuretum also habitats with a higher partial pressure of oxygen or temporary variations from anaerobic to more or less aerobic conditions.

The primary type of microbes of the biocenosis are sulfur-microbes with a relatively high number of H_2S tolerant bacteria, Cyanophyceae, Protococcales, diatoms, flagellates, rhizopods, and ciliates. The interest of microbiologists has been concentrated upon the microbes of the sulfur cycle so that our knowledge about the accompanying microbes is low; their metabolism and life conditions have not yet been thoroughly explored.

We have studied the changes of Eh, pH, rH_2 and H_2S-pressure in sulphureta which developed under marine and brackish (nearly limnic) conditions in aquaria with mud/water layers of about 75 cm height. We used a H_2S producing blackish mud its natural biocenosis of microbes and small metazoa. To the from the Baltic Sea with high content of organic matter and with

mud, we added 2% $CaSO_4 \cdot 2\ H_2O$ + 0.05% $Na_2SO_4 \cdot 10\ H_2O$ + 0.05% $MgSO_4 \cdot 7\ H_2O$. The brackish water had a salinity of 4‰; the sea water 3.5 per cent. Source of H_2S was desulfurication by desulfurizers living within the mud.

Eh and pH measurements were made *in situ* during more than one year. For pH we used the Argosonde of Dr. Ingold GmbH, Frankfurt/Main, with glass electrode, and the pocket pH meter type 54/RT of Wissenschaftl.-Technische Werkstaetten in Weilheim/Oberbayern. For Eh, we have constructed a Pt-electrode in a glass tube which could be stabbed into the sediment or lowered into the water at different levels. The conducting connection from the calomel electrode to the substrate was made by a glass tube drawn out into a capillary and filled with KCl agar. The pH meter was type MV 11 of Clamann and Granert, Dresden. For every result, only measurements which could be reproduced were evaluated.

The development of the typical biocenosis was watched through microscopic changes in the water and the sediment, by microscopic observations and by isolation of microbes in enrichment and pure culture.

About four months after the beginning of the experiments, at room temperature of about 18-20 C, the development of color in the sulfureta became visible because of red and green-sulphur microbes, Cyanophyceae, and sulfur-precipitation which were the same under marine and limnic conditions. In some of our aquaria, a pronounced horizonal stratification was present in the water above the sapropelic sediment, in others it was lacking. The specific conditions are not yet known, but we believe that small differences of temperature and thermic streamings within the waterbody may be of importance. In the first case, a bacterial plate, 4-10 mm thick, was formed some cm above the mud-surface (Fig. 1), composed mainly of Chloromicrobes, sometimes also of purple-sulfur microbes. Within the plate, Eh shifted up from low values below to about + 500 mV (Fig. 2). In the mud, rH_2 values were 6.1-6.3, within the plate 10-22 and about 10 cm above 25-32 with only small differences between limnic and marine conditions (Table 1).

[1] Brackish water from a bay of the Baltic Sea near Greifswald.

Fig. 1. Bacterial plate (about ½ natural size). 1) surface of the sediment. 2) purple sulfurmicrobes from an earlier niveau of the plate, now adhering to the wall of the aquarium. 3) bacterial plate, floating in the water.

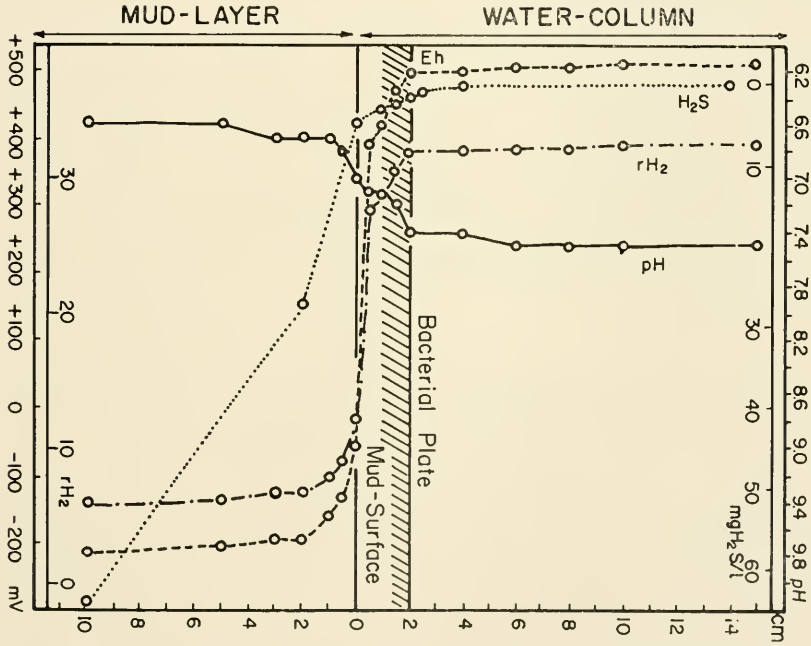

Fig. 2. Redox-conditions and bacterial plate (Aquarium no. 1/marine).

TABLE 1

pH- and rH_2- Values in Relation to the Bacterial Plate

General Conditions	*10 cm Above the Plate*		*Within the Plate*		*10 cm Below the Surface of the Mud*	
	pH	rH_2	*pH*	rH_2	*pH*	rH_2
Limnic	6.7-7.6	25-32	6.6-7.2	10-22	6.3-6.8	6.1-6.3
Marine	7.5	25-32	7.2	21	6.6	5.2

The contents of H_2S above the plate were $\pm$ 0 and in water below the plate 3-27 mg/L. The highest concentrations, up to 80 mg/L, have been found within the sediment. In aquaria without a bacterial plate, we have observed only a small shift of the values near the mud-surface, and the waterbody remained under reducing conditions (compare Fig. 3, Eh, pH and rH_2 before adding iron and copper salts). In other aquaria the shifting at the surface of the sediment was higher and the conditions within the water were aerobic.

Sulphureta may have a geomicrobiological importance at least for two geological processes, (a) sulfur deposition and genesis of sedimentary sulfur beds, (b) precipitation of iron, copper and other heavy metals as sulfides and genesis of sedimentary sulfidic ores. An example for (b) are the copper shales of Mansfeld westward of Halle and southward of the Harz mountains, Germany. Pyrite, Bornite and other minerals are embedded in dark bituminous shales of the Zechstein formation. The sediments have been settled in a shallow basin of salt water, the Zechstein Sea.

In relation to this process, we have studied the influence of iron and copper salts upon the biotope and the biocenosis, both under marine and limnic conditions. We have added (over four days) to the surface of the water, drop by drop, 1 per cent solutions, of 3gm $CuSO_4 \cdot 5\ H_2O$ and 4gm $FeCl_3 \cdot 6\ H_2O$ to each volume of 20 L water (a) undisturbed bacterial plate, and (b) with slowly stirring so that the waterbody has been mixed and the bacterial plate disappeared. The store of H_2S in the water was consumed, and important modifications took place within a short time.

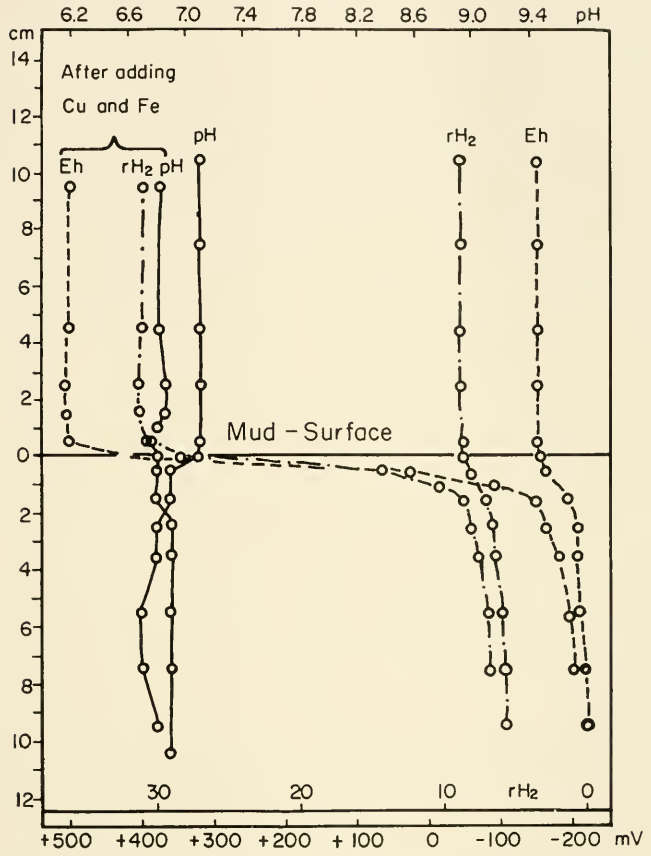

Fig. 3. Redox-conditions before/after adding copper and iron salts to the water (Aquarium no. 2/marine).

Marine Conditions *(With Stirring)*

The pH value dropped from 7.1 to 6.8, Eh rose from −160 to + 500 mV. The heavy metals have been precipitated within three days at the surface of the mud in a dark-brown, loose colloidal mud-water zone. After 12 days, normalization began and the water again contained free H_2S. The border of the upper oxydative and the lower reductive zone was about 1 cm above the surface of the sediment, with a Eh shift of about 600 mV. About 31 days after the beginning of the experiment the sulfuretum had been restored. The shifting of the potential had

disappeared and the conditions of the water were again anaerobic.

Limnic Conditions ***(With/without Stirring)***

The ecological conditions were changed in the same manner; pH was lowered and the zone of Eh shift was situated deeper than before; with stirring, it was displaced about 5 mm below the mud-surface.

With stirring, the heavy metals settled very slowly as a greenish-brown colloidal layer of basic copper carbonate and iron hydroxide, mixed with sulfides. After 25 days all the precipitated iron and copper-compounds had been reduced to a thin, more solid but still water-containing layer of sulfides, and the sulphuretum was restored.

Without stirring, the heavy metals were precipitated at first as a mixture of carbonates and hydroxides distributed in the water above the bacterial plate. When the colloidal particles sank down into the range of the bacterial plate, they were reduced to sulfides and the bacterial plate settled nearly to the surface of the sediment.

In all the experiments with heavy metals, the amount of H_2S dissolved in water and the rate of release of H_2S from the sediment was not high enough to reduce at once the heavy metals in solution to below toxic concentrations. Most of the microbes living in the water died during the first days, but but the biocenosis of the sediment had not been altered, and from the microbial reservoir of the sediment, the restitution of the sulphureta took place.

SUMMARY

The development of sulphureta has been studied under marine and brackish (nearly limnic) conditions during two years in model experiments. The wholly developed sulphureta are characterized by a shifting of pH from weakly acid to weakly alkaline values, and of Eh and rH_2 from reductive to oxidative conditions in the direction from the sediment to the water level. The shifting of rH_2 was influenced by bacterial plates and by stirring of the water. Addition of solutions of copper and iron-

salts, in concentrations that were not at once precipitated by H_2S, killed most of the microbes living in the water and changed the ecological conditions; but the biocenosis of the mud remained unaltered. Within a month, the heavy metals were transformed from carbonates or hydroxides to sulfides or directly precipitated as sulfides, and the sulphureta were restored by the microbial reservoir of the sediment. The experiments may be of significance for the reproduction of geomicrobiological processes which took place during the genesis of beds of sulfidic ores, e.g., of the copper shales of the Zechstein formation in Germany.

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Chapter 20

The Distribution of the H_2S in the Lake of Faro (Messina) with Particular Regard to the Presence of "Red Water"

Sebastiano Genovese

In the course of my previous observations (1) on the physical and chemical conditions of the brackish lakes of Ganzirri and Faro (Messina) performed in 1950 and 1951, I had noted in the latter environment, the presence of increasing amounts of hydrogen sulphide from 12 meters towards the bottom.

Later on I had isolated a strain of *Desulfovibrio desulfuricans* from the mud taken from the bottom of that lake and studied, in collaboration with Pichinoty and Senez (7), its biochemical characteristics, by Warburg's manometric method. At the same time, counts of sulphate-reducing bacteria on mud samples and waters collected at different depths were carried out.

In the course of these previous investigations, by means of laboratory cultures, I could also ascertain the presence of purple and green sulphur bacteria in the lake of Faro (6).

The morphological characteristics of above environment include a constant presence of a more or less compact layer of hydrogen sulphide from a certain depth down to the bottom; thus it seemed advisable to follow, during a full year, the changes in the quantitative distribution and stratification of the above gas, in relation to the changes in the quantity of dissolved oxygen in the superficial layer of the water.

MATERIALS AND METHODS

The brackish lake of Faro (Fig. 1), is situated in Sicily, North of Messina, near Capo Peloro. Its shape is almost circular with its main diameter towards NW-SE of 661 m; its surface is 263,600m^2. In the center, the lake has a depth of 28 m, a rather exceptional value for litoraneous brackish lakes. Such a depth

was developed at some time after the formation of the lake itself, by tectonic sinking, and therefore the bottom of the lake took on the form of a funnel.

The lake of Faro reaches the sea through two canals, one of which, named "canale degli Inglesi" was opened only a short time ago, during my investigations. It is also connected to the nearest lake, Ganzirri, through another narrow and low canal. We must also point out that the two canals connecting with the sea are generally closed for most of the year, reducing active circulation of the lake waters.

Because of these morphological characteristics, the lake of Faro represents an ideal environment for investigations on the distribution of hydrogen sulphide and on consequent microbiological problems.

My investigations were performed during a full year, from

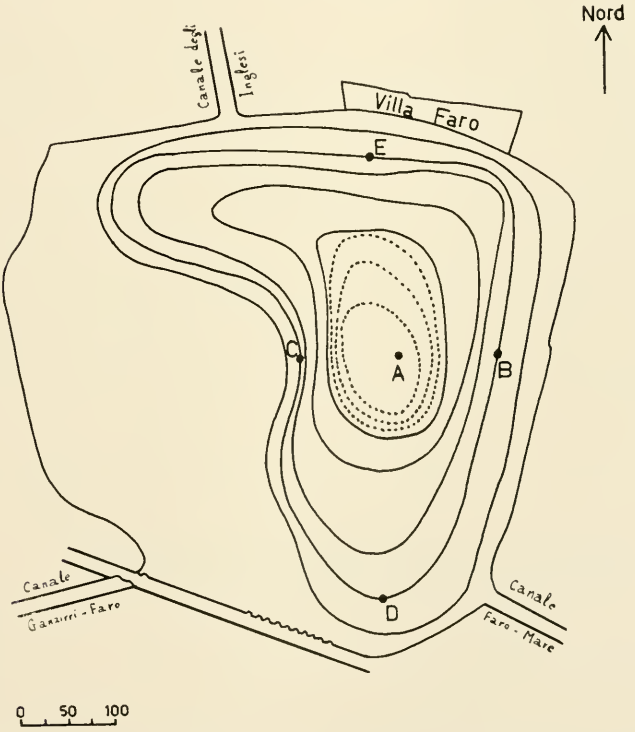

Fig. 1. Lake of Faro. Equidistance of level curves: solid lines 5 m, dotted lines 1 m (from 1).

February, 1960 to January, 1961. Every month samples of water from different depths, especially at the stations A and C were collected from the stations B, D, E (Fig. 1). Such samples were taken each 5 meters of depth. In the transition zone between the upper layer containing oxygen and the lower one containing hydrogen sulphide, samples were taken out at 1 meter or every half meter depth.

Measurements of pH, analyses of dissolved oxygen by the Winkler method, and of hydrogen sulphide by iodometric titration method, were carried out. Water temperatures were also recorded.

For counting sulphate-reducing bacteria the methods of Grossman and Postgate (8) and especially the method of Senez, Pichinoty and Geoffray (9) for the waters of Gasometers were used. I used the culture technique of 'shake' agar previously employed by Genovese, Pichinoty and Senez (7). The occurrence of the purple sulphur-oxidizing bacterial floral in the sediments and water was confirmed in laboratory by the Winogradsky culture-aquarium. The glass-stoppered cylinders containing the cultures were exposed to the sunlight at the temperature of the laboratory.

RESULTS

As already stated, I had previously noticed that the transition zone between the higher layer containing oxygen and the lower one containing hydrogen sulphide was situated at a depth of 12 m. In the course of the present investigations, the zone did not maintain a constant depth, but it had some very remarkable shifting.

During the early months of the investigation, from February up to July, the transition zone remained at a depth between 8 and 9 m (Fig. 2); in August it shifted up to level 1.5 m, and then during the remaining months of investigation, it shifted downwards again to deeper levels, varying between 11 and 14 m.

Notice that during the months from April to July, that is during the samplings made on 4 April, 4 May, 9 June and on 4 July 1960, the transition zone, at station A, stayed at a constant depth of 8.5 m. During this period the phenomenon of "red water" was observed in the zone lying immediately below. I had recently occasion to refer this phenomenon to the 17th Sym-

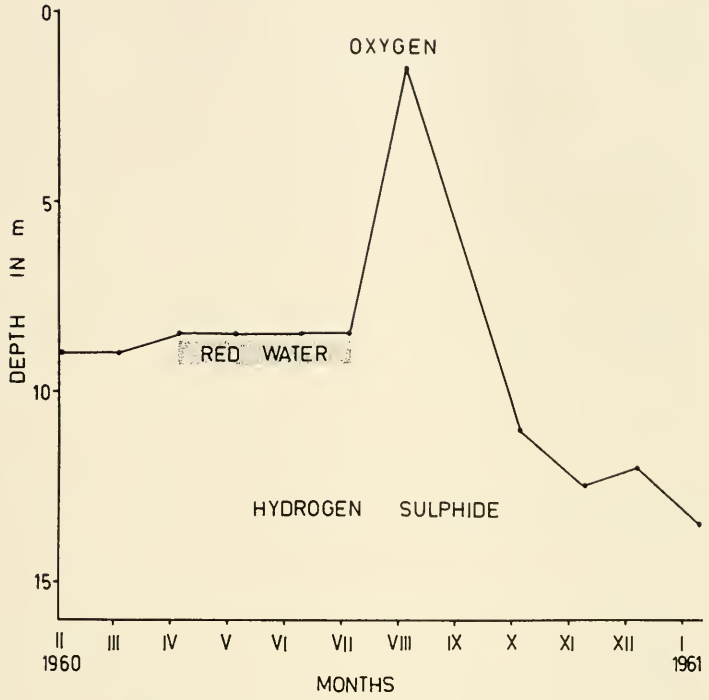

Fig. 2. Vertical variation of the transition zone between the layer containing oxygen and the lower one containing hydrogen sulphide.

posium of the "Commission Internationale pour l'exploration scientifique de la Mer Méditerranée" held in December 1960 in Monaco.

In the samples collected in the red tide zone, the water was more or less rose-colored, and was present only for about 1 m

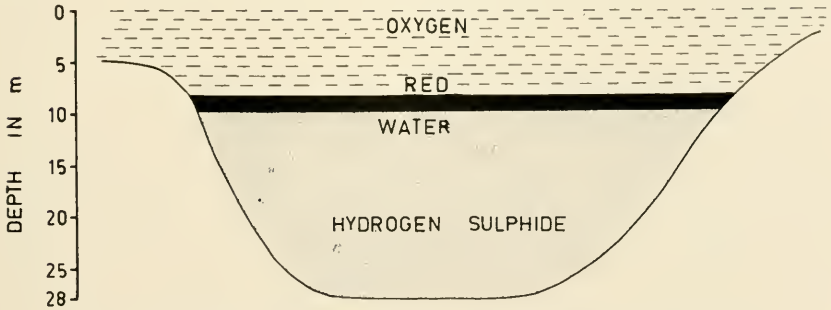

Fig. 3. Profile EW through the center of the lake of Fargo.

thickness, at the almost constant depth of 9 m (Fig. 3); only on 4 April 1960, at station C, it was encountered at 10 m.

With regard to the distribution of oxygen and hydrogen sulphide at the different levels (Fig. 4), we can state that until July the gases were present in quantities conforming with their normal stratification. At the surface the amounts of dissolved oxygen varied from a maximum of 6.54 ml/L, (5 March), to a minimum of 4.23 ml, (4 July). At 5 m, oxygen concentration was always slightly lower compared to that found on the surface; at 8 m instead, i.e., near the transition zone, oxygen oscillated from a maximum of 6.07 ml in March, to a minimum of 0.94 ml, in July.

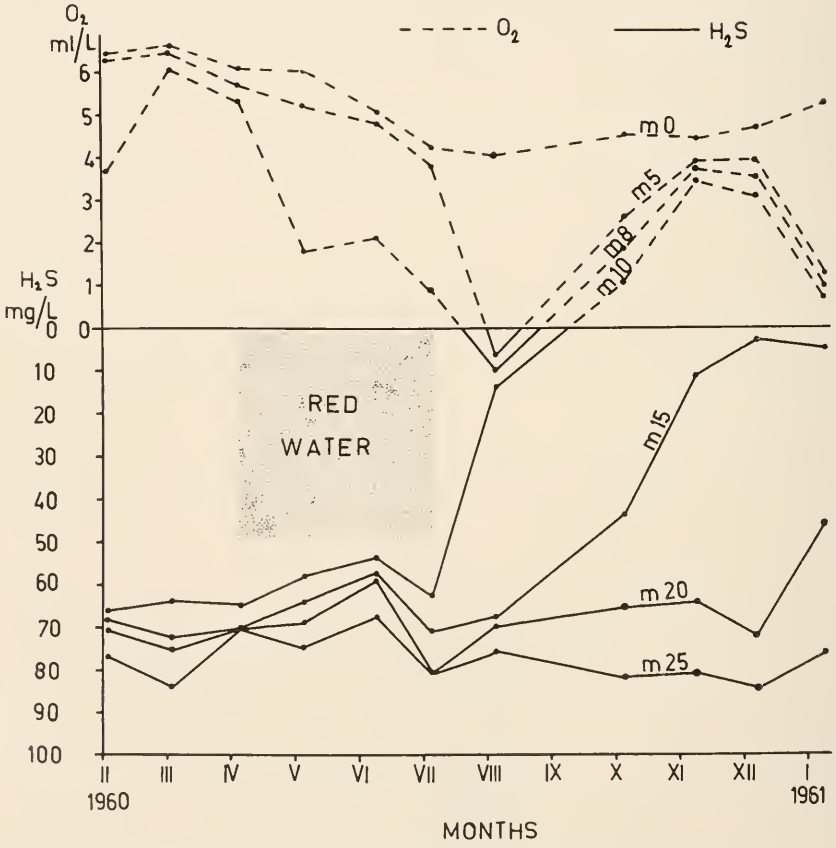

Fig. 4. Variations of oxygen and of hydrogen sulphide at station A.

During this time, the hydrogen sulphide was found in increasing quantities from the transition zone towards the bottom. At 10 m it had already reached remarkable values, between 54.74 and 66.15 mg/L. The maximum value found was 84.52 mg/L at 25 m in March.

It is to be pointed out that the quantity of H_2S contained in the "red water" was between a minimum of 0.78 mg/L, as found on 4 April at 9 m at station A, and a maximum of 49.50 mg/L, as found on the same day at 10 m at station C.

Quite exceptional values were found in samples collected on 4 August. Oxygen at the surface reached 4.08 ml/L, a value which, although low, however is to be considered as possible during that period. Oxygen disappeared almost completely at 2 m and was replaced by increasing quantities of H_2S. Up to 10 m, H_2S appeared in rather reduced limits (14.13 mg/L), while from 15 m to the bottom it showed a tendency to reach its normal values (68.26 mg/L).

In the following months, the distribution of the two gases had a tendency to progressively stabilize, according to their normal stratification. Indeed in November and December 1960, oxygen was found down to 10 m in quantities above 3 ml. Notice, however, that hydrogen sulphide at a depth of 15 m, which previously had rather high values and slightly less than the noted maximum, now had a tendency to gradually decrease (Fig. 4). On 6 December, the quantity of H_2S present was only 0.29 mg/L.

In the last month of the investigation, there took place again, to a less extent and thus with less distrophic effects, the conditions encountered in August. On 9 January 1961 at 5 m only 1.20 ml of O_2 was found, with slightly lower quantities at both 8 and 10 m.

Surface temperature (Fig. 5), was a maximum of 27 C in July, and a minimum of 10.9 C in January, and appeared to follow the changes in the atmospheric temperature. At 20 m, all through the year, there was a regimen of omotermia with temperatures between 15.5 and 16 C. At 10 m, during the early period of investigation, up to July, the temperature followed the changes in the temperature of the deep layers, while in the remaining months, it followed the changes of the surface temper-

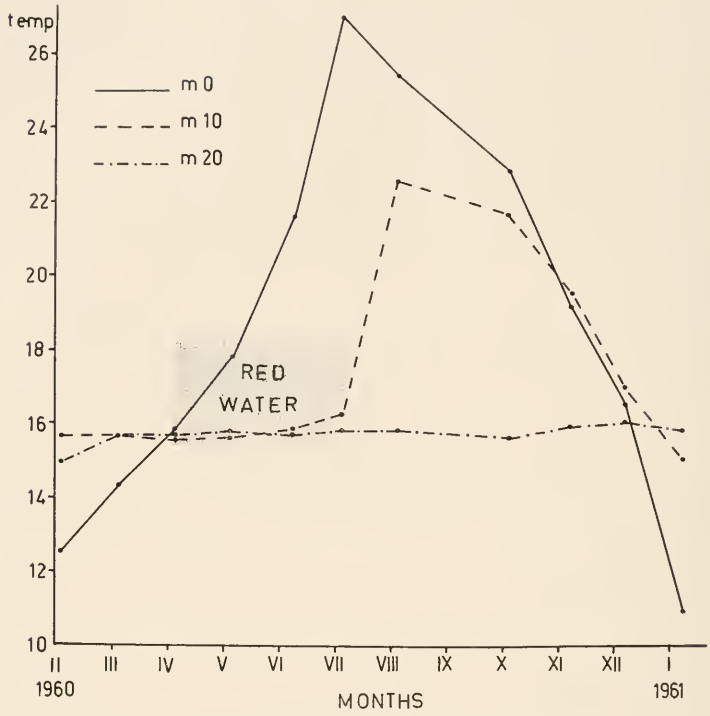

Fig. 5. Variations of temperature at station A.

ature. Particularly interesting is the omotermic regimen at a depth of 10 m, where occurred "red water," the temperature of which oscillated within rather narrow limits.

As it was to be expected, pH showed decreasing values from the surface to the bottom (Fig. 6). Notice the completely irregular behaviour of the O m curve compared to the 20 m one, showing limited changes, between 6.90 and 7.10. At a depth of 10 m pH behaviour was irregular which showed some connection with changes in the temperature and in the contents of H_2S observed at the same depth. The pH values of the "red water" are between 7.48, as seen on 4 April at 9 m at station A, and 7.00, noticed on the same day at 10 m at station C, and a minimum and maximum of H_2S respectively.

The preliminary results of counts of sulphate-reducing bacteria confirmed the ones previously obtained (7). In the water

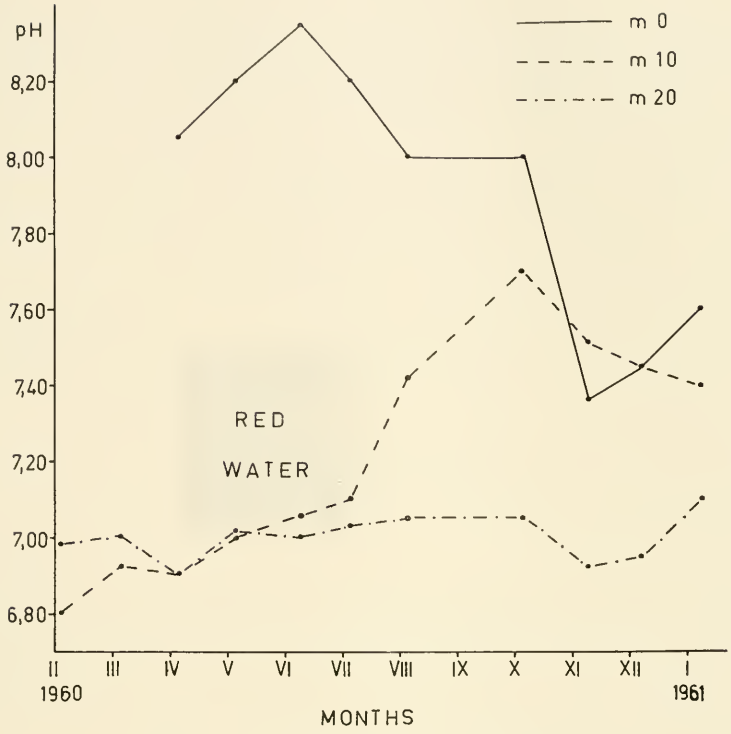

Fig. 6. Variations of pH at station A.

collected at a depth of 25 m and in the mud taken at bottom at station A, the bacteria counted were respectively in the order of 10^4 per liter of water and of 10^6 per kg of mud.

The bacteriological test of the Winogradsky cultures- aquarium showed the presence of *Thiopolycoccus ruber* colonies and, in a lesser quantity, of forms belonging to the genera *Cromatium* and *Thiocystis*.

DISCUSSION

The occurrence of "red water" had previously been observed by several authors in the Mediterranean brackish waters. Cerruti (3) reported that in August 1938 the water of Mar Piccolo, in Taranto, took on a deep dark-reddish colour, and he connected this phenomenon with contemporary presence of hydrogen sulphide at a depth of 12.5 m. In 1953 Cviic encountered such

water in the lake "Malo Jezero" (4) and, successively, in 1954, in the lake "Veliko Jezero" (5). Both brackish lakes, situated in the island of Mljet in Jugoslavia, present morphological and chemical-physical characteristics quite similar to those ascertained in the lake of Faro.

Such a peculiar sulphur-oxidizing bacterial flora, providing the above colour, is to be expected in environments showing the same ecological characteristics as the lake of Faro. However, it must be pointed out that, in order to describe the phenomenon, I carried out a careful and detailed exploration of whole column of water, meter by meter, while, for any other hydrobiological research, samples of more distant levels would satisfy. It may be supposed that such environments more frequently would show the occurrence of "red water," if examined in more detailed way, considering the limited thickness that such a layer of water sometimes has.

It must also be pointed out that in such environments the phenomenon is in relation to the existence of determined ideal conditions such as an adequate temperature and a normal stratification of the water, causing especially a rather constant quantity of H_2S at the layer immediately underneath the one where the "red water" is found.

Under such conditions, even pH would maintain within lower limits, although according to Van Niel (10) and Baas Becking and Ferguson Wood (2), the purple bacteria can develop within much higher limits of pH.

If such environmental stability which is the one conditioning the presence of the "red water" should fail, then as a consequence the fundamental basis for the occurrence of the phenomenon in nature would fail.

These conditions, in reality, occurred in the lake of Faro during the course of these investigations. The exceptional situation which arose in August, followed a first period of normal stratification of the water which lasted until July and during which time the phenomenon of "red water" was observed. In fact, in the sampling carried out on 4 August, the hydrogen sulphide, present at a short distance from the surface, produced a distrophic crisis, causing a remarkable damage to mussel farm-

ing. In this respect, we must point out that the new canal named "degli Inglesi" connecting the lake to the Tyrrhenian Sea had just been opened a few days before. It must therefore be supposed that such a crisis was due to the new supply of sea-water, which, entering at a certain rate, caused a mixing in the middle layer of the water. It is quite probable that such mixing and not an abnormal production of H_2S, caused the above crisis, considering that, in the deepest layers, the quantity of H_2S remained almost constant during all the period of investigations. In confirmation of this, notice that we had the same phenomenon, although on a minor scale, in January 1961, by opening of the canal, previously closed for a long time because of rough sea.

SUMMARY

The changes in the quantitative distribution and stratification of hydrogen sulphide, present in the brackish lake of Faro from a certain depth down to the bottom, were followed, during a full year. In the period between April and July 1960 the transition zone between the higher layer containing O_2 and the lower one containing H_2S remained at an almost constant depth of 9 m. In this period, in the zone lying immediately below, the "red water," about 1 m thickness, was constantly found. Data were obtained also on counts of sulphate-reducing bacteria and on bacteriological tests with the Winogradsky culture-aquarium.

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Chapter 21

The Distribution of Major Organic Nutrients in Marine Sediments

Gordon P. Lindblom

A part of our research program, designed to interpret the contribution of heterotrophic microbes to the organic geochemistry of marine sediments consists of three major divisions. These are 1) study of the distribution of organisms with depth in various types of recent sediments; 2) determination of the concentration and distribution of the major organic fractions in association with the microflora; and 3) study of the complex ecology of the groups of organisms found at various depths.

The latter study requires considerable attention to so-called "natural conditions" in devising laboratory experiments. Most microbiologists recognize that such attention has, to date, not been at all adequate in any attempt to artificially reconstruct and control a given natural environment. The methods of analysis of static or continuous mixed cultures, for instance, must be learned before such experiments can be effectively interpreted.

Basic to any such studies (especially those relating to nutrient limits) is the medium in which the reactions take place. Therefore, the analysis of the sediment for organic nutrients assumes particular importance.

Reports of general biochemical analysis (other than elemental analyses) of marine muds are few, and the samples are scattered and almost always are taken from the topmost sediments. Most of these data have been obtained by Russian workers (4, 5, 6, 7) (see Table 4). The field has been reviewed in detail by Vallentyne (8).

It seemed, therefore, expedient to determine the changes in the concentrations of the major organic fractions (proteins, carbohydrates, and lipids) with depth in various sediments, this

work to be followed by more detailed identification of molecular components of the extracts.

EXPERIMENTAL METHODS

The sediments were sampled immediately after collection (usually by piston-coring equipment) or were taken from cores which had been frozen. A few analyses were made on cores preserved by sealing (without air) in a plastic bag and stored in metal tubes.

Protein was determined by an adaptation of the method of Krey (2) for protein in marine plankton. The sediment samples were treated for twenty-four hours at room temperature with 8 per cent sodium hydroxide, filtered and diluted. Color was developed in the filtrate with the Folin reagent and measured by a spectrophotometer.

Carbohydrates were extracted with hot 80 per cent ethanol (approximately 3 ml per gm of sample). After evaporation of solvent, the extract was taken up in water. Determination of reducing sugars was by the method of Folin and Malmros (1).

Lipids were extracted with ether from ground, air-dried samples in a standard Soxhlet apparatus.

"Bound" lipid was determined by a second ether extraction after treatment with 0.1N HCl, drying, and re-grinding.

RESULTS

Recently, we reported (3) some of our preliminary data from these studies. Analysis of sediments from the Orinoco Delta (Venezuela) showed that proteins and carbohydrates decrease rapidly with depth to about ten feet, from which point the decrease is much slower. Protein decreases by 22.8 μg per gm of dry sediment per foot as determined by the slope of a regression line through the points. The correlation coefficient (r) of -0.435 is significant at the 99.9 per cent level. The carbohydrate decrease is 0.648 μg/gm/ft ($r = -0.468$, $p = 0.001$).

Ether-extractable lipids, on the other hand, show a slight increase below ten feet, after the initial rapid decrease. The increase of 1.224 ppm/ft is independent of lithology and is significant at the 99 per cent level ($r = +0.434$). When the sedi-

ments were treated with acid and again extracted with ether, a second fraction (the so-called "bound lipid") was obtained. This fraction was greater in amount than the "free lipid," and showed a slightly greater increase with depth.

Certain other environments have been studied in less detail. Cores from the Gulf of California have had the greatest amount of organic matter of any sediments we have yet examined. The samples have been from short cores, and therefore no deep trends can be seen. However, the per cent of lipid in "bound" form increases strikingly with depth (Table 1).

TABLE 1

BIOCHEMICAL ANALYSES—GULF OF CALIFORNIA SAMPLES

Core	*Depth (ft)*	*Protein (mg/g)*	*Sugars (μg/g)*	*Lipids (ppm)* *Free*	*Bound*	*Total*	*% Bound*
1	1	10.17	464	1101	204	1305	15.6
(24°25′N,	2	6.39	311	322	206	528	39.0
108°37.9′W)							
2	1	17.02	554	1306	340	1646	20.7
(26°54.5′N,	3	11.72	264	1298	467	1765	26.5
110°57′W)	4	13.63	345	1016	527	1543	34.2

All figures are on a dry-weight basis.

Sediments from nineteen cores from the Cariaco Trench are currently being analyzed. Table 2 shows some results to date on four of these cores. The correlations between these samples are quite complex, so no definite interpretation of trends has yet been made. Of particular interest is Core 2 below eleven feet. This core penetrated an oxidized layer (possibly Pleistocene) wherein the organic matter was markedly diminished. This older layer, however, continues to show the increase in per cent bound lipids noted before.

Further interesting results have been obtained from analysis of two cores from Norwegian waters. One core, NT-1 (23 ft in length), was taken in 372 fathoms in Voldenfjord about 40 km south of Ålesund. The other core, NT-2 (14 ft.), is from the mouth of the Norwegian Trough in the North Sea. The water depth here was 250 fathoms. Data from these cores are shown in Table 3. The concentrations of the biochemical fractions in

TABLE 2

BIOCHEMICAL ANALYSES—CARIACO TRENCH SAMPLES

Core	Depth (ft)	Protein (mg/g)	Carbohydrate (μg/g)	Lipids (ppm) Free	Bound	Total	% bound
1	3	22.92	—	521	31	552	5.6
(10°30.8′N,	6	8.67	51.7	591	—	—	—
64°40′W)	8	9.88	—	506	—	—	—
	13	9.44	41.5	580	310	890	34.7
	16	7.02	—	460	—	—	—
	20	8.33	48.3	709	227	936	21.9
	25	5.22	36.7	372	—	—	—
	28	6.70	—	422	41	463	8.9
	30	3.88	36.7	342	—	—	—
2	0.5	10.00	—	745	168	913	18.4
(10°26.2′N,	3	4.66	70.5	357	—	—	—
64°41.2W)	7	5.20	—	548	138	686	20.1
	10	7.57	44.7	520	—	—	—
	12	0.85	—	93	78	171	45.6
	14	0.50	19.6	94	—	—	—
	21	0.65	22.0	28	—	—	—
	24	0.89	—	189	196	385	50.9
	28	1.17	43.2	70	—	—	—
3	4	8.88	74.3	687	—	—	—
(10°38.7′N,	12	4.62	51.6	631	—	—	—
65°4.5′W)	18	8.85	115.2	715	—	—	—
	23	5.96	82.8	550	—	—	—
4	6	8.13	56.4	461	—	—	—
(10°57′N,	10	6.80	49.3	686	—	—	—
64°38.5′W)	15	9.02	96.1	1250	—	—	—
	21	6.82	58.3	614	—	—	—
	27	5.09	67.7	397	—	—	—
	34	2.92	93.4	835	—	—	—

All figures are on a dry weight basis.

the samples from NT-2 were the lowest yet seen in our work, and may approach the lower limits for heterotroph survival in muds. The lipid increase was seen only in the Voldenfjord core (NT-1) below eighteen feet. Data are not yet available on the "bound" lipids in these cores.

The absolute amount of any given fraction depends on the environment of deposition, the source of the organic matter, the geologic history of the sediment, and the age and depth of burial of the sample. More detailed analyses (both chemical and

TABLE 3

BIOCHEMICAL ANALYSES—VOLDENFJORD AND NORTH SEA SAMLPES

Core	*Depth (ft)*	*Protein (mg/g)*	*Carbohydrate (μg/g)*	*Free lipids (ppm)*
Voldenfjord,	1	4.02	48.4	142
Norway	2	3.92	87.8	—
NT-1	3	2.87	69.7	168
(62°10′N,	5	1.66	31.7	95
5°59′E)	7	0.33	57.9	102
	10	1.23	43.5	92
	12	0.51	16.9	96
	15	0.28	22.7	91
	16	0.36	32.2	—
	18	0.49	61.4	64
	21	1.50	58.9	150
	23	1.98	59.6	270
North Sea	1	0.13	110.6	391
NT-2	2	0.33	146.4	—
(62°25′N,	3	0.48	23.7	361
1°58′E)	5	0.03	39.8	176
	8	0.07	56.7	138
	10	0.19	26.1	90
	12	0.03	15.0	103
	14	0.02	7.4	93

All figures are on a dry weight basis.

geological) are necessary for a total evaluation of the meaning of the observed trends. Also, determination of the molecular composition of the various extracts must be linked with further microbiological studies. However, the present data do point out that before one can assume that "natural" conditions exist in a particular experiment involving organisms from marine sediments, strict attention must be given to the true nutrient levels in the vicinity of the particular microbial community under study. This is illustrated by Table 4, which shows the concentrations (in gm/L of wet sediment) of proteins, carbohydrates, and ether-extractable lipids for several depths in each of the cores described. These figures have been obtained from the original dry weight data by calculations taking into consideration the per cent moisture of the sample and the density of the dry minerals. The ratios of protein to carbohydrate, protein to lipid, and lipid to carbohydrate are also shown. For comparison, figures calculated from the results of Shabarova (6) are also given.

TABLE 4

VARIATIONS IN NUTRIENT LEVELS WITH DEPTH AND WITH TYPE AND LOCATION OF SEDIMENT

Core	Depth (ft)	(g/L Wet Sediment) Protein (P)	Carbohydrate (C)	Lipid (free) (L)	P/C	P/L	L/C
Cariaco Trench							
1	6	5.81	0.034	0.40	170.9	14.5	11.8
	30	2.60	0.027	0.23	96.3	11.3	8.5
2	3	3.17	0.048	0.24	66.0	13.2	5.0
	10	5.15	0.027	0.35	54.4	14.7	13.0
	14	0.57	0.023	0.10	24.8	5.7	4.3
	21	0.68	0.021	0.03	32.4	22.7	1.4
3	12	2.82	0.031	0.38	91.0	7.4	12.3
4	6	5.12	0.038	0.29	132.1	17.7	7.6
	15	5.05	0.056	0.70	90.2	7.2	12.5
	27	2.95	0.041	0.23	62.2	12.8	5.6
Orinoco Delta							
C9	10	2.58	0.165	0.19	15.6	13.6	1.2
	51	2.44	0.025	0.14	97.6	17.4	5.6
	101	1.49	0.010	0.22	149.0	6.8	22.0
	135	0.90	0.007	0.43	128.6	2.1	61.4
Voldenfjord, Norway							
(NT-1)	5	1.00	0.018	0.06	55.6	16.7	3.3
	15	0.35	0.029	0.09	12.1	3.7	3.1
	21	1.01	0.040	0.10	15.3	10.1	2.5
North Sea							
(NT-2)	3	0.45	0.019	0.34	23.7	1.3	17.9
	12	0.04	0.013	0.13	3.1	0.3	10.0
Gulf of California							
1	2	3.90	0.189	0.67	15.3	5.8	3.5
2	1	6.30	0.204	0.48	30.9	13.1	2.4
	4	6.54	0.230	0.49	28.4	13.3	2.1
*Far East							
(opean ocean)	Surface	0.025	0	0.02	—	1.3	—
*Black Sea	Surface	0.019	0	0.013	—	1.5	—
*Caspian Sea	Surface	0.040	0.002	0.039	20.0	1.0	19.5
*White Sea	Surface	0.014	0.007	0.007	2.0	2.0	1.0
Medium 2216E	—	5.2	0.005	0.0022	1040.0	2363.6	0.44

*—Average data from Shabarova, (6) (1965a), calculated on basis of 60 per cent moisture.

It can be seen that wide variations exist (both in absolute values and in ratios) within single cores as well as between cores in the same or different environments. For reference, Table 4 also shows the same kind of analysis on a common medium often used for studies of marine organisms, Medium 2216 E of ZoBell (9).

ACKNOWLEDGMENTS

Cores from the Gulf of California were provided by Dr. Tj. H. van Andel of Scripps Institution of Oceanography. Those from the Cariaco Trench were sampled at Woods Hole Oceanographic Institution through the courtesy of Dr. J. M. Zeigler. The Norwegian samples were obtained while the author was a guest member of the scientific party aboard WHOI *R/V Chain* cruise No. 13, Dr. J. B. Hersey, chief scientist.

Technical assistance during these investigations was provided by Mrs. Marcia D. Lupton, Dr. C. V. Campbell, Mr. K. V. Davis, and various members of the scientific party and crew of *R/V Chain*.

SUMMARY

A wide variation in the local concentrations of the major biochemical fractions (proteins, carbohydrates, and ether-extractable lipids) exists within different marine sediments. This variation is in ratios of the three fractions as well as in absolute amounts.

Interesting trends of protein and carbohydrate decrease and lipid increase have been observed in deeper sediments from some areas. Further research is needed to clarify the mechanisms concerned, and to determine whether microbial reactions are involved.

An implication of these results is that some previous studies of the activity of marine sediment microbes may have involved use of "overfed" or "nutritionally unbalanced" cultures. Further work in this field should consider nutrient levels of primary importance in design of experiments in which attempts are made to duplicate a natural environment.

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Part 3
Ecology of Algae, Protozoa and Fungi and Viruses

Chapter 22

Density of Flagellates and Myxophyceae in the Heterotrophic Layers Related to Environment

Francis Bernard

INTRODUCTION AND HISTORY

The paucity of accounts on the density of unicellular organisms in the aphotic zone is surprising in the present state of oceanography. More than 30 important cruises have prospected great depths, from the *Challenger* (1873) to 1960. Between 1903 and 1913, H. Lohmann (10) discovered the nannoplankton, showing the importance of Coccolithophorids in warm seas, but, even today, nannoplankton specialists are scarce, and determinations are often limited to the photic zone. Many cruises have not caught single samples of deep phytoplankton.

In the study of deep plankton, German workers are in the lead. In the central and southern Atlantic (*Deutschland* cruise, 1921) deep samples were studied by Lohmann (10), and (*Meteor* cruise, 1933-34) by Hentschel (7), but their samples were centrifuged, and the quantitative results are uncertain. Steemann-Nielsen (1933-38), using Utermohl's method of slow sedimentation, has shown that centrifugation causes at least a loss of one-third of total cells, several species being entirely lost.

Ercegovic (1936) followed Steemann-Nielsen in using the inverted microscope, and made interesting studies in the Adriatic Sea off Split, but chiefly in the euphotic zone.

Hulburt, Ryther and Guillard (8) have prospected the Sargasso Sea which is rather poor, finding quantities of *Coccolithus huxleyi* at great depths, a species rarely so common at such levels in other warm seas.

The aim of the present paper is to give recent data on deep

pelagic fertility, chiefly in the Mediterranean, but also in the Indian and tropical Atlantic Oceans. The experience acquired from assaying 1,210 deep water samples has convinced me that *the number of flagellates per liter in the aphotic zone is often of the same order as, or even greater than, in the photic layer.*

These curious results seem particularly clear in the Mediterranean, where oxygen and pH show relatively high values at great depths. However, some of our stations off Senegal and in the central Indian Ocean are also richer below 200 m than in the euphotic zone. It is not necessary to say much about the regions studied, which are summarized in Table 1.

TABLE 1

NUMBER OF WATER SAMPLES, PRESERVED WITH NEUTRAL FORMALIN, IN WHICH FLAGELLATES WERE COUNTED BY THE AUTHOR IN SEVERAL SEAS, FROM 1936 TO 1960 (APHOTIC LAYERS, FROM 20 TO 4,000 METERS).

Region	*Dates*	*Depths in Aphotic Zone*	*Number of Stations*	*Number of Samples*	*Observations on Plankton*
Off Monaco	1936–39	200–2,000	93	202	Very poor
Off Toulon	IX. 1957	200–1,500	2	10	Samples taken from bathyscaphe
Off Banyuls	1936–37	200–500	17	30	Rather poor
Off western Algeria, 3 to 5 miles off coast	1943–53	200–1,000	81	382	Often rich between 300 and 500 m.
Gibraltar straits	VI. 1954	200-500	7	31	Rather poor, very much sand
"Calypso" cruise from Marseilles to Greece	VII. 1955	200–4,000	29	335	Often rich between 500 and 3,000 m.
"Calypso" cruise from Algiers to Lisbon	VII. 1960	200–2,500	26	116	Often rich between 200 and 500 m.
"Norsel" cruise in Indian Ocean	1955–56	200–1,000	17	86	Very rich at 200 and 1,000 m.
"President Th. Tissier" cruise off Senegal	VI. 1936	200–500	9	18	5 times richer than Indian Ocean
Total	7 regions	200–4,000	281	1,210	

With the exception of Monaco (which has low phosphates and light values), all these regions have calcareous flagellates of the genus *Cyclococcolithus* as prevailing and perennial organisms.

PREVAILING ORGANISMS—CHARACTERISTIC QUANTITATIVE RELATIONS

In deep layers, nannoplankton is less variable and poorer in species than in the euphotic zone. Unfortunately, very little is known of its distribution in Pacific and in cold seas. However, it is possible to summarize here the valuations in the southern Atlantic (*Meteor*), in the Mediterranean, and in the central Indian Ocean (6).

The absolute number of cells per ml or liter is the most solid basis for comparisons, but individual cell volume is variable, from 30 μ^3 (smallest flagellates) to 50,000 or more (largest Peridinians and Diatoms). For instance, a palmelloid cell of *Cyclococcolithus* (mean volume 3600 μ^3) is probably able to give 100 times more food to a Copepod than a small *Chromulina* (30 to 45 μ^3). The following percentages, therefore, indicate the mean approximate proportion in the total volume of unicellular organisms: all groups are listed according to decreasing values of that mean volumetric percentage, only in the aphotic zone:

1. Coccolithaceae of the Genus *Cyclococcolithus (Fragilis* Group)

Very common and perennial, from the surface of warm seas to their greatest depths. Numerically: 3 to 4,000 cells per ml, the mean being between 50 and 250 cells. Volume: 65 to 95 per cent of the total phytoplankton, prevalent almost everywhere. Only very rarely did water samples contain no trace of *Cyclococcolithus* (of 1,000 samples in the deep Mediterranean, only one had no trace: at 3,000 m in the Matapan trench, near Greece). *C. fragilis* occur in water from 70 to 105 per cent oxygen saturation.

2. Syracosphaeraceae, and True *Coccolithus (Pelagicus* Group)

Common, with at least 30 species and 7 genera in the deep sea, but, generally, they make up only 10 to 25 per cent of the volume, and 5 to 400 cells/ml. They are much more oligotrophic

and fragile than *Cyclococcolithus*, and unable to build internal resting spores. Rare when oxygen is below 90 per cent of saturation. Examples: *Thoracosphaera heimii* (Lohm), *Corisphaera fagei* (Bernard, etc.)

3. Small Naked Flagellates

In majority, less than 13 μ long. Various species, belonging chiefly to Volvocales and Chrysomonads (five other orders are concerned but are not common). They are often colorless, but I have seen, in the Mediterranean at 1,000 to 2,500 m depth, several cells with colored plastids. Rarely more than 12 per cent of the total volume: numbers of cells are sometimes large (300 to 1,000/ml), but their size is small.

Naked flagellates are much more abundant in low salinities (Gibraltar, Atlantic current off Algeria) and ten times rarer at the highest salinities (38.6 to 39‰) of the oriental Mediterranean.

4. Myxophyceae

The prevailing form everywhere is a small *Nostoc*, of 4 to 6 μ, near *N. planctonicum* Poretzky from Carelian lakes: the cysts in the Indian Ocean are quite similar to those off Monaco and Algeria, often entirely lacking, but when present, sometimes very numerous (400 to 12,000 elements/ml). There is no evidence of upwelling of bottom mud: regions of upwelling, such as Senegal and Greece, are almost devoid of Myxophyceae. The genera *Dactyliococcopsis* and *Microcystis* are uncommon: generally half of the small flagellate value by volume (5 to 6% of total).

5. Dinophyceae

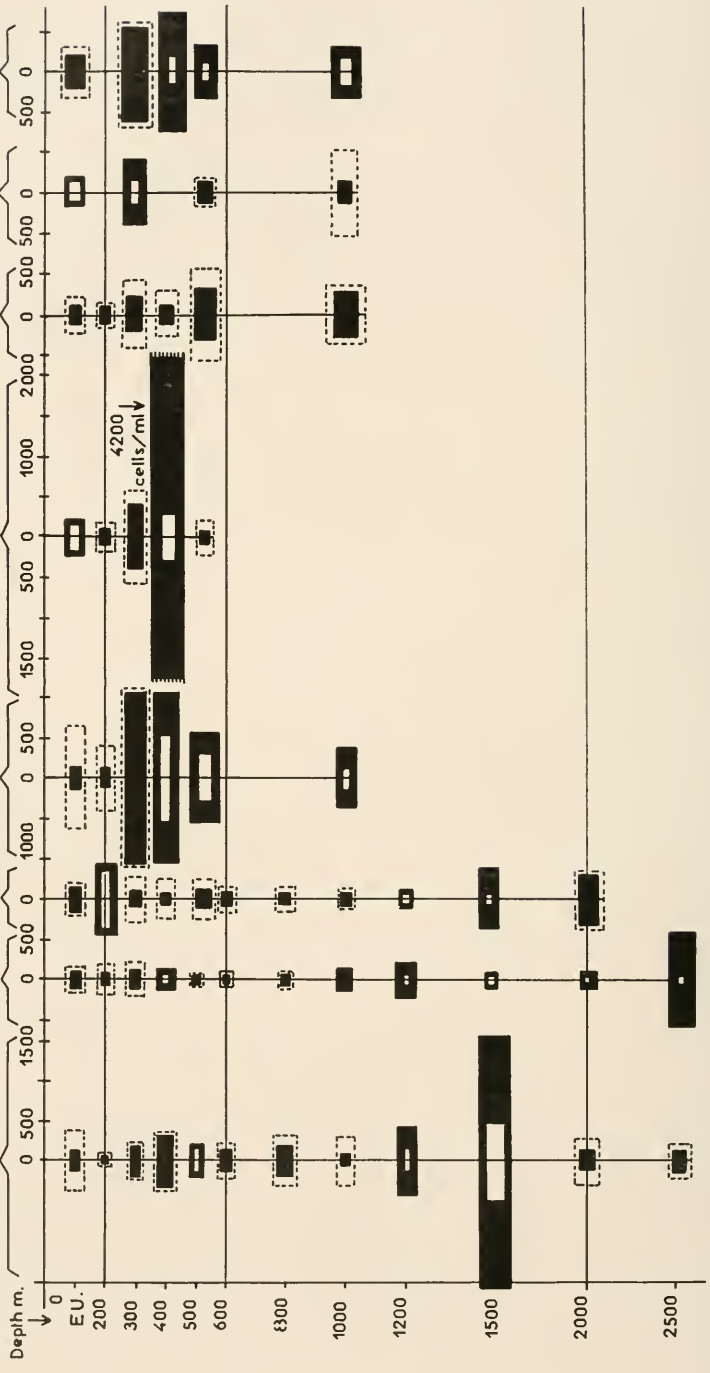
Very numerous (with *Exuviella, Ceratium* and *Gymnodinium*) only in the uppermost photic layer (0-25 m in the Mediterranean). Generally subordinate in the deep sea, with exception of some stations off Lybia, where *Exuviella* were swarming at 500 and 800 m depth. At mean aphotic value, Dinoflagellates do not make up a volume equal to the Myxophyceae.

It is possible to calculate some habitual characteristic quantitative relations between the above named groups of unicellular organisms. Each region of sea shows relations rather different

TABLE 2

Relations Between the Numbers of Cells/ml. for Several Prevailing Groups in the Aphotic Zone. The Values are, for each Marine Region, the Mean Result for all Water Samples (Number of Samples Given on First Line). See Table 1 for the Dates of the Cruises

Value	*Indian Ocean*	*Off Senegal*	*Off Monaco*	*"Calypso" Cruise, July 1955 Off Greece*	*Off Lybia*	*6-Occidental Stations*	*Off western Algeria June-July, 1950-1953*	*Observations*
Number of deep samples	86	18	202	103	154	78	281	
Per cent of calcareous Flagellates in nannoplankton	37%	17.5%	73%	40.6%	36.2%	43.5%	32%	As relative *volume* always predominant
Per cent of *Cyclococcolithus* in total Coccolithophorids	64%	88%	21%	77%	87%	88%	89%	Monaco waters too poor for *Cyclococcolithus*
Cyclococcolithus Syracosphaeraceae	1.8	7.5	0.27	3.3	6.9	7.7	7.8	Calm waters off Monaco and Greece suitable for Syracosphaeraceae, also those in Indian Ocean.
Cyclococcolithus Exuviella	22.5	42.0	26.7	4.5	3.1	22.0	25.2	Oriental Mediterranean and Dead Sea very rich with *Exuviella*
Nostoc Cyclococcolithus	0.94	0.04	0.8	0.01	0.33	0.22	0.22	Only Monaco and central Indian Ocean are clear enough, without mud, for *Nostoc*.


St 6 "Calypso"
St 16 "Calypso" St 24
Off Western Algéria 1952
Central Indian Ocean 1956
(off Sardinia)
(off Lybia) (off Crête)
Ténès 23 VI
Cherchell 28 VI
Dellys 11 XII
St 26
St 29
4200 cells/ml
Depth m.
E.U.

from those of other regions. Table 2 gives several examples of such facts.

Of course, Table 2 is provisional and has only a seasonal value: off Algeria and Lybia only results for June and July are known. However, several facts seem obvious and interesting:

The percentage of calcareous flagellates is greater than 30 per cent throughout the Mediterranean (Adriatic excepted) and in the central Indian Ocean. It is lower in the Strait of Gibraltar and in the Atlantic, owing to the large local numbers of naked flagellates and Peridinians.

Nostoc is very sensitive to mud in suspension, and perennial only when water is very pure, as off Monaco and in the central Indian Ocean. The same fact was discovered by Hentschel (7) for "olivgrünzellen" (*Microcystis*) in the deep southern Atlantic.

EXAMPLES OF STATIONS RICHER IN THE APHOTIC ZONE (Fig. 1)

Among the 281 stations examined by us, we have chosen (Fig. 1) eight of the most remarkable places where the euphotic zone is, more or less, poorer in cells/ml than the deep sea. Sometimes (see stations 6, 16, and off Ténès and Cherchell, Algeria) a considerable abundance of palmelloid *Cyclococcolithus* occurs only in aphotic layers.

Nevertheless, one must maintain that, taking a mean value of all stations, the deep zone is never extremely poor. It contains generally one-fourth to two-thirds of the density of cells observed in euphotic layers. For *Cyclococcolithus*, Gymnodinians and naked flagellates, the commonest relation is about one-half of the surface value between 200 and 400 m, about one-third below.

The scheme in Figure 1 represents only actual fertility (cells/ml) for *Cyclococcolithus* (black lines) and naked flagellates (dotted lines). For the other kinds of cells, forming gen-

←

Fig. 1. Density per ml of palmelloid cells of *Cyclococcolithus* (black drawings) and small naked flagellates (dotted lines) in eight stations of Mediterranean and of Indian Ocean. Those stations are chosen for their deep richness. The density of *Cyclococcolithus*, off Cherchell (W. Algeria), at 400 m depth, is today the world maximum of deep fertility (4,200 cells/ml. See text for other comments.

TABLE 3

Comparison of the Euphotic Layer with the Layers Known in Aphotic Zone for the Eight Stations of Figure 1. Each Number Gives the Ratio: Mean Aphotic Density-ml/Mean Euphotic Density-ml

Region	*"Calypso" cruise, July 1955*			*Off Western Algeria*			*Central Indian Ocean*	
Station	*Between Sicilia and Sardinia 6*	*Off Lybia, 100 miles from coast. 16*	*Between Crete and Greece 24*	*25 miles off Ténès 23.VI.52*	*6 miles off Cherchell 28.VI.52*	*12 miles off Dellys 11.XII.52*	*"Norsel" St. 26*	*"Norsel" St. 29*
Cyclococcolithus (palmelloid cells)	2.7	1.5	1.5	5.7	4.8	2.2	1.36	1.86
Naked Flagellates	0.68	0.67	1.4	0.97	1.3	1.45	2.1	0.94
Myxophyceae (chiefly *Nostoc*)	0.07	0.05	3	4.05	1.0	1.54	7.3	2.5
Richest layer	1,500 m	2,500 m	200 m	250 m 300 m	400 m	500 m	1,000 m	300 m

erally less than 50 per cent of the preceding organisms, we can only give in few words an outline of their distribution:

Syracosphaeraceae, which have 15 to 40 species in the Mediterranean euphotic layer, have only two to nine species below it (for instance: *Thoracosphaera heimii* Lohm., *Syracosphaera profunda* Bernard, *Corisphaera fagei* Bernard . . .). They consist generally of one-third of the number of coccolithaceae (*Coccolithus wallichi* is, by far, less common than *C. fragilis*).

Dinoflagellates are represented chiefly by small Gymnodinians, not very abundant (one-tenth of the naked flagellates). Here and there, *Exuviella* (cf. *marina* Cienk.) is more common and prevails in the oriental Mediterranean (sometimes 300 to 4,000 cells/ml at 500 and 800 m depth). It is not certain that the deep form is the same *Exuviella* as that at surface. The electron microscope is required to show the details of the shells for separation.

Nostoc is rare when water is polluted by suspended mud. In very clean water, far from the bottom and from the coasts, we can have thousands of *Nostoc* per ml; as in the Indian Ocean.

Off western Algeria (Fig. 1) the saline oriental current, running between 250 and 500 m depth, is sometimes very rich. In the oriental basin, and at station six (between Sardinia and Sicilia) deeper fertilities seem to be caused by local currents, estimated in the following pages.

In the Indian Ocean, 200 m is often the richest layer, and 1,000 m has more cells than 400 to 600 m, and much more *Nostoc*.

Table 3 gives the mean deep numbers of organisms, compared with the mean numbers in the euphotic zone, for the eight stations described.

The aphotic zone is very rich, except for *Nostoc*. The densities of flagellates are often greater than at the surface, and represent at least two-thirds of the euphotic densities. In poorer stations (not included) the values are at least one-third of the euphotic value.

HYPOTHESIS ON THE CAUSES OF DEEP SEA FERTILITY

It is actually impossible to be certain of the real factors in the deep sea. Heterotrophy is the only possible process of metab-

olism below 200 m. But we do not know anything about the concentration of organic substances in the aphotic layer, and very little about the kinds of substances that small flagellates prefer.

Another important and necessary factor is the occurrence of deep currents, renewing nutrients and carrying oxygen. Unfortunately, these are rarely measured, and theoretical calculations give only some probabilities on such currents. We shall try a very provisional reconstruction on the influence of currents in the deep Mediterranean.

Only general hypotheses are available now, but several facts, based on observations of more than 1,000 water samples, seem to confirm our theoretical views.

Heterotrophic Nutrition

I have observed several times (3 times off Monaco and seven times off Algeria) the presence of young *Cyclococcolithus* (palmelloid stages of 6 to 12 μ) on decaying dead Copepods or Tunicates suspended in the sea. Using the inverted microscope, I thought at first that some dead body had fallen under free palmelloid flagellates, but these cells were firmly attached to the Copepod body. It seems almost sure that young stages of *Cyclococcolithus* are able to assimilate organic substances of seston. They may also consume bacteria, as several flagellates are known to do.

Figure 2 shows a probable relation between the density of seston (particles of decaying matter) and the density of flagellates:

Our figure is based upon 252 water-samples, collected by the *Calypso* in July 1955, between 200 and 4,000 m. Seston, per ml of water, is estimated by counting after sedimentation, using an inverted microscope. Of course, the large masses of seston are not present in a small volume, but the little particles (4 to 90 μ) are dense enough and regularly distributed; their number per ml varying from 3 to 1,230 in *Calypso* samples.

It seems clear that, when seston is rare (3 to 10 particles/ml), flagellates are also rather rare. Probably dissolved organic matter is much more abundant when particulate organic pellets are numerous.

Fig. 2. Percentages of samples rich with *Cyclococcolithus* (black lines) or with naked flagellates (dotted lines) for several categories of the seston density, between 0 and 1,230 cells/ml. It is clear that rich nannoplankton does not coexist with poor seston, probably according to less dissolved or suspended organic matters.

In the aphotic zone, quite analogous results may be shown in 382 deep water samples off Algeria, and 86 samples in central Indian Ocean.

Role of Probable Deep Currents

Food is not quickly renewed in great depths, except when permanent strong currents are able to carry organic matter. In the northern occidental basin of the Mediterranean, such currents are rare or absent, and deep plankton is poor, but in the southern Mediterranean, there is a great deep current coming from Asia. Current appears to be maximum in the layer 290 to 450 m in the occidental basin, and in the layer 500 to 2,500 m in the oriental basin (see correlation with plankton at station 6 and 16 in Fig. 1). The current of 0.7 to 4 knots, measured in the Strait of Gibraltar, seems rather fast.

Table 4 gives, very approximately, a possible distinction between five kinds of waters in the aphotic zone of the southern Mediterranean. When the surface Atlantic current dives to 200 to 400 m (rarely, in very calm weather only), its deep waters are not rich in *Cyclococcolithus*. Mixed Mediterranean and Atlantic waters are sometimes rich, but have 61 per cent rather poor samples. Pure Mediterranean occidental waters are generally not rich. It is the oriental high salinity current (38.4 to 39.1‰) which sometimes shows the greatest production of *Cyclococcolithus* (700 to 4,200 cells/ml).

In the abyssal layer (2,000 to 4,000 m), the effect of probable currents is particularly clear. Our eighteen samples with theoretical currents (warmer and more saline) have a mean of 2.3 times more *Cyclococcolithus,* 3.3 times more *Exuviella,* and 2.6 times more naked flagellates than our 52 samples probably taken outside the oriental current. Our observations are suggestive rather than certain.

In conclusion, the frequent richness of deep water is obvious. At several Mediterranean stations the aphotic zone contains more flagellates and Myxophyceae than the euphotic layer does. The Mediterranean has the advantages of high oxygen and pH values in the deep sea. Even in the Indian Ocean (and probably in the Atlantic, according to the *Meteor* results), great densities of uni-

TABLE 4

PERCENTAGES OF *Cyclococcolithus* (PALMELLAE) IN DIFFERENT KIND OF WATERS, FROM 200 TO 2,000 METERS (APHOTIC ZONE) (SOUTHERN OCCIDENTAL MEDITERRANEAN CRUISES OFF ALGERIA, 1949-53) AND "CALYPSO" CRUISE, 1955 - 337 SAMPLES

Kind of Water	*Atlantic Current off Algeria*		*Mixed Waters*		*Pure Mediterranean Occidental Waters*		*Deep Oriental Current More or Less Mixed*		*Oriental Current Pure*		*Total*	
Salinities in Aphotic Zone	*36.49 to 37.2*		*37.3 to 37.90*		*37.91 to 38.40*		*38.41 to 38.66*		*38.67 to 39.1*			
Palmelloid cells of *Cyclococcolithus* per ml: extremely rich	cells	Per cent of the water category	cells	Per cent of the water category	cells	Per cent of the water category	cells	Per cent of the water category	cells	Per cent of the water category	cells	Per cent of the water category
(1200-4200)	0	0	0	0	0		3	2	2	2.9	5	1.5
Very rich (600-1200)	0	0	1	3.6	0		2	1.3	3	4.3	6	1.8
Rather rich (300-600)	0	0	0	0	3	3.8	14	9.2	3	4.3	20	5.9
Mean value (100-300)	6	60	10	36	27	34.5	59	39	19	27.5	121	36
Rather poor (25-100)	4	40	10	36	37	47.4	63	41.5	29	42	143	42.5
Very poor (3-25)	0	0	7	25	11	14	11	7.2	13	19	42	12.5
Total	10	3	28	8.3	78	23.1	152	45	69	20.5	337	

cellular organisms are often observed between 200 and 2,000 m. But another problem, as yet scarcely resolved, is to explain those local fertilities. The only sure fact is that deep cells of *Cyclococcolithus* have exactly the same skeletons as surface specimens; i.e., rather heavy (probable density: 1.23), such palmelloid flagellates can dive, but adopt an heterotrophic physiology when light is failing.

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Chapter 23

Heterotrophy in Marine Diatoms*†

Joyce C. Lewin

INTRODUCTION

Diatoms can generally grow autotrophically by photosynthesis, manufacturing from CO_2 and H_2O in the presence of light all or almost all of the organic matter that they need for their life activities. It is of some ecological interest to know whether or not marine diatoms can also grow heterotrophically, i.e., in darkness in the presence of a suitable carbon source. If such heterotrophic growth can take place, then some diatom species might actually be able to multiply in ocean depths where light does not penetrate, beneath the surface of mud, etc. Approximately 39 species (60 isolates) of marine diatoms now maintained in bacteria-free cultures in the laboratory have been tested for their ability to multiply under heterotrophic conditions; the results are presented below.

Isolates Studied

Forty-four isolates of marine littoral diatoms, comprising species of pennate diatoms such as *Nitzschia, Navicula,* and *Amphora* spp. and including one centric species, were tested for their ability to grow heterotrophically (4). In addition, some interesting and ecologically important strains isolated by Dr. R. Guillard (Woods Hole Oceanographic Institution), comprising marine littoral and planktonic centric species, subsequently also have been tested for heterotrophic growth.

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* Contribution from Scripps Institution of Oceanography. New Series.

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Substrates Employed

In a general but limited survey of this type, the choice of potential substrates is important, since with so many isolates it is impracticable to test a wide variety of substrates. Since previous investigations showed that glucose was preferred for heterotrophic growth by many freshwater diatoms (2), and that lactate was readily oxidized by respiration in the dark (3), these two compounds were chosen as the substrates most likely to support heterotrophic growth in marine diatoms.

Most marine diatoms in illuminated cultures grow better when the medium is supplemented not only with nitrate but also with an organic nitrogen source, such as Tryptone. (Other organic sources such as glycine or Casamino acids serve equally well.) For those species stimulated by Tryptone in the light, Tryptone was also added to the media in tests for heterotrophic growth in the dark.

The general procedure was to transfer cells of each isolate into: (a) sea-water medium enriched with nitrate, phosphate, silicate, trace elements, vitamin B_1, vitamin B_{12}, and generally also with 0.1 per cent Tryptone; (b) the same medium + glucose (0.5%); (c) the same medium + lactate (0.2%). The cultures were incubated both in the light and in the dark. At the end of two weeks the presence or absence of growth in darkness was noted; in most cases the result was unequivocal.

RESULTS

Species Capable of Heterotrophic Growth

The species tested and the results obtained are summarized in Table I, from which certain trends and generalizations emerge. Half of the twenty-four species of littoral pennate diatoms tested included strains capable of heterotrophic growth. Among the fifteen species of centric diatoms tested, so far only one isolate has been found capable of heterotrophic growth. This is a small species of *Cyclotella* isolated from Martha's Vineyard, Mass., by Dr. R. Guillard. None of the seven isolates of centric diatoms obtained by Guillard from the Sargasso Sea grew heterotrophically.

TABLE 1

SPECIES OF MARINE DIATOMS TESTED FOR HETEROTROPHIC GROWTH UTILIZING GLUCOSE OR LACTATE[1]

Species Found Capable of Heterotrophic Growth	*Species Found Incapable of Heterotrophic Growth*
Pennate	*Pennate*
Amphora coffaeiformis (8 isolates)	*Achnanthes brevipes*
Navicula incerta	*Amphipleura rutilans*
Nitzschia angularis	*Amphiprora paludosa*
Nitzschia filiformis	*Amphora coffaeiformis* (2 isolates)
Nitzschia frustulum (3 isolates)	*Amphora lineolata*
Nitzschia laevis (2 isolates)	*Navicula menisculus*
Nitzschia curvilineata	*Navicula* sp.
Nitzschia marginata	*Nitzschia hybridaeformis*
Nitzschia punctata	*Nitzschia frustulum* (3 isolates)
Nitzschia tenuissima	*Nitzschia obtusa* v. *scalpelliformis*
Nitzschia closterium (4 isolates)	*Nitzschia ovalis*
Nitzschia thermalis	*Nitzschia* sp.
	Synedra affinis
	Stauroneis amphoroides
Centric	*Centric*
Cyclotella sp.	*Chaetoceros pseudocrinitus* (?)
	Chaetoceros ceratosporum (?)
	Chaetoceros pelagicus (?)
	Chaetoceros (2 unidentified sp.)
	Coscinodiscus asteromphalus
	Cyclotella nana (3 isolates)
	Cyclotella caspia
	Melosira nummuloides
	Melosira sp.
	Skeletonema costatum
	Thalassiosira fluviatilis
	Unidentified (2 species)

[1] Some of these data have been published elsewhere (4).

Necessity of an Organic Nitrogen Source for Heterotrophic Growth

All of the tests carried out and reported in Table I involved media containing Tryptone in addition to the organic substrate (except in the case of four species of centric diatoms that were inhibited by Tryptone in the light). The nutritive value of Tryptone for heterotrophic growth was assessed independently by incubating heterotrophic species in the dark in media containing

(a) enriched sea water, (b) enriched sea water + glucose, (c) enriched sea water + Tryptone, and (d) enriched sea water + glucose + Tryptone. The results are summarized in Table 2.

TABLE 2

Effect of Tryptone on Heterotrophic Growth of Marine Diatoms

	Glucose	*Tryptone*	*Glucose + Tryptone*
Amphora coffaeiformis	—	—	+
Nitzschia angularis	—	—	+
Nitzschia thermalis	—	—	+
Nitzschia frustulum	—	—	+
Nitzschia closterium	—	+*	+
Nitzschia marginata	+	+*	+
Nitzschia laevis	+	—	+
Navicula incerta	+	—	+
Cyclotella sp.	+	—	+

* Cells thin, pale

Rate of Growth of Diatoms Under Heterotrophic Conditions

The experiments reported above were of a qualitative nature, telling us only whether heterotrophic growth was possible. In order to determine actual rates of growth under heterotrophic conditions, the generation (doubling) times of three species were determined both in the dark and in the light. The results, shown in Table 3, indicate a wide variation between the species. *Nitzschia marginata* can multiply as rapidly in the dark as in the light. The generation time of *Cyclotella* sp. in the dark was more than twice as long, while the generation time of *Nitzschia clos-*

TABLE 3

Generation Times of Three Species of Marine Diatoms in Light and Darkness at 20 C (The Basal Medium Consisted of Sea Water, Supplemented with Nutrient Minerals, Vitamins and 0.1% Tryptone)

	Autotrophic Growth	*Heterotrophic Growth on:*	
	(3000 lux)	glucose	lactate
Cyclotella sp.	18 hrs.	30 hrs.	
Nitzschia closterium	12 hrs.		78 hrs.
Nitzschia marginata	12 hrs.	12 hrs.	

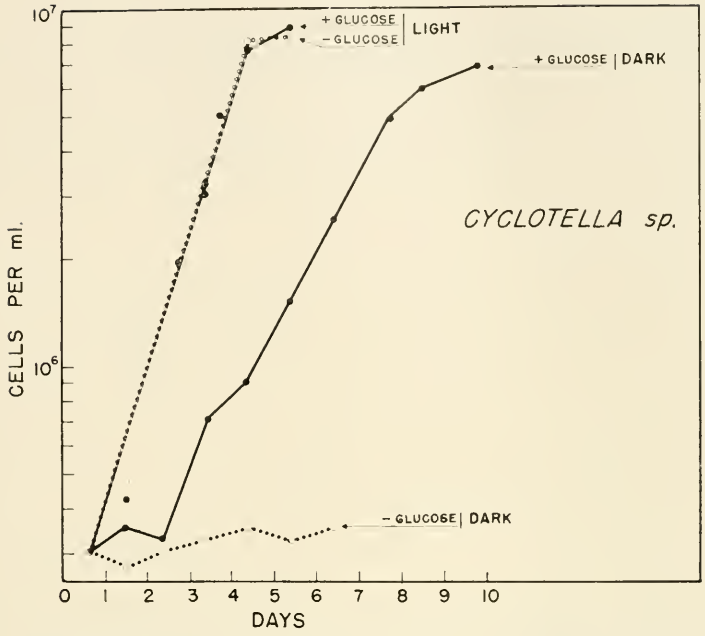

Fig. 1. Autotrophic and heterotrophic growth of *Cyclotella* sp. in aerated cultures. (Light intensity, 300 lux; temperature 20C).

terium in the dark was about six times as long as that in the light. The growth curves for the *Cyclotella* shown in Figure 1 illustrate that in the light the presence of glucose in the medium had no effect on the generation time.

DISCUSSION

The results obtained so far seem to follow the pattern that one would have predicted, i.e., among marine littoral pennate diatoms, that often inhabit environments rich in organic nutrients, a higher proportion of species are capable of using such nutrients for heterotrophic growth than among the centric diatoms that generally inhabit neritic or pelagic waters with much lower concentrations of organic nutrients.

Some diatom species can multiply in the dark in the presence of glucose and nitrate, while others require an additional factor present in Tryptone. It remains to be determined which of the components of Tryptone is active, and whether it can be replaced

by ammonia. Diatoms having such a double requirement for reduced carbon and nitrogen sources presumably have less opportunity for heterotrophic growth in natural environments than do species with only a single requirement. Two pennate species were found that could multiply in the dark on Tryptone alone, although the cells growing under these conditions were unusually thin and pale.

We now know something about the dissolved organic substances that occur in marine environments. For instance, free sugars (sucrose, glucose, fructose, galactose, arabinose, xylose) have been demonstrated in deep-sea sediments (5), and organic acids (acetic, formic, lactic, glycolic) have been demonstrated in sea-water samples from the Pacific Ocean (1). It is still unknown to what extent diatom cells can make use of such dissolved substances for heterotrophic growth in natural environments, where they must compete with obligately heterotrophic micro-organisms such as bacteria, fungi, and colorless flagellates. Mud samples collected from the ocean floor at depths as great as 7,400 m contained diatoms, some still with cell contents (6, 7). Some cells of pennate forms from shallow waters (60-120 m) were actively motile, but it remains to be determined whether they had been actually growing at these depths. (See Chapter 22.)

ACKNOWLEDGMENTS

The author is grateful to Dr. Robert Guillard, Woods Hole Oceanographic Institution, for cultures of several marine centric diatoms, and to Irma Pintner, Haskins Laboratories, who isolated the strain of *Coscinodiscus asteromphalus*.

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Chapter 24

The Relative Importance of Groups of Protozoa and Algae in Marine Environments of the Southwest Pacific and East Indian Oceans

E. J. Ferguson Wood

This paper is a report of current work, so the conclusions will be speculative rather than positive. Studies have been made of living phytoplankton in the Coral and Tasman Seas to the east of Australia, in the North-east Indian Ocean, and the seas among the islands of Indonesia, including the Arafura Sea. These studies, especially in the Indian Ocean, will be extended in the near future, particularly in association with the oceanographic program of SCOR.

Examination of the living phytoplankton at sea has highlighted the following points: the varied ratio between the so-called net-phytoplankton (diatoms, dinoflagellates etc.) and the nannoplankton (smaller forms which usually escape the finest nets that can be used); the frequent occurrence of large numbers of colorless flagellates, the large numbers of small, naked dinoflagellates, the importance of the Myxophyceae in tropical waters in these regions and the increase of the ratio of diatoms to dinoflagellates closer inshore and in cooler waters.

RATIOS OF NET PHYTOPLANKTON TO NANNOPLANKTON

The substitution of containing samplers for nets in catching phytoplankton has demonstrated the importance in the oceans of phytoplankters ranging in size down to 1 micron, and these smaller forms are known as nannoplankton, ultraplankton, etc. The division between these small elements and the larger ones that were usually caught in nets with a mesh size between 150 and

200 per inch is arbitrary, as cells of less diameter than the net pores may be caught, while organisms of the same group or even of the same species may differ greatly in size, sufficiently to be classed as nannoplankton in one case and net-phytoplankton in another. The diatoms *Skeletonema costatum* and species of *Chaetoceros* are cases in point. However, if an arbitrary limit of 20μ in greatest dimension is chosen, most of the net-phytoplankton will be above this.

Wood and Davis (3) found that the nannoplankton greatly outnumbered the net phytoplankton in the onshore waters east of Cronulla. In the Coral and North Tasman Seas, where counts were recently made from a large number of stations, and at depths to 150 m, the nannoplankton is, on the average about 13 times as numerous as the phytoplankton over 20μ in diameter, and ranges from 1 to 100 times as numerous, except on rare occasions when the larger organisms may outnumber the nannoplankton. In the Southern Tasman, however, the ratio of nannoplankton to net phytoplankton ranges from 140 to 1 to 1 to 15, and the net phytoplankton exceeded the nannoplankton at 35 per cent

TABLE 1

Composition of Phytoplankton in Random Samples from (a) Coral Sea, (b) Southern Tasman Sea

	Microflagellates 1-5μ	*Gymnodinium*, etc. 5-10μ	*Diatoms and Larger Dinoflagellates* 110μ
a.	10	5	2
	19	2	1
	16	0	1
	30	0	2
	10	1	1
	20	5	0
b.	10	0	1
	30	4	2
	3	0.4	5
	6	0	1
	8	0.6	1.5
	10	2	5
	0.3	1	6

of the stations worked, 5 per cent of these stations being within 120 miles of the coast. On one occasion in the Coral Sea (Quickmatch Station 1), there were about 5×10^8 nannoplankton organisms (*Chromulina*) per liter and no larger phytoplankton.

Table 1 shows the composition (organisms per field) of (a) Samples taken at random from oceanic stations in the Coral Sea; and (b) samples also taken at random from the Southern Tasman Sea.

THE IMPORTANCE OF COLORLESS FLAGELLATES

The ratio of chlorophyll-bearing to chlorophyll-less microorganisms has been studied by first counting the number showing the autofluorescence of chlorophyll in blue-violet light, and then counting those giving a green fluorescence induced by acridine orange. The same samples were used for both counts. As both types of organism stain with acridine orange, the chlorophyll count was subtracted from the acridine orange count. At the on-shore Cronulla stations, the ratio ranged from 3:1 to 1:3, while the oceanic stations ranged from 1:2 to 1:100. Most phytoplankton counts do not differentiate between these potential autotrophs and the heterotrophs, and such differentiation becomes important if one wishes to correlate phytoplankton numbers with productivity, nutrients, etc.

THE RELATIVE IMPORTANCE OF NAKED DINOFLAGELLATES

The dearth of naked dinoflagellates in formalized material is reflected in the taxonomic paper of Wood, (2) where only eleven unarmored species were described out of a total of 280, though many smaller forms were missed by the nets used for those studies. A study of living phytoplankton from the Coral Sea resulted in the recording of 130 species of naked dinoflagellates, and, at times, these forms made up about 50 per cent of the total phytoplankton. In the Southern Tasman, the naked flagellates were not so important, but they did not completely disappear even at the 11C isotherm.

MYXOPHYCEAE IN TROPICAL WATERS

Bernard and Lecal (1) record the importance of the

Nostocaceae in the phytoplankton of the Indian Ocean, while *Oscillatoria erythraea* (*Trichodesmium*), *O. thiebaultii, O. bonnemaisonii, O. hildebrandtii, Katagnymene spiralis* and *Halarachne lenticularis* are frequently important members of the phytoplankton in the Coral Sea, Arafura Sea, Timor Sea, and Northeast Indian Ocean in spring and summer. *O. erythraea* in particular may form vast sheets or windrows extending for many miles.

THE RATIO OF DIATOMS TO DINOFLAGELLATES

In the Indian Ocean and the Coral Sea, the highest ratio of diatom to dinoflagellate species occurs in restricted waters such as Macassar, Sunda, Lombok and Vitiaz Straits, and in the low salinity waters off the southwest coast of New Guinea, where the ratio is as high as 96 per cent diatoms in some instances. The lowest ratios were found in the southern Coral and Tasman Seas (0-5%) in late summer. In the Antarctic, and south of the subtropical convergence, however, the ratio of diatoms to dinoflagellates is high, even in oceanic waters. High diatom ratios have also been found associated with upwelling of cold water of sub-Antarctic origin north and east of North Cape, New Zealand.

SIGNIFICANCE OF OTHER GROUPS OF ORGANISMS

In the Coral and North Tasman Seas, the Coccolithophores occur frequently but not in great numbers, while Chrysomonads and Cryptomonads are relatively common. In the northeast Indian Ocean, however, Coccolithophores are dominant on occasion. Silicoflagellates are to be observed frequently but are never numerous.

CONCLUSIONS

The present stage in our investigations of phytoplankton in Australian waters and the adjacent seas is still one of exploration and speculation. One may conclude, however that the nannoplankton is of greater importance in warmer than in cool waters; that colorless flagellates are sufficiently numerous to make it necessary to distinguish between these and the photosynthonts when studying the catches of smaller plankton elements; that the naked dinoflagellates, myxophyceae, and chrysomonads are important members of the phytoplankton in the waters round

Australia, while the Coccolithophores are important numerically in the Indian Ocean, but not in the Southwestern Pacific seas; and that the diatoms are more numerous in inshore waters, dinoflagellates in offshore waters, except in colder waters south of the sub-tropical convergence and possibly in regions of upwelling.

Because of the frequency of occurrence of non-phototrophic, and facultatively heterotrophic microorganisms in phytoplankton catches, the term "Protoplankton" is suggested to include the net-phytoplankton, nannoplankton and the non-photosynthetic protozoa and algae.

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Chapter 25

Size Fractionation of C^{14}-Labeled Natural Phytoplankton Communities*

Robert W. Holmes and George C. Anderson

INTRODUCTION

Plankton nets of the finest available mesh are much too coarse to permit quantitative assessment of the abundance and species composition of standing stock of marine phytoplankton. This is concluded from comparisons of species abundance obtained with phytoplankton net catches and with water samples concentrated by centrifugation or sedimentation techniques (8, 11). Furthermore, estimates of species abundance obtained by centrifugation of water samples have been found to be considerably lower than estimates obtained with sedimentation techniques (11, 14). Species recovery by centrifugation becomes as large as by gravity settling when inorganic precipitates are deliberately formed in the water during centrifugation (13). Serial dilution techniques have been found to give even lower estimates of nannoplankton abundance in sea water samples than did centrifuge methods (1).

The results of the above investigators apply only to the standing crop of phytoplankton. It might also be asked how, under natural conditions, the rate of carbon dioxide assimilation is distributed among various size categories of the standing crop? Preliminary observations by Holmes *et al.* (5) in the eastern tropical Pacific were not conclusive, but the data suggested that organisms responsible for most of the primary production pass through a fine nylon net (mesh size 30 x 30 μ). In lakes, Rodhe *et al.* (10) and Rodhe (9) have convincingly demonstrated that

* Contribution No. 246, from the Department of Oceanography, University of Washington.

a low but somewhat variable percentage of C^{14}-labelled phytoplankton passes through the meshes of a No. 25 plankton net (mesh size about 70 μ). More recently, Yentsch and Ryther (16) working in Vineyard Sound, showed that the net portion (aperture size: 65 μ) of the phytoplankton made up on the average a small portion of the total population: 9 per cent of total cell numbers, 8 per cent of the chlorophyll and 2 per cent of the photosynthesis.

Carbon dioxide uptake by phytoplankton in the water sample, after inoculation with C^{14} and suitable incubation in the light, is measured by collection of the plankton on a fine filter and the radioactivity retained measured with a suitable counter. Most investigators employ membrane or molecular filters with pore sizes falling between 1 and about 0.4 μ. Goldberg *et al.* (3) examined microscopically the size distribution of all preserved organisms on an HA Millipore filter in two sea water samples and concluded that the filter gave complete retention of all plankton above a size of 0.5 μ. Lasker and Holmes (7), however, pointed out that there may be some leakage of radioactive material through filters in this pore-size range, even though the experimental organism, *Dunaliella primolecta*, is considerably larger than the filter pore size. A similar phenomenon was also observed by Guillard and Wangersky (4).

During the spring and summer of 1960, the authors attempted to assess the approximate size distribution of photosynthetically active planktonic organisms in the waters around San Juan Island, Washington, by inoculating sea water samples with C^{14}, and after incubation, measuring the C^{14} activity of the organisms retained on a size-graded series of nets and membrane filters. Data are reported from two locations: Friday Harbor, 48°32.7′ N, 123°00.6′ W, an area of vigorous tidal mixing; and East Sound, 48°39.7′ N, 122°53.9′ W, a sheltered arm extending into Orcas Island with little tidal mixing and pronounced stratification during summer.

Financial support was provided by the Friday Harbor Laboratories, by which the senior author was employed as instructor and investigator during the summer session of 1960, and by the

Atomic Energy Commission, Contract No. AT (45-1)-1425. It is a pleasure to acknowledge the cooperation of Dr. Robert L. Fernald, Director, Friday Harbor Laboratories and the assistance of Miss Rita Horner, Department of Oceanography.

METHODS

Water samples were collected with plastic buckets or modified plastic samplers (2, 15) and placed in clean 0.5 or 1 L Pyrex glass stoppered bottles. A hypodermic syringe, containing 10-40 μc of C^{14}-labelled sodium carbonate solution (12) was used for inoculation. After suitable agitation to insure complete mixing, the sample was incubated in a water bath cooled with circulating surface sea water, or in a plastic bucket in which the water was frequently changed, under natural illumination for two to six hours.

At the end of the experimental period, the samples were filtered in one of two ways. In the April-June series, the entire sample was passed in succession through each piece of net and filter, beginning with the coarsest and ending with the finest. In the July-August series, the sample was shaken and an equal volume (100 ml) passed through each filter in the graded series (Table 1). Filtration volumes were kept small to avoid progressively increasing retention by the filters due to clogging. Dark controls in which the C^{14} concentration was quadrupled, were run occasionally. The samples were filtered, using the second, or aliquot filtration, procedure. After filtration, 10 ml of 0.001 N hydrochloric acid in 3 per cent sodium chloride solution was passed through each filter to remove inorganic radioactive carbonate solution retained on the filter.

The radioactivity on the dried filters was measured with a thin-window gas flow counter tube (Nuclear Chicago D-47) mounted in a manual changer (Nuclear Chicago M-5) and an associated scaler (Nuclear Chicago 161A).

RESULTS AND DISCUSSION

The results of 28 sets of observations obtained during the spring and summer of 1960 are presented in Figures 1 and 2. In both figures, the C^{14} activity retained by the HA Millipore filter

(pore size 0.45 ± 0.02 μ) has been assigned a value of 100. This method of expression was used because this porosity is now widely used, and our interest is centered on retention by smaller and larger porosities in comparison with this standard size.

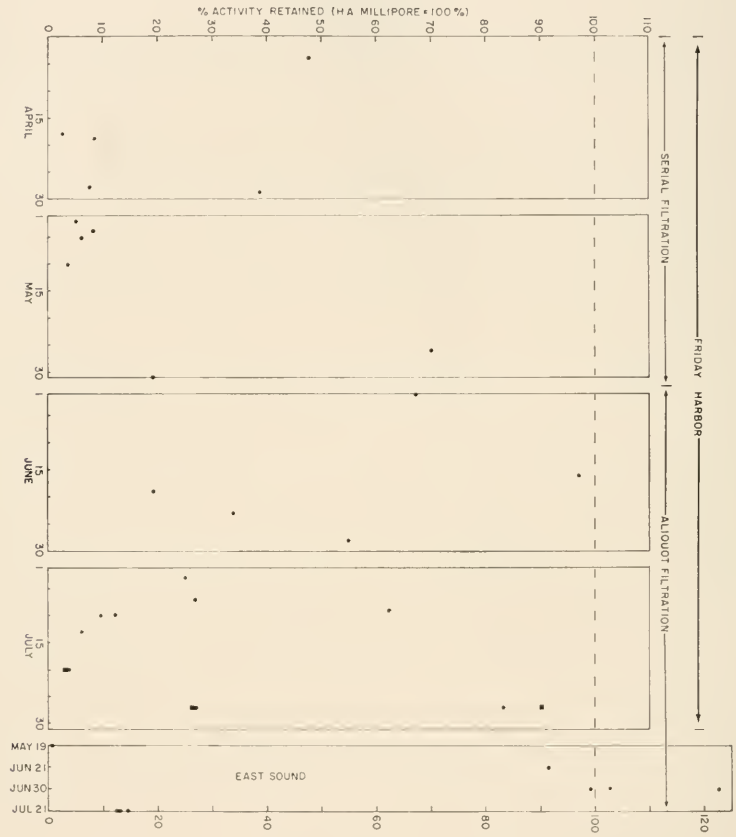

Fig. 1. The retention by nets (● 35 x 35 μ mesh; □ 106 x 106 μ mesh) of natural phytoplankton communities labelled with C^{14} compared to the activity retained by 0.45 μ±0.02 μ filters.

In Figure 1 the relative retention of net material and of the HA Millipore filter is compared. With the exception of two observations in East Sound, the nets retained less activity than the 0.45 μ filter. There is no obvious trend in the relationship—in fact, the scatter is considerable, with approximately two-thirds

of the values falling below 50 per cent retention. This scatter may reflect the changing species composition of the phytoplankton although during the months of June and July qualitative estimations of species abundance made with the inverted microscope led us to believe that large chain-forming and non-chain-forming diatom species were dominant. If this impression is correct, the passage of labelled organic matter through the 35 μ net material could arise largely from fragmentation of intact cells due to filtration. On the other hand, it is possible that less conspicuous, small organisms made up an important fraction of the photosynthesizing biomass and that these escaped notice during the qualitative examination of the Lugol's iodine-preserved material. The two samples in which the C^{14} activity of the net sample exceeded that of the 0.45 μ filter argues against the fragmentation hypothesis. It seems more likely that our first impression of the dominance of the conspicuous chain-forming dia-

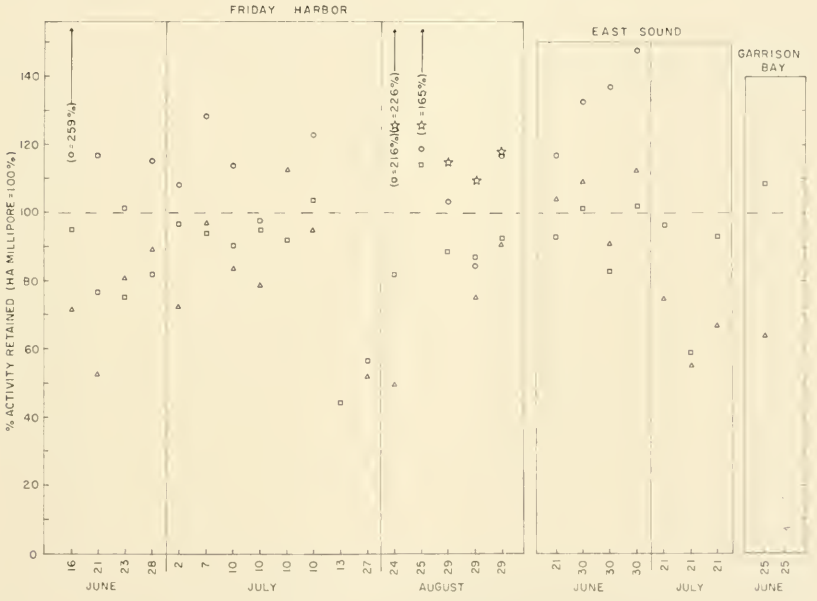

Fig. 2. The relative retention of C^{14}-labelled phytoplankton by filters, compared with the retention of a 0.45 μ filter (Δ = 5 $\pm$ 1.2 μ; $\square$ = 0.80 $\pm$ 0.05 μ; O = 0.30 $\pm$ 0.02 μ; $\star$ = 0.22 μ (pore size variability not given by manufacturer)).

TABLE 1

C^{14} Uptake (c/m)
(Concentration of C^{14} in the Dark Bottles was Quadrupled)

Filter	*Pore Size*	*July 2*		*July 7*		*July 10*		*July 13*		*July 20*		*July 27*		*Aug. 24*		*Aug. 25*	
		Light	*Dark*	*Light*	*Dark*	*Light*	*Dark*	*Light*	*Dark*	*Light*	*Dark*	*Light*	*Dark*	*Light*	*Dark*	*Light*	*Dark*
No. 20 net	106x106μ	—	—	—	—	—	—	—	—	34	2	284	19	—	—	—	—
35μ net	35x35μ	59	4	274	11	55	7	10	7	41	4	284	17	30	—	—	—
5μ	5.0 $\pm$ 1.2μ	171	8	999	18	488	15	163	8	—	5	554	8	197	8	(1033?)	19
AA	0.80 $\pm$ 0.05μ	229	5	965	19	529	16	178	7	477	26	602	15	324	3	545	9
HA	0.45 $\pm$ 0.02μ	236	7	1020	27	582	21	171	7	1070	46	1057	21	392	6	476	8
PH	0.30 $\pm$ 0.02μ	256	5	1020	32	664	20	211	20	—	—	—	—	849	4	565	9
GS	0.22*	—	—	—	—	—	—	—	—	—	—	—	—	888	4	783	9

* Pore size variability not given by manufacturer.

toms was incorrect and that a significant fraction of the photosynthesizing biomass is contained in cells with one dimension smaller than the net mesh size. Whichever explanation is correct, it can be stated with certainty that even the finest nets should not be used for the collection of phytoplankton for studies of primary production.

The results obtained with the graded Millipore filter series are presented in Figure 2. Again, a few points fall out of the expected order; in six instances the retention by coarser filters exceeded the retention by finer ones. These aberrant points reflect the difficulty in obtaining representative aliquots of a sample containing an abundance of chain-forming organisms (6).

These data show that on the average, finer porosity filters retain more activity than coarser ones even though the pores of the finer filters are smaller in diameter than any described photosynthetic organism (Figure 3). It is also apparent that

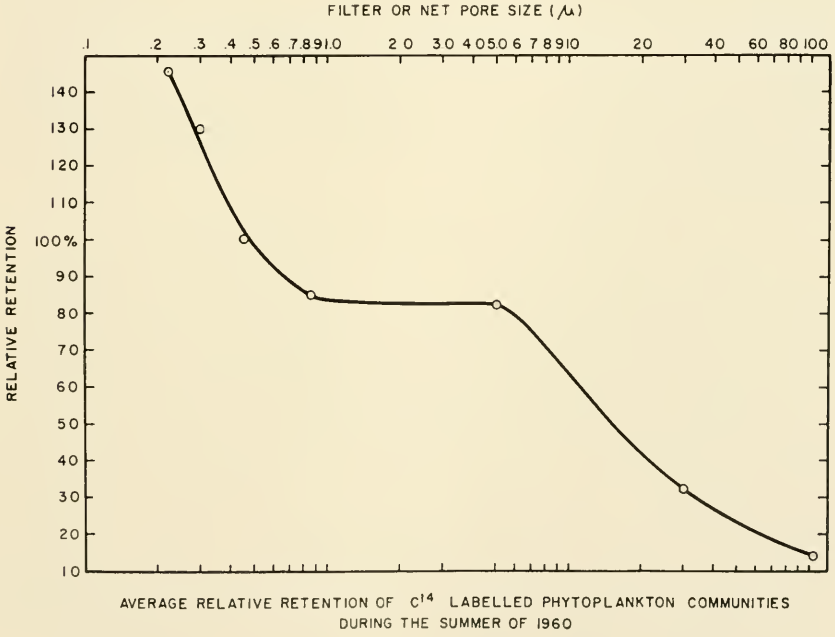

Fig. 3. Average relative retention of C^{14} labelled phytoplankton communities during the summer of 1960.

when results of other investigations are compared, the porosities of filters employed must be considered. In view of the significant loss of activity through HA filters, measurements of marine primary production at the University of Washington and Scripps Institution of Oceanography are now made with PH filters.

Although only eight filtration series have dark bottle replicates (Table 1), there is no indication that dark uptake by bacteria or other heterotrophic organisms can account for the greater amount of activity retained by the finer porosity filters. We believe there are three possible explanations which account for the results obtained; these should not be considered mutually exclusive. The first is that finer porosity filters prevent the passage of radioactive fragments and/or exudates resulting from mechanical fragmentation of cells during filtration. The second explanation supposes the presence in sea water of exceedingly minute autotrophic organisms which pass through all but the finest filters. The last and least likely possibility would assume the existence of bacterial symbionts which may live in close association with the phytoplankton. Such symbionts could become labelled in the light by incorporating into their protoplasm radioactive substances excreted by the photosynthesizing phytoplankton. Such bacteria might pass through the coarser filters and be retained by the finer filters.

SUMMARY AND CONCLUSIONS

Recovery of C^{14}-labelled natural phytoplankton communities on fine plankton nets and on membrane filters of graded porosities was measured successively, using waters collected near San Juan Island in Puget Sound. The finest plankton net (35 x 35 μ mesh) usually failed to retain half of the assimilated C^{14} that was recovered on the 0.45 μ filter, and in some cases, recovery with the plankton nets was below 10 per cent. More than half of the photosynthesis, therefore, was usually effected by algae that pass through a 35 μ net. The range of membrane filter porosities used in this study extended well below the known dimensions of the smallest marine algae, yet recovery of assimilated C^{14} was commonly greater with a pore size of 0.22 μ than 0.4 μ or larger. Greater retention of assimilated organic matter on the

finest membrane filters may result from the recovery of fragments of fragile algae that are broken during filtration. From experimental results in dark bottle C^{14} uptake, small heterotrophic organisms such as bacteria did not seem to be responsible for the higher recoveries of assimilated C^{14} on the finest membrane filters. The algae which accounted for the bulk of the C^{14} assimilation observed in this study were smaller than 30 μ. The possible existence in the sea of minute autotrophic organisms (<1 μ) cannot be ignored, and this possibility should be examined further.

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Chapter 26

Light Assimilation Curves of Surface Phytoplankton in the North Pacific 42° N — 61° N*

Sigeru Motoda and Teruyoshi Kawamura

Although the activity of phytoplankton may be studied for a single species by placing its pure culture under various artificial conditions, observations on the activity of a natural population are also useful, because a species in pure culture might act differently from that existing in natural environments in the sea. Moreover, in experiments using pure cultures, the density of population is often far greater than in nature. Observations on natural populations when composed almost exclusively of one species would be most helpful.

During the Trans-Pacific cruise through the Bering Sea by the training ship, *Oshoro Maru* of Hokkaido University from June to August, 1960, the present writers had opportunity to make experiments on photosynthetic activity of phytoplankton at fifteen stations which were scattered in the area ranging from 42°N to 61°N and from 151°E to 168°W (Fig. 1). The temperature of the surface water varied with stations Sta's 1-11, from 5.5 C to 7.7 C on the eastbound course, and stations Sta's 12 and 13, from 13.4 C to 16.4 C on the westbound course. At these stations, standing crops of phytoplankton in the surface water were studied by counting the number of cells separately by species, and also by measuring photometrically their chlorophyll content. Diatoms comprised the major part of the phytoplankton population in the samples. Microplants other than the diatoms were almost non-existent.

* This work was supported by the financial assistance provided on Contract AT (04-3)-15 with the U. S. Atomic Energy Commission through Dr. Maxwell S. Doty of the University of Hawaii, and also by funds from the Japanese National Commission for UNESCO.

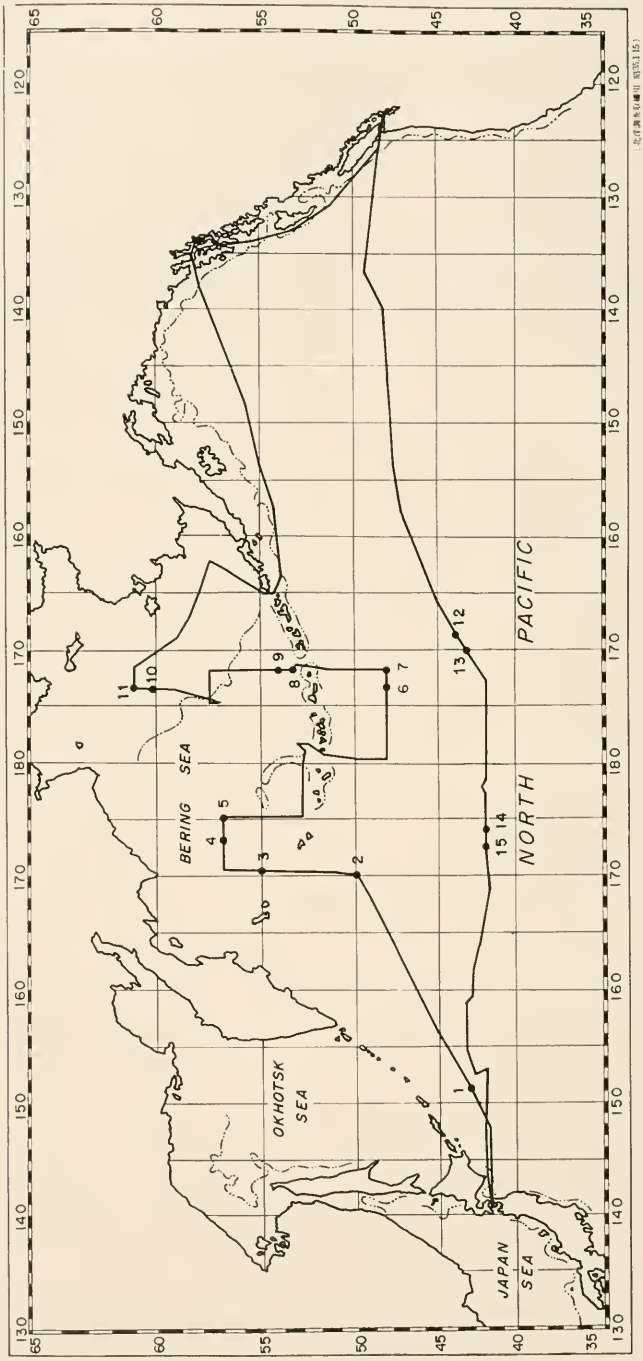


Fig. 1. Track of the cruise of the "Oshoro Maru" from June to August 1960, and stations at which experiments on light assimilation curves of phytoplankton were made.

The water sample was also used for measurement of photosynthesis by means of the carbon fourteen isotope method. Because of lack of any appropriate non-metal closing sampler, materials in the subsurface zone were not examined. The carbon[14] productivity experiments followed the method of Doty and Oguri (2). A dark bottle and four light bottles each of 250 cc capacity were placed in an assimilation box in the laboratory on shipboard. These four light bottles received 10, 25, 50 and 100 per cent of light respectively in assimilation box. No neutral filters were available on this cruise, so the bottles had to be covered with one to three layers of metal sieves instead of neutral filters. These possibly let the light pass through into the bottles with minor change of wave length composition. The bottles were kept at the temperature of the sea surface by running sea water around the bottles. Samples were taken from the sea in the morning or at midday and they were exposed to the light in the box for five hours from about 6:00 a.m. to about 11:00 a.m. or from about 1:00 p.m. to 6:00 p.m. For illumination in the assimilation box, two cool white fluorescent tubes of 20 watts were set at each side of the bottles. Each fluorescent tube emitted as much as 4000 lux at 100 volts as measured with a selenium photocell which is sensitive to 400-600 mμ wave length. Therefore, the light intensity emitted by four tubes would be roughly 16000 lux. However, unfortunately, the plastic wall of the tank in which the bottles were set was not absolutely transparent. It was found that the light was reduced by about one-half after it had passed through a plastic plate and again a glass plate having similar thickness to that of the experimental bottle. Accordingly, the maximum intensity of light which reached the sample water was supposedly only about 8000 lux. This intensity was undoubtedly insufficient for the experiments; the results obtained therefore are limited to a certain extent.

Table 1 shows the total cell number and chlorophyll content of phytoplankton, in the surface water at fifteen stations. Relative abundance of cells of leading species in the population and amount of carbon taken by plant organisms are also presented. Despite the fact that the location of samplings in most cases was far from land, most of the species contained in the samples were

TABLE 1

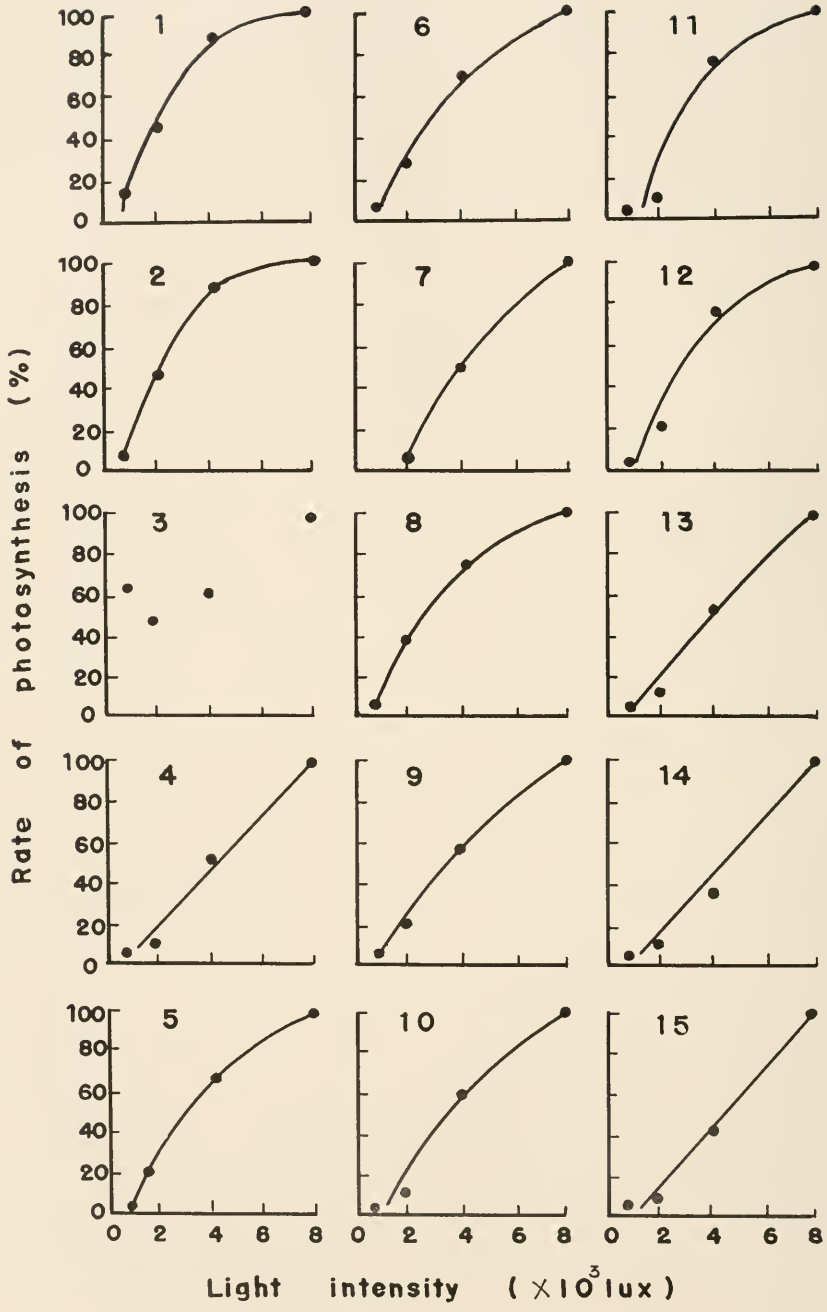
PHYTOPLANKTON STANDING CROP, PHOTOSYNTHETIC ACTIVITY, AND DOMINANT SPECIES IN THE POPULATION

Station	*Cells/m³*	*mg Chlorophyll -a/m³*	*mgC/hr/m³ at max. Light Intensity (8000 lux)*	*mg C/mg Chl/hr*	*mg C/ Cell/hr*	*Dominant Species in Population (Percentage in Cell Number)*
1	9186×10^4	2.89	3.0	1.0	0.3×10^{-7}	*Chaetoceros debilis* (77%), *Chaetoceros convolutus* (11%)
2	424×10^4	0.24	0.5	2.1	1.2×10^{-7}	*Chaetoceros debilis* (85%), *Chaetoceros convolutus* (60%)
3	224×10^4	0.44	0.7	1.6	3.1×10^{-7}	*Denticula marina* (82%), *Fragilaria oceanica* (7%)
4	176×10^4	0.93	1.3	1.4	7.4×10^{-7}	*Chaetoceros atlanticus* (55%), *Asteromphalus heptactis* (27%)
5	320×10^4	0.78	0.6	0.8	1.9×10^{-7}	*Denticula marina* (63%), *Asteromphalus heptactis* (10%)
6	184×10^4	0.30	0.5	1.7	2.7×10^{-7}	*Thalassiothrix longissima* (22%), *Fragilaria striatula* (17%)
7	268×10^4	0.17	0.5	2.9	1.9×10^{-7}	*Thalassiothrix longissima* (21%), *Nitzschia seriata* (16%)
8	1504×10^4	0.34	0.7	2.1	0.5×10^{-7}	*Fragilaria striatula* (78%), *Denticula marina* (13%)
9	180×10^4	0.18	0.9	5.0	5.0×10^{-7}	*Fragilaria striatula* (69%), *Denticula marina* (27%)
10	448×10^4	0.14	0.4	2.9	0.9×10^{-7}	*Fragilaria striatula* (43%), *Chaetoceros convolutus* (36%)
11	112×10^4	0.26	0.2	0.8	1.8×10^{-7}	*Fragilaria striatula* (93%)
12	176×10^4	0.44	0.7	1.6	4.0×10^{-7}	*Fragilaria striatula* (68%), *Nitzschia closterium* (18%)
13	120×10^4	0.41	0.7	1.7	5.8×10^{-7}	*Nitzschia seriata* (67%), *Asteromphalus heptactis* (20%)
14	2512×10^4	0.17	1.2	7.1	0.5×10^{-7}	*Coscinodiscus subtilis* (96%)
15	260×10^4	0.17	0.5	2.9	1.9×10^{-7}	*Fragilaria striatula* (42%), *Coscinodiscus lineatus* (15%)

neritic forms. Oceanic forms, such as *Chaetoceros atlanticus* and *Thalassiothrix longissima*, were found only in a few samples.

Carbon uptake by one mg of chlorophyll-*a* per hour varied from 0.8 mg to 7.1 mg, and that by one diatom cell varied from 0.3×10^{-7} mg to 7.4×10^{-7} mg. The variation of carbon uptake by unit weight of chlorophyll or one cell of diatoms was thus great from station to station. No correlation was shown between the amount of carbon uptake and characteristics of population or of environments.

Figure 2 illustrates the light assimilation curves (photosynthesis-light intensity curves) obtained by fourteen experiments made at different locations. In this figure the light intensity is put on the abscissa taking the half of values of light intensity emitted by four fluorescent tubes because of the reduction of light by the plastic and the glass plates. The rate of photosynthesis is plotted in percentage on the ordinate taking the photosynthesis at the maximum light intensity (8000 lux) as 100 per cent. Since the maximum intensity of light provided was pretty low, most of the assimilation curves did not reach the light saturated region in photosynthesis. Photosynthesis became light saturated only at about 5000 lux at Sta's 1 and 2, and around 8000 lux at Sta's 8, 11 and 12. So far as these five results are concerned, the optimum intensity of light in photosynthesis was far lower than in phytoplankton population reported in other areas by previous workers. Steemann Nielsen and Jensen (5) reported that phytoplankton photosynthesis became light saturated at about 30000 lux in the tropical water, at 20000 lux in the Tasman Sea (46°S) in the summer, at about 15000 lux in the North Atlantic (62°N). Steemann Nielsen and Hansen (6) again reported that light saturation in photosynthesis in the Atlantic was shown at about 13000 lux at 69°40′N, at about 10000 lux at 63°35′N and at 6000-7000 lux at 62°00′N. In Davis Strait in the North Atlantic (64°N) it was 11000 lux (7). Dim light of the northern North Pacific in spite of length of daylight time in the summer might have been responsible for the low intensity of optimum light in photosynthesis. In the Bering Sea and Aleutian region, and also western North Pacific misty weather is often met with in the summer; phytoplankton in the surface water


Rate of photosynthesis (%)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0
20
40
60
80
100
0
2
4
6
8
Light intensity (×10³ lux)

may not usually be exposed to bright sunlight.

This may suggest the fact of adaptation of phytoplankton in their photosynthesis to the dim daylight in their habitat as stated by Ryther and Menzel (4) and by Steemann Nielsen and Hansen (7). Ichimura (3) experimented on adaptation to light by lake plankton and found that the effect of past history of light conditions did not affect the photosynthetic character of algae for long after the algae were transferred to a habitat with different light conditions. Steemann Nielsen and Hansen (7) stated that the rate of the enzymatic process in photosynthesis increases with increase in temperature, and that adaptation is brought about simply by establishing a higher or lower concentration of the enzymes active in photosynthesis.

Populations in which a single leading species occupied more than 60 per cent of the total cells were found at Sta's 1, 2, 5, 8, 9, 11, 12, 13 and 14 (Table 1). The population at Sta's 1 and 2 was mainly composed of *Chaetoceros debilis* which is known as a boreal neritic species. The photosynthetic activity of this population reached the maximum at the light intensity of about 5000 lux, while the optimum light intensity for photosynthesis of the population at Sta's 8, 11 and 12 which was mainly composed of a boreal neritic form, *Fragilaria striatula*, was at about 8000 lux or more; a similar population (*Fragilaria*) at Sta. 9 did not reach light-saturated region at 8000 lux. The photosynthesis of the population mainly composed of a cold water form *Denticula marina* at Sta. 5 seemed to be almost light-saturated at 8000 lux. The mixed population with a boreal oceanic species, *Chaetoceros atlanticus*, and an oceanic temperate species,

Fig. 2. Light assimilation curves obtained at 14 stations in the North Pacific 42°N - 61°N. 1) 43°13′N 151°05′E (morning sample), 2) 50° 09′N 170°00′E (morning sample), 3) 54°56′N 170°10′E (morning sample), 4) 57°00′N 173°19′E (morning sample), 5) 57°00′N 174°50′E (midday sample), 6) 48°24′N 173°11′W (morning sample), 7) 48°22′N 172°00′ W (midday sample), 8) 53°09′N 172°00′W (morning sample), 9) 53°31′ N 171°55′W (midday sample), 10) 60°14′N 173°24′W (morning sample), 11) 61°01′N 173°24′W (midday sample), 12) 44°06′N 168°40′W (morning sample), 13) 43°20′N 170°03′W (midday sample), 14) 49° 19′N 174°08′E (morning sample), and 15) 42°19′N 172°52′E (midday sample).

Asteromphalus heptactis, at Sta. 4 did not show any indication of light saturation at 8000 lux. The photosynthesis of this population seemed to require much higher intensity of light. This was also true of the mixed population with a boreal oceanic species, *Thalassiothrix longissima* and a neritic species, *Fragilaria striatula*, at Sta. 6, of the mixed population with *Thalassiothrix longissima* and a neritic species *Nitzschia seriata* at Sta. 7, and of the mixed population with *Fragilaria striatula* and an oceanic cold water species, *Chaetoceros convolutus*, at Sta. 10. The photosynthesis of the population mainly composed of a neritic species, *Nitzschia seriata*, at Sta. 13, of the population almost exclusively composed of a cosmopolitic species, *Coscinodiscus subtilis*, at Sta. 14, and of the mixed population with *Fragilaria striatula* and a widespread species, *Coscinodiscus lineatus*, at Sta. 15, did not show any retardation under increasing intensity of light up to 8000 lux.

From the observations made in the course of this study the conclusion can be drawn that under the environments in the northern North Pacific, *Chaetoceros debilis* would be light saturated in its photosynthesis at about 5000 lux. Talling (8) proposed to use the value, *Ik* which is equivalent to the light intensity at which in a photosynthesis-light intensity curve, the extrapolation of the initial linear region and the light saturated region intersect (7). *Ik* of *Chaetoceros debilis* in the present case corresponds to about 3800 lux. The photosynthesis of *Fragilaria striatula* and *Denticula marina* would be saturated with light at about 8000 lux, while *Nitzschia seriata* and *Coscinodiscus subtilis* require much more intensive light than 8000 lux to attain the maximum activity of photosynthesis.

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Chapter 27

Succession of Phytoplankton, and the Ocean as an Holocoenotic Environment*

Theodore J. Smayda

Numerous investigations of the annual phytoplankton cycle in marine waters have revealed 1) a conspicuous recurrent succession of species, and 2) a marked cyclic abundance distinguished by one or more well defined maxima. Although traditionally treated as separate phenomena, there is little doubt that succession and the attendant seasonal population dynamics are inextricably related, even though the nature of this interdependence has yet to be adequately defined.

Dating from Brandt's (5) application of Liebig's Law of the Minimum to the sea, marine ecologists have sought to attribute the annual quantitative fluctuations of phytoplankton to some environmental "limiting factors." Consistent with Liebig's Law, nutrient limitation is frequently ascribed (19), although the limiting factor hypothesis has been extended to include those conditions which control the initiation of growth as well: inadequate light (7) or water mass stability (4).

Such specific environmental regulation has not satisfactorily accounted for succession, however. While the magnitude of the light, nutrient and temperature levels appears to determine how much a community will produce, community re-organization frequently begins before the productive limit is reached for that stage. Accordingly, it does not necessarily follow that the factor(s) limiting production, such as nutrients, also regulate the observed succession. Succession is accompanied by gradual seasonal changes in the temperature, light and nutrient cycles, a

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condition which also obscures the underlying causes of this phenomenon.

Culture studies have demonstrated that phytoplankters have specific temperature, light, salinity and nutrient requirements, indicating that these factors are involved in succession. One can easily apply biogeographical data to the problem of succession on the probably valid supposition that those environmental factors governing species distribution also influence their temporal occurrence within an area. Accordingly, temperature may regulate succession[1] since the distribution of many species, such as the arctic diatoms *Thalassiosira hyalina* and *Bacterosira fragilis*, appears to be temperature-dependent (48).

The salinity-dependent distribution of many organisms (54, 48) may be reflected in the succession of populations occurring in environments where significant variations in dilution may occur.

Phytoplankton species can be classified as "sun" (heliophilic) or "shade" (umbriphilic) loving species, suggesting that light may also be important in succession. Among the pelagic arctic diatoms, *Fragilaria oceanica, Achnanthes taeniata* and *Nitzschia frigida* have been found in active growth on the underside of ice (*vide* 49). Steemann Neilsen (51) distinguished between oligophotic, mesophotic and euphotic species of *Ceratium* on the basis of their vertical distribution. Findenegg (11) demonstrated that light determined both the vertical distribution and seasonal occurrence of certain limnetic species.

Rodhe (42) has shown experimentally that certain limnetic phytoplankters vary in their tolerance to levels of basic nutrient concentration. This observation lends credence to Peter's (32) belief that the distribution of certain marine *Ceratium* species is controlled by phosphate and nitrate concentrations. Accordingly, nutrient levels and the degree of "saprobism" characteristic of the species may influence succession.

The inadequacy of reliably ascribing a given successional change to any of the above factors, despite their involvement, suggests that only in extreme instances is succession governed

[1] Succession will refer to the appearance or disappearance of a species or community.

exclusively by a single factor. Rather, the responses of cultured and natural populations and the gradual seasonal changes in growth parameters suggest that phytoplankters have specific tolerances as defined by Shelford's Law of Tolerance (31, p. 26). This is reflected in the classification of phytoplankton as eurythermal or stenothermal, euryhaline or stenohaline, and so forth.

Biological interrelationships are also potentially important in succession. Lucas (25, 26, 27, 28) has instanced numerous examples of biotic interplay in formulating an "ectocrine" theory which assigns a general ecological role to organic substances in the ocean, including the possible mediation of phytoplankton succession. This important concept has stimulated many discoveries of the ecological role of metabolites (45), including the well-documented observations of Droop and Provasoli that many phytoplankton species require vitamins. Metabolic products, therefore, may be involved in succession through their growth-regulatory properties which would determine the presence or absence of species.

The ultimate influence of metabolites, as with other growth factors, is regulation of cell division irrespective of whether they are phyto-toxic or phyto-stimulatory. It remains to be determined, however, whether such metabolites act independently of other growth factors, or whether metabolites are only one of a combination of variables whose collective, simultaneous and interdependent operations govern succession and the cyclic abundance of phytoplankton. Much of the following discussion will concern the application of the ectocrine concept to succession primarily because it is particularly amenable to bringing out certain aspects of environmental regulation of succession and growth variables in general.

SUCCESSION AND NATURAL CYCLES

Only succession generated by the autochthonous production of organic substances, or physical environmental changes associated with natural and normally occurring climatic and biological cycles will be considered. Succession induced by extraneous factors such as domestic pollutants (2) or the nitrogenous-rich effluent of duck farms (44) are of relatively local interest and

represent a drastic, unnatural modification of the flora and its ensuing dynamics.

Gross disruptions of historically established phytoplankton cycles (14) are more readily attributable to sequential (15) or more protracted hydrographic disturbances than to the vagaries of antibiotic production. Minor departures from the customary pattern of succession can likewise frequently be related to unusual climatic conditions (7). "Red tides" and other blooms of extraordinary intensity and duration, however, may represent instances of environmental imbalance resulting from unchecked organic substances. However the water mass size and its degree of physical insulation appear to be important in the generation and maintenance of such blooms (47).

It should also be noted that the nature, rate of succession and the species involved generally appear to be relatively constant irrespective of annual fluctuations in the magnitude of the standing crop. Whereas if metabolites or changes in nutrient concentrations were the mainspring of succession, one would expect the rate of succession and the degree of change in species composition to be approximately proportional to the density of the populations producing these metabolites, or consuming the nutrients.

It is revealing, then, that the most marked communal disruptions accompany the incursion of water masses, climatic deviations which may affect light intensity, water temperature and stability, and "red tide" blooms which are accompanied by local "atypical" hydrographic conditions. In other words, these are environmental disturbances which produce changes in many growth variables upsetting the previous equilibrium and leading to community re-organization. These observations suggest that rigorous environmental control of growth parameters normally occurs preventing a given factor from disrupting phytoplankton cycles. This control may account for the orderly, predictable process of succession characteristic of a given area, rather than an erratic, unpredictable succession that would be expected if metabolites, light or nutrients solely determined succession.

Let us examine what environmental controls may be operative in counteracting the unilateral regulation of succession assuming metabolites are involved.

LIBERATION AND ENVIRONMENTAL CONTROL OF METABOLITES

It is uncertain just how conservative natural phytoplankton populations are in the liberation of metabolites during active growth (13, 12). It is known that the production and liberation of certain organic substances by healthy populations are governed by the prevailing environmental conditions, including light intensity (12), temperature and nutrients (23). The regulation of metabolite production and liberation, apparently existing during active growth when cells would appear to be especially vulnerable to such substances, progressively declines, resulting in an increased liberation during senescence (37; among others). Indeed, Duursma (10) has concluded that dissolved organic substances emanate primarily from dead cells.

Although the data are sparse, they consistently suggest that the production and secretion of metabolites are regulated by environmental conditions and are related to cell vigour—increasing with senescence and followed by copious liberation upon death. The ecological implications of this will be returned to.

Not only does the production and liberation of organic substances appear to be regulated during certain growth stages, but following their secretion as well. There is presumptive evidence that environmental forces restrict the accumulation and attenuate, if not completely neutralize, the potential biological effects of metabolites. Accordingly, the potential activity of a metabolite as a nutrient or growth regulator appears to be determined, *inter alia*, by its: 1) degree of intrinsic stability, 2) degree of non-adsorptiveness, and 3) resistance against biological degradation—all being coupled with the capacity of the watermass to minimize loss through dispersion (4, 47).

Stability

It is obvious that a substance which is thermo- or photolabile under the environmental conditions encountered upon liberation may become less potent. It appears that metabolites are particularly amenable to environmental modification. Kashiwada *et al.* (24) observed that 50-81 per cent of vitamin B_{12} decomposed in the upper 20m upon exposure to solar irradiation.

Vishniac and Riley (55) mention the potential non-biological destruction of thiamine induced by pH and temperature. The photo-oxidation of unsaturated fatty acids present in inert *Chlorella* extracts leading to antibacterial activity is a notable example of environmentally induced alteration of organic substances (50).

The discovery that many soil antibiotics are highly unstable under the prevailing pH conditions (22) is undoubtedly representative of the marine environment as well. Fatty acids appear to be the toxic principles involved in unequivocal cases of algal and bacterial antibiosis (50, 35, 46). These investigators have shown that the toxicity is very pH dependent, short-chained fatty acids becoming highly active at low pH, while alkaline conditions spurred the activity of others. The toxicity of these compounds is markedly reduced near neutrality. Consequently, the high buffering capacity of sea water, which needs no elaboration here, probably represents a most formidable environmental constraint upon the potential activity of this group of compounds.

Adsorption

There are numerous substances in natural waters which function as adsorbents, including gels, colloids, terrigenous particles and organisms (43, 16). Admittedly, sorption reactions are dependent upon numerous environmental factors (6) and probably vary in importance throughout a growth cycle. ZoBell (56) has shown that inert solids adsorb measurable quantities, 2 to 27 per cent, of organic matter in sea water. Adsorption, which may be considered analogous to an activated charcoal (self-purification) process, not only reduces the soluble concentration of organic substances, but increases the likelihood of bacteriological degradation.

Biological Degradation

It is well established that marine bacteria require a suitable substrate for development, and that the concentration of organic matter is a principal factor affecting heterotrophic bacterial growth (56). Although adsorbed organic material in clay lattice may be somewhat resistant to biological decomposition (16), ad-

sorbed organics generally provide convenient microcosms for bacterial activity. It is not to be inferred, however, that only adsorbed organic matter can be degraded. There is evidence that variable pelagic bacterial communities exist (56). Although dead phytoplankters are readily decomposed by bacteria (56), increased activity of natural phytoplankton communities is likewise accompanied by a heightened bacterial response (56, 34). Indeed, Pratt and Berkson suggest that this increased bacterial activity may facilitate further phytoplankton growth through nutrient regeneration. These data, coupled with the remarkable facility of marine bacteria to degrade a vast spectrum of organic types (56), suggest that the susceptibility of metabolites to bacterial degradation may severely attenuate their inherent biological potency. It is difficult to forebear the conclusion that a metabolite must also be markedly bacteriostatic (although the bacteria are capable of altering metabolites and producing new ones) beyond that normally attributed to sea water and algal metabolites in order to exert a major influence in succession.

NATURE OF METABOLITES AND ROLE IN SUCCESSION

Algal metabolites may be biologically inert, or active as nutrients (36) or more subtle growth regulators (45), being either phyto-toxic or phyto-stimulatory in the latter instance. As growth regulators, they are also auto- or hetero-regulatory. But regardless of their biological influence their ultimate effect is regulation of cell division.

It must be remembered that the production of active metabolites is ecologically significant to both the producer and its competitors. Assume a species produces a metabolite which "chemically" affects only its competitors. It is apparent that this species may be ecologically auto-inhibited or auto-stimulated, depending on the physical environmental conditions, in that its abundance and duration of occurrence may be curtailed or promoted. The nature and regularity of succession suggest, then, that if ectocrine substances are important in this phenomenon they must be intimately related to the temperature, light and nutrient regimes enabling a consistent, predictable ecological response of the producers and reactants alike. Otherwise, an irregular, non-

predictable succession might be expected if metabolites were produced and operated independently of physical growth parameters.

It is obvious that the impact of a growth regulator upon a community or species is one of degree. For example, lethal phytotoxins are potentially more important in succession than those only moderately toxic. Yet it is significant that antagonism between species is related to their relative population densities and the extent of acclimatization. Rice (37) has demonstrated that a species suddenly exposed to an antagonist is usually more intensely inhibited than if both species had grown together over an extended period. Lucas (26) appropriately points out that evolutionary adaptation to metabolites must have occurred between species growing together over interminable generations. That phytoplankton species adapt to diverse environmental conditions is demonstrated by the responses of natural communities (52) and the diatom *Biddulphia mobiliensis* (18) to various light levels. Additionally, it is commonly experienced that past environmental conditions determine the growth of species upon sub-culture. It might be that species adaptation to metabolites represents an additional control over the exclusive regulation of succession by these potent substances. A similar conclusion might apply to the other growth parameters. It follows that should a "wild" metabolite be introduced into, or emanate within, an historically established community, a floristic catastrophe such as a "red tide" or *Phaeocystis* bloom may occur—an abnormal disruption of succession (27).

Let us now consider the nature of the various growth regulators and their potential role in succession. There are two major groupings of phyto-stimulatory substances: I) those that only supplement existing growth, i.e., *not* required for normal growth; and II) those substances required for growth. Since growth progresses independently of the availability of substances in category I, they are probably of minor significance in succession. For example, it is doubtful that an auto-stimulated species can significantly influence succession. It does not create a niche for itself since it already occupies it. Therefore, it might prolong its presence and/or promote abundance through the competitively

awkward sequence of: nutrient assimilation followed by the production and liberation of a metabolite which may then be assimilated or favorably alter the physical environment.

The second group of phyto-stimulatory compounds comprises those absolutely required for growth, such as vitamin B_{12} or thiamine (9, 36). Since a requirement, or its lack, for these substances is absolute (9, 36), as it is for nitrogen or phosphorus, minimal quantities may limit growth because of depletion, not as a phyto-toxin, while high concentrations may prolong growth, but not as a phyto-stimulant in the sense of the ectocrine theory as it is applied to succession. As accessory substances they fall within the realm of potential limiting factors along with inorganic nutrients.

What are some consequences of inhibitory substances on succession? Assume Species A produces hetero-inhibitory substances during its advancing senility. Adherence to the ectocrine theory might suggest that Species A is protecting its dominance through inhibition of competitors. But since senility undoubtedly results from an inadequate environment, Species A enjoys no real competitive advantage, for inhibition of its competitors will neither permit its own rejuvenation nor alleviate the conditions inducing senility.

It is recognized that phytoplankters are characterized by intrinsic differences in specific growth rates (3) which undoubtedly vary with environmental conditions. Assume now that vigorous cells of Species A liberate hetero-toxic substances, probably in less copious amounts than during senescence. Nothwithstanding these metabolites, or even stimulatory substances, it is obvious that Species A, or a stimulated species, may prolong its "chemically" induced dominance provided its basic metabolic requirements are met, and the environment favors its intrinsic growth rate over that of its competitors. But recall that light, temperature, nutrients and the general environmental regimes are likewise changing during the temporal progression of succession, i.e., conditions also causing simultaneous alterations in the growth rates of all species. Once again it seems likely that unless a metabolite is produced in prodigious amounts its influence on

succession is readily tempered and applied through interactions with physical growth factors.

HOLOCOENOTIC CONCEPT OF PHYTOPLANKTON SUCCESSION AND DYNAMICS

Ectocrine substances undoubtedly influence succession. However, the over-riding influence of the environment normally preventing the independent action of such growth regulators has been demonstrated. This environmental control is exerted along the following lines: 1) the production and liberation of metabolites are governed by the prevailing environmental conditions; 2) metabolite liberation appears to be most copious when least likely to provide competitive advantages; 3) stringent physical and biological restraints tend to curb the accumulation and attenuate metabolites upon liberation; and 4) evolutionary adaptation of naturally occurring communities has probably moderated the capacity of species for "chemical warfare."

Although the role of ectocrine substances in succession was emphasized in the previous section, essentially the same conclusions result if the unilateral regulation of succession by light, temperature or nutrients is applied. Environmental control of these physical growth factors is most apparent in the frequent coupling of variables resulting in certain physiological or ecological responses. Some of these interactions, and their effects include:

Temperature—Salinity

Ritchie (41) has demonstrated that the growth of marine fungi is related to the combined influence of temperature and salinity. This interaction also governs the distribution of higher algae (8) and dinoflagellates (30).

Temperature—Light

There are numerous examples of temperature–light interactions. Findenegg (11) clearly demonstrated that the seasonal occurrence of certain limnetic phytoplankton species is determined by this interaction, permitting the following classification:

	Schwachlichtformen	*Starklichtformen*
Kälteformen:	*Winterplankter*	*Frühjahrsplankter*
Wärmeformen:	*Herbstplankter*	*Sommerplankter*

A similar interaction determines the seasonal occurrence of many marine species, as demonstrated in Conover's (7) experiments with natural phytoplankton populations in Long Island Sound. Experiments with light adapted cultures of the diatom *Biddulphia mobiliensis* suggest that upon exposure to other intensities the growth rate is also a function of temperature (18).

Halldal (17) has observed that pigment formation in the limnetic phytoplankter *Anacystis nidulans* is also dependent on the simultaneous action of temperature and light intensity. Finally, it is well known that photosynthesis is determined by the combined action of temperature and light above certain light intensities (20).

Temperature—Nutrients

Hutner *et al.* (21) have demonstrated that the thiamine and vitamin B_{12} requirements of the chrysophycean *Ochromonas malhamensis* are related to temperature; indeed, there appears to be a general relationship between temperature tolerance of natural communities and nutrient availability (48). The marine diatom *Thalassiosira nordenskioldii* attains a maximum at 2° to 3° C in Nature and disappears at slightly higher temperatures (15, 7, 33). Yet this diatom has exhibited prolific growth in culture at 10° C (1) when nutrients presumably were more concentrated than normally encountered in Nature. Braarud (2) has observed boreal diatoms in active growth at 20° C in polluted areas of the Oslofjord (Norway) whereas growth was negligible in non-polluted areas.

McCombie (29) observed that upon doubling the nutrient concentration (Chu No. 10 medium) the lower limit of temperature tolerance of *Chlamydomonas reinhardi* shifted from 6° to 12° C and the optimum dropped from 28° to 18° C. The magnitude of the decrease in photosynthesis and growth of natural populations caused by phosphate depletion is determined by temperature (39). The limiting effects of low phosphate supplies become less pronounced as the temperature increases.

There are other examples of an interaction between growth factors. Sverdrup's (53) "critical depth" theory demonstrates that light intensity coupled with water mass stability is a critical factor governing the inception of growth in natural populations after the winter minimum. Significantly, Riley's (38, 39, 40) statistical assessment of the factors influencing the dynamics of natural populations has been more successful when these variables have been treated collectively rather than singly.

This diverse but consistent body of evidence indicates that many factors are simultaneously operative in determining phytoplankton growth. The manifold environmental interactions of growth variables reveal the ocean to be a *holocoenotic* environment, that is, an environment in which the observed phytoplankton dynamics are the *resultants* of the collective, simultaneous and interdependent action of many parameters. The combined action of these variables is expressed in the recurrent succession and cyclic abundance of marine phytoplankton.

This conclusion is not new, although its significance appears to have been frequently overlooked in the design of field and experimental studies. Yet a scientific approach based on the holocoenotic concept has merit in being more representative of natural conditions, which enhances its prospects of contributing significantly to the development of a quantitative theory of phytoplankton dynamics.

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Chapter 28

Some Relationships of Phytoplankton to Environment

E. J. Ferguson Wood

In my laboratory, attention is directed at the moment to the study of phytoplankton in situ, advantage being taken of the furnishing by the Royal Australian Navy of two frigates fitted out as oceanographic research ships. Using these ships, it is possible to collect samples for immediate examination, and at the same time to obtain chemical and physical data as required.

This paper gives some results of work to date, and is not intended to present a coordinated picture of phytoplankton ecology.

THE RELATION OF PHYTOPLANKTON TO TURBIDITY IN THE OPEN OCEAN

A striking feature of a study of fresh phytoplankton samples collected by centrifugation of 5 liter samples is the large amount of particulate matter which they contain. In the southwest Pacific and East Indian Oceans and adjacent seas, these particles consist of two types,—small (1-3 μ) opaque, and larger (10-30 μ) transparent or translucent particles. The ratio of the number of particles to the number of phytoplankters ranges from about 3:1 to 2,300:1, with a mean of 213:1 in the Coral Sea.

Figure 1 shows plots from an oceanic station in the Coral Sea in which, at left, turbidity (extinction coefficient, EK) is plotted against depth, while at right particle and phytoplankton numbers are plotted (numbers per liter). The shape of the curves suggests that turbidity at the surface is related to the total number of particles and that the hump in the turbidity curve at 75 m is due to the increase of phytoplankton at that depth. The central graph shows T-S relationships.

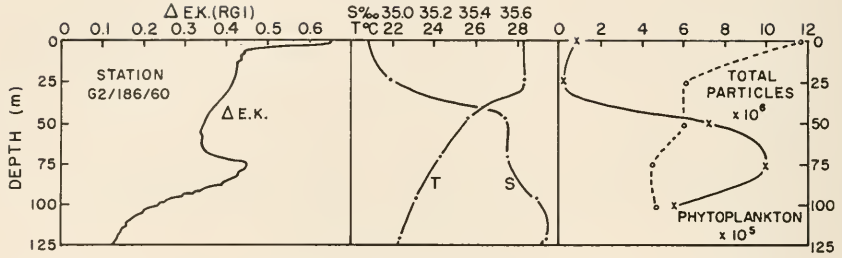

Fig. 1. Relations of extinction coefficient (EK) (at left) to particle and phytoplankton numbers (at right).

In one instance, a bloom of *Prorocentrum micans* was detected by a sharp increase in turbidity at 4 m and confirmed by a microscopic count of an intermediate sample taken at that depth.

Turbidity appears to depend on the number of particles irrespective of size, so it would appear that interesting relationships between turbidity, particle numbers and phytoplankton numbers may be determined by attempts to correlate these.

SORPTION AS A FACTOR

Fluorescence microscopy is used in our laboratory and at sea for the routine counting of phytoplankton, taking advantage of the autofluorescence of chlorophyll (5). This fluorescence can be observed through the transparent particles, and frequently several organisms can be observed as sorbed on one of the larger particles. Small phytoplankters sorbed on the smaller opaque particles emit a red halo. The relative number of sorbed organisms is greater when the catch consists largely of nannoplankton, and therefore differs widely in different catches. The presence of large numbers of particles also allows for the adsorption of nutrients and metabolites, and so may affect the population of microorganisms in several ways (4).

VERTICAL DISTRIBUTION

Samples taken at fixed stations on the continental shelf outside Port Hacking (Cronulla) over several years have shown that the greatest concentration of phytoplankton rarely occurs

at the surface, but more usually at 25 m and frequently at 50 m. In the open sea, in the Coral and North Tasman Seas, the highest concentration occurs between 50 and 100 m, frequently extending through this depth range, and occasionally at 150 m, at which depth phytoplankton is frequently quite plentiful. In the Southern Tasman, however, phytoplankton is more concentrated in 25 to 50 m of water, and the maximum has been found at the surface, at some stations. (see cruise data Gascoyne 2/60 and 1/61 C.S.I.R.O. Station Lists).

Both vertical distribution and total numbers of phytoplankters may vary diurnally (Tables 1 and 2), with a maximum in the daytime, not necessarily at the same hour, and a minimum at night. Table 1 shows that this variation is not restricted to photo-

TABLE 1

Fluorescent and Total Counts of Phytoplankton Collected at Depths of 1, 25 and 50 m in 60 m of Water to Study Diurnal Variation

Time	1700h	2100h	0100h	0500h	0900h	1300h	1700h	2100h	0500h
Fluor. count									
0m	10	3	2	2	4	0	2	4	4
25m	12	8	3	1	5	16	12	6	11
50m	12	10	10	8	2	10	7	5	1
Total at all Depths	34	21	15	11	11	26	21	15	16
Total count									
0m	57	31	23	32	60	120	120	27	25
25m	66	50	33	50	90	300	35	99	60
50m	125	58	51	54	30	86	128	73	30
Total at all Depths	248	139	107	136	180	506	283	199	115

Coefficient of variation approximately 36 per cent.

TABLE 2

Fluorescence Counts of Phytoplankton from Surface Samples Taken from a Ship at Anchor

Time	0700h	1115h	1615h	2030h	0700h
Mean count	3.6	27.6	72.3	0.7	1.3

synthetic organisms but also applies to the non-photosynthetic ones, which microscopy shows to consist chiefly of colorless flagellates. The samples for these tables were taken with the bottom at 30 fathoms in each case, so it may be concluded that there is a variation in the total number of microorganisms in the photic zone. This variation may be due to grazing, to rhythms in reproduction, or both.

HORIZONAL DISTRIBUTION

Horizontal distribution of phytoplankton has been studied along the edge of the continental shelf in waters derived from the Coral Sea, and in the southern Coral Sea between Lord Howe Island and Sydney. The results are given in Tables 3 and 4.

TABLE 3

PHYTOPLANKTON COUNTS OF SURFACE SAMPLES TAKEN AT THE EDGE OF THE CONTINENTAL SHELF FROM A SHIP STEAMING AT 12 KNOTS

Time	0830h	0900h	0930h	1420h	1500h	1530h
Mean count per field	4.05	4.15	4.30	7.0	7.75	7.45
Standard error	1.81	1.81	1.71	1.34	1.36	1.04

TABLE 4

PHYTOPLANKTON COUNTS AND TOTAL MICROORGANISMS OCCURRING ON THE SURFACE DURING A RUN FROM LORD HOWE ISLAND TO SYDNEY

Time	0845	0915	1945	1015	1145	1315	1415	1525	1625	1715	0645	0815
Fluor.	10	13	10	108	12	14	20	15	17	6	20	12
Total	95	93	88	102	71	84	150	135	114	71	200	350

In Table 3, the mean count of phytoplankton per field is of the order of four in the morning, and of seven in the afternoon and the difference at stations twelve miles apart is negligible. The difference between morning and afternoon stations is of the order of that found in studies of diurnal variation, and could be due entirely to this. In this case, the population studied in Table 3 could be regarded as uniform, and, in fact, it consisted of the same plant association, *Schroederella delicatula, Chaetoceros secundum, Gymnodinium simplex.*

Table 4, which shows a continuous run of 100 miles (from 0845 to 1715 hours), the population would once again appear uniform both as regards phytoplankters (fluorescent organisms) and total microorganisms except for diurnal variation. The higher counts on the second morning were probably influenced by proximity to land as they were taken on the continental shelf. Once again the species were uniform.

Studies of the phytoplankton counts from cruises of the research vessels referred to, show that, in general, in the Coral Sea, Arafura Sea and North-east Indian Ocean, the greatest concentrations of phytoplankton occur near land masses (see Cruise Reports of Gascoyne 1 and 2/60, 1/61 and Diamantina 2/60). Increases of population also occur in the vicinity of upwellings.

TROPICAL RED TIDES

An important feature of tropical waters in the South Pacific and Indian Oceans is the "red tide" due to *Oscillatoria* (*Trichodesmium*) *erythraea*. This blue-green alga occurs at times, especially in spring, in vast sheets on the surface. I have flown over such a red tide with an area of the order of 20,000 sq. miles. The concentration of the alga was confined to 0.5 m from the surface, with the greatest amount at the surface. It is usually in long windrows in the direction of the prevailing wind, giving the impression from the air of a series of sandbanks, shallow on one side, steep on the other. Although *Oscillatoria erythraea* normally behaves like other members of the phytoplankton, in the red tide stage it adheres to samplers and collecting bottles and cannot be retained by centrifugation. These *Oscillatoria* blooms are not confined to neritic areas, but occur in the open ocean. I have seen one such occurrence about 500 miles northwest of Fremantle in the Indian Ocean. In this case, the requisite concentration of nutrients could have been brought about by a divergence which exists west of Western Australia.

SALINITY AND TEMPERATURE

Determinative studies have now been made of phytoplankton from a large number of stations in the Southwest Pacific and northeast Indian Oceans, and the species have been recorded

on punched cards together with salinity, temperature, latitude, longitude, date, time, depth, etc. Sorting of these cards has just begun, but it has been found that delimiting phytoplankton organisms by salinity and temperature maxima and minima gives a reasonable means of determining "indicator species" and their significance, and thus expressing the ecological picture of the Australasian region with some accuracy.

A preliminary examination of the data suggests that T-S optima in the Pacific and Indian Oceans differ for certain species in a manner which may indicate different populations of the

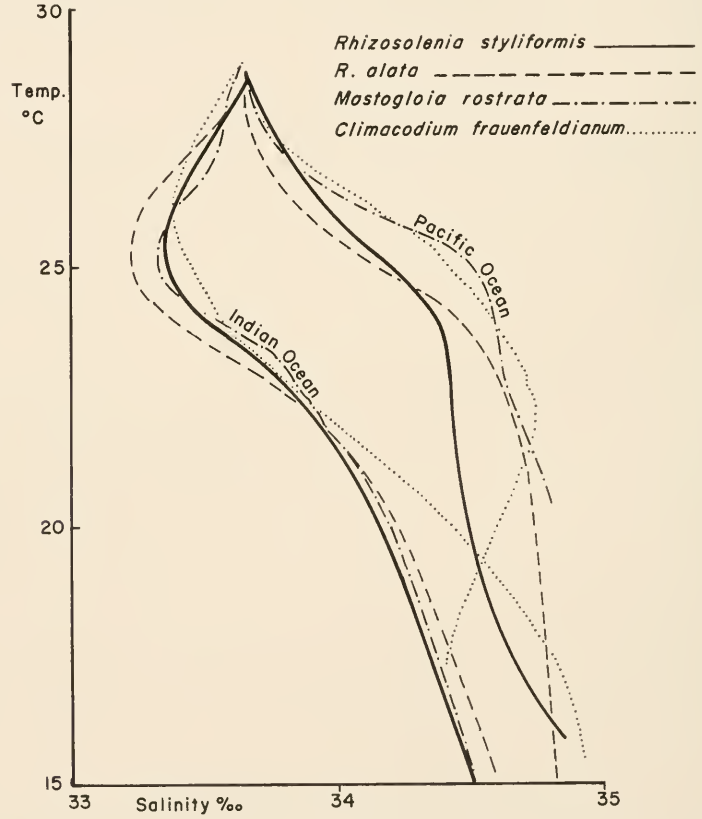

Fig. 2. Salinity-temperature relationships of certain species of diatom phytoplankton in the Indian (left-hand curve) and Pacific Oceans (right-hand curve).

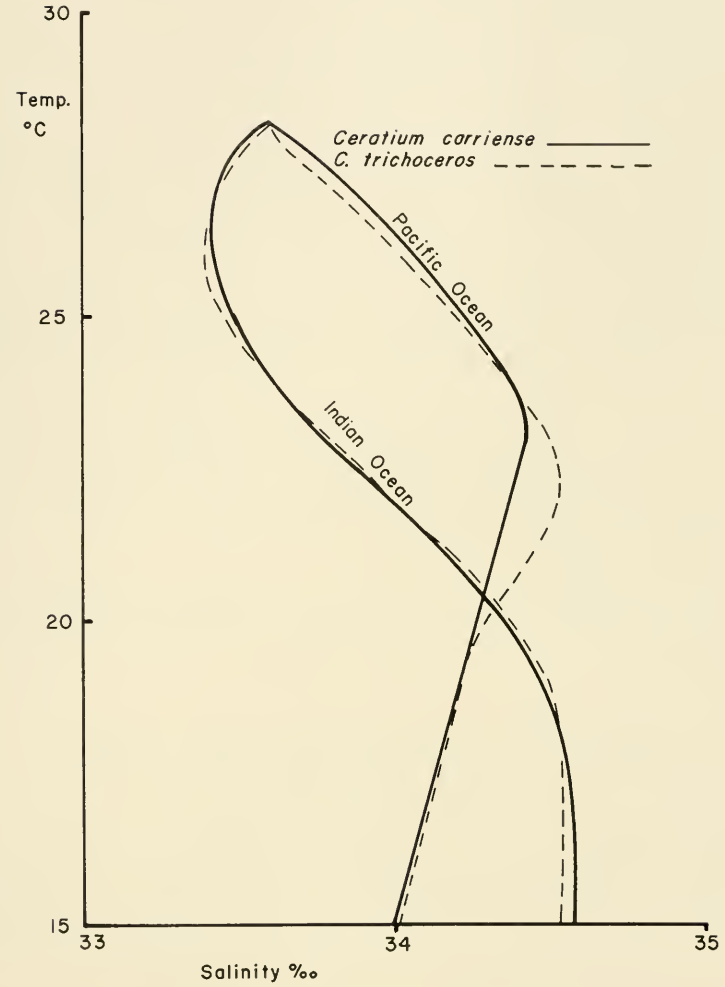

Fig. 3. Salinity-temperature relations of certain dinoflagellates in the Indian and Pacific Oceans.

species in the two oceans. It is too early yet to draw definite conclusions, but the work indicates that this method of study (Figs. 2, 3) may be productive of ecological information for many plankton species, and may be useful in delimiting the influence of certain biological water masses.

An interesting discovery is that of certain antarctic diatom

species (*Biddulphia weissflogii, Rhizosolenia chunii* and *Actinocyclus janus*) in the close vicinity of upwellings on the south coast of New South Wales. It is considered that these organisms could only have come with antarctic water which passes under the warmer water of the East Australian Current, i.e., below the photic zone, and that they must therefore have remained for a considerable time (years) in a spore or resting stage.

Other instances are the occurrence of several diatoms characteristic of the Arafura Sea (low salinity water) (*Chaetoceros messanense, Ch. laeve, Ceratium dens*) in a patch of low salinity water off New South Wales in 1958; and of cold water species (*Chaetoceros criophilum, Corethron criophilum, Chaetoceros convolutum*) near the 17° isotherm north of New Zealand. If we can fix the confidence limits of the salinity-temperature relationships of a number of phytoplankters, any occurrence outside the known regimen will demand an explanation, which must fit known hydrological data.

I would suggest that phytoplankton ecology may become an important tool in oceanography, quite apart from its importance in productivity studies.

THE EFFECT OF ORGANIC SUBSTANCES ON PHYTOPLANKTON

The existence of extracellular organic substances in the sea raises the question of the effect of such substances on the growth and photosynthesis of phytoplankton. Although *in vitro* experiments cannot be regarded as a true indication of *in vivo* activities, they may give valuable clues, especially in instances such as this where *in vivo* experiments are out of the question.

Methods. *Thalassiosira aestivalis, Skeletonema costatum, Dunaliella marina* and an *Isochrysis* were isolated from local waters in bacteria-free culture, *Dunaliella* by plating on agar, the others by dilution and washing after the method of Pringsheim (2). The cultures were made in large vessels, in Allen and Nelson's seawater (1), and divided for test into a number of aliquots. Counting was done using a Petroff Hausser bacterial counting chamber and a fluorescence microscope, taking advantage of the auto-fluorescence of chlorophyll. Dark and light

controls were set aside and the required nutrients added to the test flasks to bring the final dilutions of the test substances to 1×10^{-4}, 1×10^{-5} and 1×10^{-6} g/liter. The cultures were incubated in a water bath at 20° C, and illuminated by fluorescent lamps giving 300 lux. Counts were made at intervals, and the oxygen given off by photosynthesis was estimated by polarography under nitrogen, using a rotating platinum electrode. Results:— Figures 4 and 5 are graphs of typical results. In these cases asparagin caused an increase in the rate of reproduction of both *Dunaliella* and *Skeletonema*, but in the latter, the death rate was later accelerated. This may have been due to exhaustion of nutrients. With *Dunaliella*, asparagin increased the total oxygen production, but with *Skeletonema*, any increase is probably not significant. Of the substances tested, urea did not have any appreciable effect on any of the test organisms, cysteine caused increases in growth rate and oxygen production in *Dunaliella* and *Skeletonema*, but decreases in *Thalassiosira*. Asparagin, inulin and galactose gave increases in all cultures; arginine was bene-

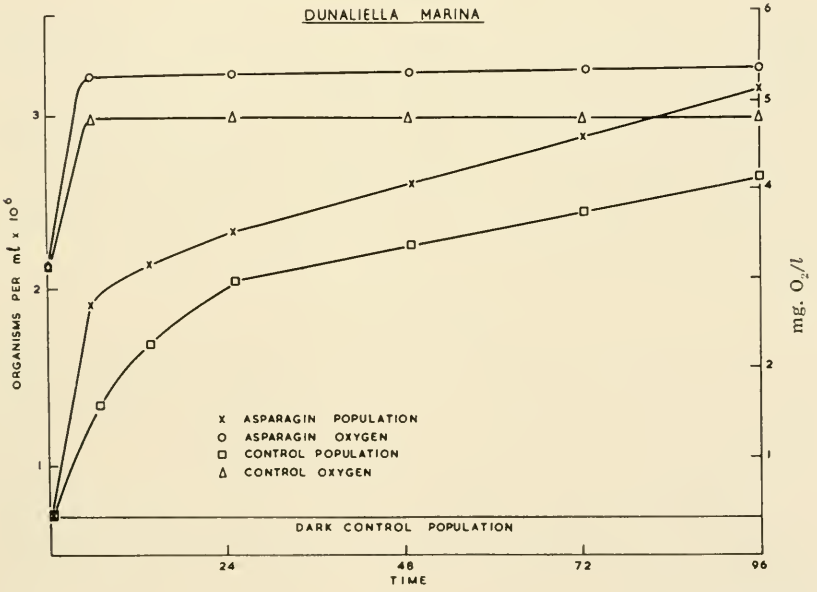

Fig. 4. Effect of asparagin on oxygen production and growth of *Dunaliella marina*.

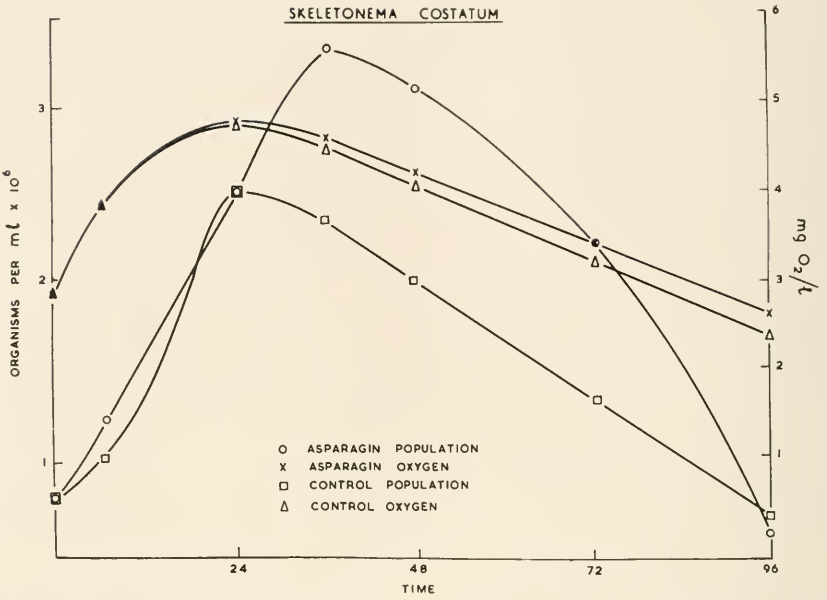

Fig. 5. Effect of asparagin on oxygen production and growth of *Skeletonema costatum.*

ficial for *Dunaliella* but adverse for *Skeletonema* and glucose and glycine seemed slightly inhibitory.

In each case, where beneficial effects were observed, these increased with concentration up to 1×10^{-4} g per liter.

These experiments, admittedly of a preliminary character, do suggest possible reasons for the selective growth of certain species such as is observed in phytoplankton blooms.

CONCLUSIONS

The presence in the open ocean of numerous particles could be an important ecological factor in phytoplankton distribution, due to sorption phenomena associated with the smaller microorganisms, with nutrients and with metabolites.

There is evidence that, in uniform water, horizontal distribution of microorganisms may be reasonably uniform, but that there is a diurnal variation in numbers, and possibly in their vertical distribution.

Salinity-temperature relationships of many organisms are

useful indicators of their distribution. The beneficial or restrictive effects of certain external metabolites may effect the growth rates and photosynthesis of phytoplankton organisms and may contribute to the biological selection of organisms occurring in blooms.

This paper is given in the hope that useful criticisms and suggestions may be forthcoming. It is fully realized that each section presents a very incomplete picture, and poses more problems than it solves, but it is a picture of research in progress, not of an accomplished program. Moreover, the program has to be fitted to the facilities available, and these in oceanography are usually a compromise.

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Chapter 29

The Effects of Osmotic and Nutritional Variation on Growth of a Salt-Tolerant Fungus, *Zalerion eistla* *

Don Ritchie and Myra K. Jacobsohn

The "optimum" salinity for vegetative growth in several marine fungi has been found to be a fluctuating value, shifting as temperature shifts. In a marine *Phoma,* for example, the salinity optimum varies from about 20 S‰ (20 parts total solids by weight per thousand parts of solution) at an incubation temperature of 16 C to about 48 ‰ at 37 C (7). This *Phoma* pattern has also been found in marine imperfect fungi of the genera *Pestalotia, Curvularia,* and *Zalerion.* It is irregular in some genera (e.g., *Helicoma*) and absent in others (*Alternaria, Pullularia, Spicaria,* etc.). Fungi isolated from terrestrial habitats (*Mucor, Aspergillus*) and tested did not exhibit the *Phoma* pattern (8, and Ritchie, unpublished). These data tell only that an increasing temperature is accompanied by an increasing salinity "optimum," but they give no clue as to whether that optimum reflects an increasing tolerance or an increasing salt demand, nor do they afford any indication of the mechanism involved.

Phoma herbarum did not grow at all at 16 C in a medium containing 90 S‰ while it attained a colony diameter of 36 mm at 25 S per mille. At the other extreme, a marine *Curvularia* did not grow at all at 37 C in a medium containing 10 S‰ while it attained a colony diameter of 10 mm at 100 S‰. Thus an increasing temperature is accompanied by an increasing salinity "optimum," but the data do not show whether that optimum reflects an increasing tolerance or an increasing salt demand, nor is the mechanism involved indicated.

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Several modes of action appear possible. Inasmuch as many of the components of sea water are usable in metabolism, the heightened salinity optimum at higher temperatures might be a nutritional requirement, and if it is, the salt or salts concerned might be replaced by others. If the phenomenon is due to the action of some specific enzyme or enymes, the metals especially would be critical components of the medium. The effect might also be due to membrane activity, in which case specific salts might again be critical. Still another possibility is that the phenomenon is primarily an osmotic one, in which the salt could be essentially a diluent of water. If that is so, one should, by using any non-toxic solute, be able to produce growth curves similar to those obtained from saline cultures.

The experiments described in the present paper were performed in an effort to clarify the problem of why incubation temperature and salinity are interrelated, and to find what other factors, if any, are involved in the laboratory culture of marine fungi.

MATERIALS AND METHODS

The present investigations were conducted with a culture of *Zalerion eistla* Moore and Meyers, from Argentia, Newfoundland, supplied by Dr. S. P. Meyers of the University of Miami Marine Laboratory. The fungus was routinely cultured on a medium containing 0.3 g $NaNO_3$, 0.05 g KCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.001 g ferrous acetate, 0.1 g Difco yeast extract, 0.5 g glucose, and 1.5 g agar per 100 ml, a mixture which will be referred to as the basic medium.

In the first experiment, which demonstrated the response of *Z. eistla* to temperature and salinity, we used artificial sea water made up according to Provasoli *et al.* (6), with total salt content, by weight, of 1.0 per cent, 2.5 per cent, 3.4 per cent, 5.5 per cent, 8.0 per cent, and 10.2 per cent. The medium in each run contained 0.1 per cent yeast extract, 0.5 per cent glucose, and 1.5 per cent agar. Petri dish cultures were incubated at 6 C, 15 C, 20 C, 25 C, 30 C, and 37 C. The 6 and 37 incubators varied about 1 C, the others less. Although the plates in the 30 and 37 incubators were kept in covered containers lined with moist paper, some drying of the media occurred. Salt concentrations at

the end of a run were consequently higher than at the start.

When Z. *eistla* had been shown to have a *Phoma* type response to varied temperature and salinity, other substances were substituted for the constituents of sea water in order to determine whether control of the growth rate at a given temperature is a function of salinity specifically or of osmotic pressure in general.

Sodium chloride was chosen for one series of experiments. Media were prepared containing 0.17 M, 0.48 M, 0.58 M, 0.95 M, 1.38 M, and 1.74 M NaCl. These concentrations provide an amount of salt, as NaCl, equal to the total amount of mixed salts present in the sea water experiment. Other salts, yeast extract, and glucose were added in the same amounts as in the basic medium while in other experiments, other compounds replaced NaCl. In one series we used glucose as an example of a non-ionized, metabolically active compound capable of providing an osmotic gradient.

Glycerol, when incorporated in the medium, produced no better growth than controls containing 0.1 per cent yeast extract as the only other carbon source. It was consequently used in one experiment as an example of a non-ionic, non-metabolizable, osmotically active compound. Media were prepared with glycerol in the same molar concentrations as NaCl in the experiment described above, with glucose, yeast extract, and salts, solidified with 0.85 per cent Oxoid Ionagar. In the last three experiments described here, cultures were incubated at the full range of temperatures.

An incomplete experiment was performed using pentaerythritol as a non-ionic, non-metabolizable, osmotically active compound to which cell membranes are impermeable (1). It was added to the usual glucose-yeast-extract medium in concentrations of 0.07 M, 0.18 M, 0.25 M, and 0.40 M. Pentaerythritol, being insoluble in water beyond 0.40 M, did not allow a complete series for comparison.

Growth was plotted against osmotic pressures in atmospheres. Figures for the osmotic pressures of sea water are from Pearse and Gunter (5); glucose equivalents are from Morse *et al.* (4). Experimental values for glycerol and erythritol were not available, but as both are non-electrolytes, we worked on the assump-

tion that the osmotic values of their solutions are the same as those of equimolar glucose solutions.

Osmotic value changes due to temperature changes would never have exceeded 5 per cent over the range of temperatures used. Because of this, plus the fact that the measurement of colony diameters suffers as great or greater error, temperature corrections have not been included. Even with temperature adjustment, however, no great differences in the curves result.

Because calcium is a normal constituent of sea water, and because its presence is known to affect plasma membranes, its concentration was considered as a possible factor in the *Phoma* pattern. A basic medium containing the calcium in the 0.1 per cent yeast extract or present as contamination was prepared with solutions to which were added 0, 25, 50, 100, 250, 500 and 1000 parts per million of calcium, solidified with specially purified Oxoid Ionagar and poured into plastic petri dishes. Cultures were incubated at 25 C ond 30 C.

Various concentrations of yeast extract, peptone, and casamino acids were incorporated into media in order to determine whether certain nutrient factors might affect the *Phoma* pattern. To the basic medium, solidified with 0.85 per cent Oxoid Ionagar, we added Difco yeast extract in concentrations of 0 per cent, 0.1 per cent, 0.2 per cent, 0.4 per cent, 1.6 per cent, 3.2 per cent, and 5.0 per cent. Peptone and Difco casamino acids were each made up in the same way in concentrations of 0 per cent, 0.05 per cent, 0.1 per cent, 0.2 per cent, 0.4 per cent, 0.8 per cent, and 1.6 per cent. These three runs were incubated at only two temperatures, 25 C and 30 C, because these temperatures were known from previous experiments to be instructive.

A subsistence nutrient medium was worked out to study the effect of limited food supply on the *Phoma* pattern and was arrived at by measuring the growth of the fungus on a number of media which contained salts and agar in the usual amounts, and all possible combinations of the following percentages of yeast extract and of glucose: Yeast Extract: 0 per cent, 0.0125 per cent, 0.025 per cent, 0.05 per cent; Glucose: 0 per cent, 0.0125 per cent, 0.025 per cent, 0.05 per cent, 0.1 per cent, 0.25 per cent. Since 25 C is generally the optimum temperature for growth of

the fungus, the subsistence level at this temperature was presumed to be practically the same as the subsistence level at other temperatures. The "subsistence medium" was defined as that combination of glucose and yeast extract which at minimal concentrations would support apparently normal morphology and sporulation. *Z. eistla* has some growth on a medium which has some yeast extract, but never grew on a medium containing glucose only. When no glucose was present with the yeast extract, colony diameter increased rapidly, but the mycelium was sparse. When the lowest concentration of glucose (0.0125%) was added with the lowest level of yeast extract (0.0125%), colony diameter increased at the same rate as when the greatest amounts of glucose (0.25%) and yeast extract (0.05%) were added, but sporulation was weak. Spore formation, varying with the amount of glucose supplied, was satisfactory on 0.036 per cent glucose. Although vegetative growth measurably lessened if the level of yeast extract was reduced to 0.007 per cent, it did not completely stop. The final medium for testing the effect of subsistence nutrient conditions on the *Phoma* pattern consisted of artificial sea water as in the first experiment, 0.007 per cent yeast extract, 0.036 per cent glucose, and 0.85 per cent Ionagar. A full series of cultures was incubated over the entire temperature range.

The criterion in all experiments was increase in colony diameter, measured to the nearest millimeter across the least dimension. After the first five or six days, readings were made every two days as long as the growth rate remained steady or until the mycelium of the fastest growing culture reached the edge of the dish. "No growth," for the purposes of these experiments, was defined as no visible mycelial production after three weeks of incubation. All experiments were made in triplicate.

RESULTS

Z. eistla showed a typical *Phoma* pattern, with the best low-temperature growth at low sea water salinity, and the best high-temperature growth at high salinity. At 6 C, 16 C, and 20 C, the peak growth range varied from 0 S‰ to 25 S‰. At 25 C the peak was at about 28 S‰; at 30 C, about 58 S‰; and at 37 C it was between 55 and 80 S‰ (Fig. 1). Although colony diameters

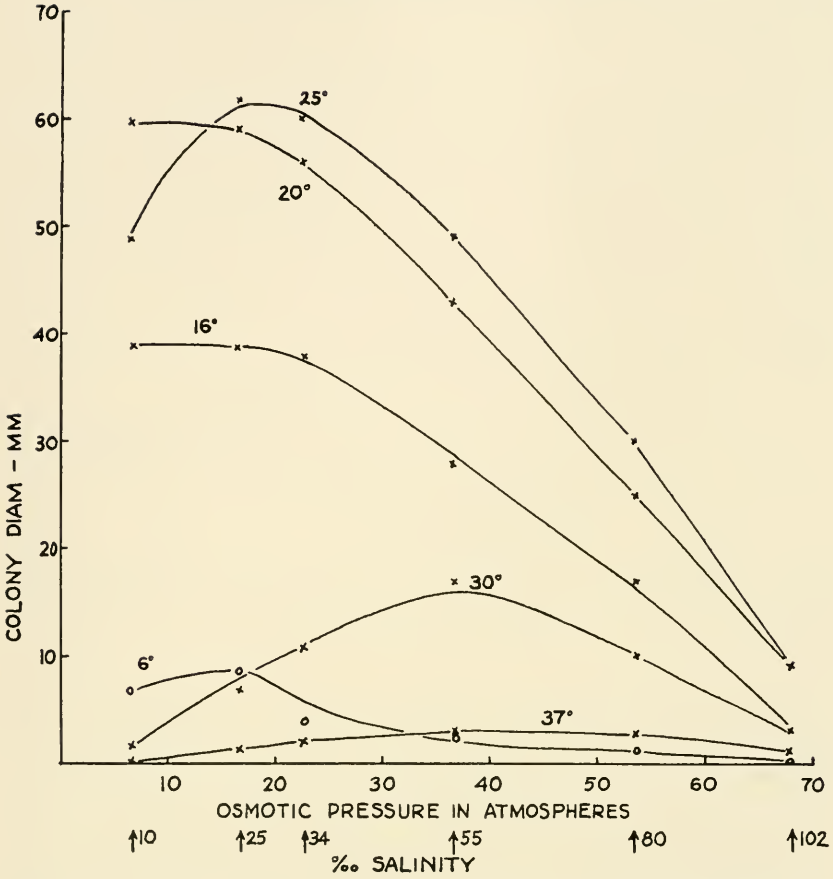

Fig. 1. Growth of *Z. eistla* at different temperatures on basic glucose-yeast-extract medium and artificial sea water in concentrations from 10 to 102 S‰, 6 to 70 Atm osmotic pressure.

at 6 C and 37 C were small, differences were measurable.

When the fungus was cultivated on media in which an osmotic gradient was provided by NaCl, the growth rate at all temperatures dropped with each increase in salt concentration (Fig. 2). The fungus did not grow at 37 C. These results, probably due the effect of toxicity of the single salt, indicate that the presence of NaCl in sea water is not the specific causal factor for the *Phoma* pattern in this organism.

Substitution of glucose for salts produced growth rates

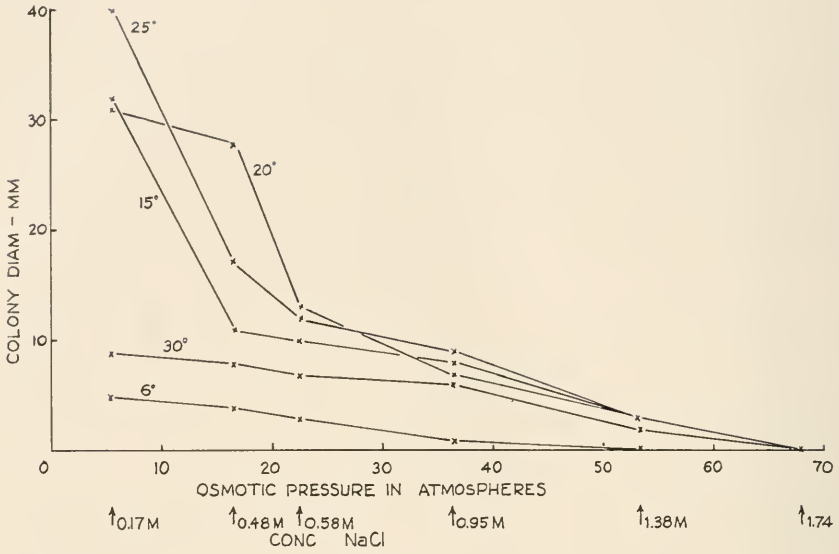

Fig. 2. Growth of Z. *cistla* at different temperatures on basic glucose-yeast-extract medium containing NaCl in concentrations from 0.17 M to 1.74 M. No growth at 37 C.

whose curves are similar to those from sea water cultures (Fig. 3). For simplicity, only the curves for 25 C and 30 C are given, but in fact at each temperature except 37 C the peak of the glucose curve was in about the same osmotic range as that of the salinity curve. As with NaCl medium, the plant did not grow on glucose at 37 C.

With pentaerythritol, the concentrations which could be made, supported growth in such a way as to suggest that this compound acts like the other osmotically active materials. The resulting curves are in fact like abbreviated copies of the glycerol curves, with negative slope at low and positive slope at high temperature (Fig. 4).

Calcium chloride was used in metabolically active quantities, but in the test concentrations it had no visible effect on the growth pattern of Z. *cistla* at 25 C or 30 C.

Increasing amounts of nitrogenous nutrients, supplied as yeast extract, had no effect on growth at 25 C when present in concentration above 0.05 per cent. From here to the maximum

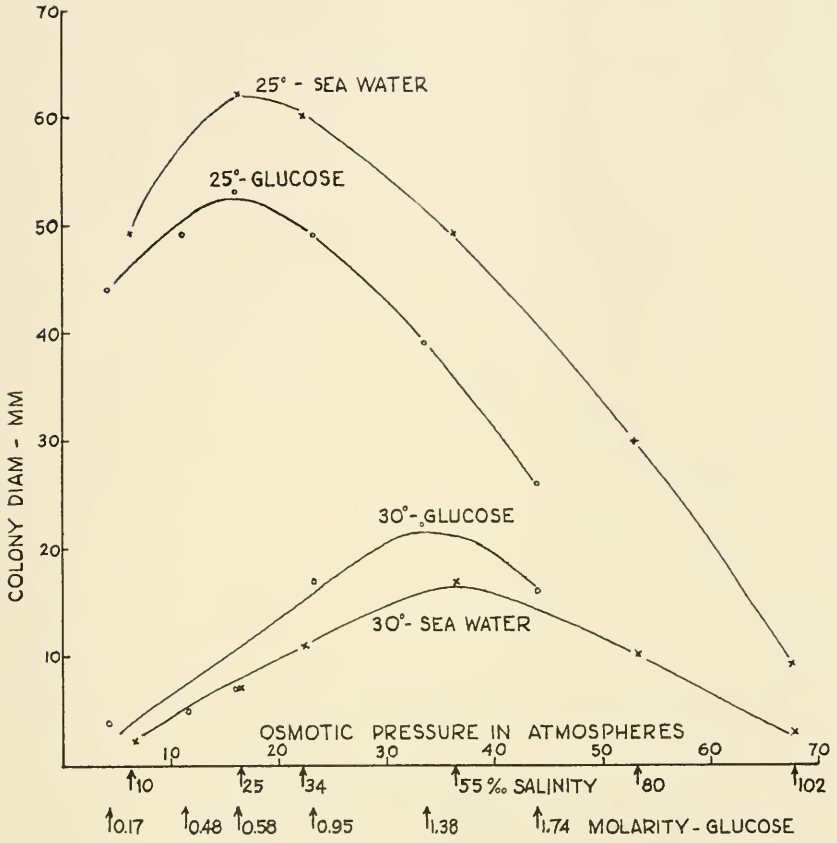

Fig. 3. Growth of Z. *eistla* at 25 and 30 C on basic medium plus glucose compared with that on basic medium plus sea water, with osmotic pressures of the two made equal.

of 0.5 per cent, colonies grew at the same rate, regardless of the amount of yeast extract available. At 30 C, however, little or no growth occurred on media which contained more than 0.1 per cent yeast extract. This was not a pH effect, inasmuch as the pH of all media remained between 5 and 6.

Total growth on a subsistence medium naturally was less than on a richer nutrient, and the optimum temperature for growth, regardless of solute content, was reduced to about 20 C. Aside from these effects, however, the relation between increasing temperature and increasing salinity optima on sub-

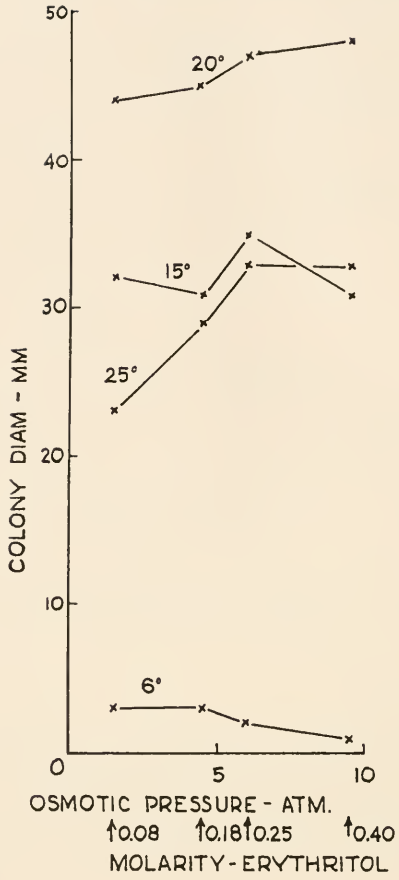

Fig. 4. Growth of Z. *eistla* at different temperatures on basic medium plus pentaerythritol in concentration from 0.18 M. to 0.40 M. See text for explanation of sharp cut-off. No growth at 30 C or 37 C.

sistence media was similar to that found for other media (Fig. 6). The osmotic pressure optimum for each temperature was the same as in previous experiments. Again no growth was observed at 37 C.

DISCUSSION

These experiments show that the relation between salinity optima and temperature does not necessarily result from gradients of strictly ionic solutes, as might have been suspected from the

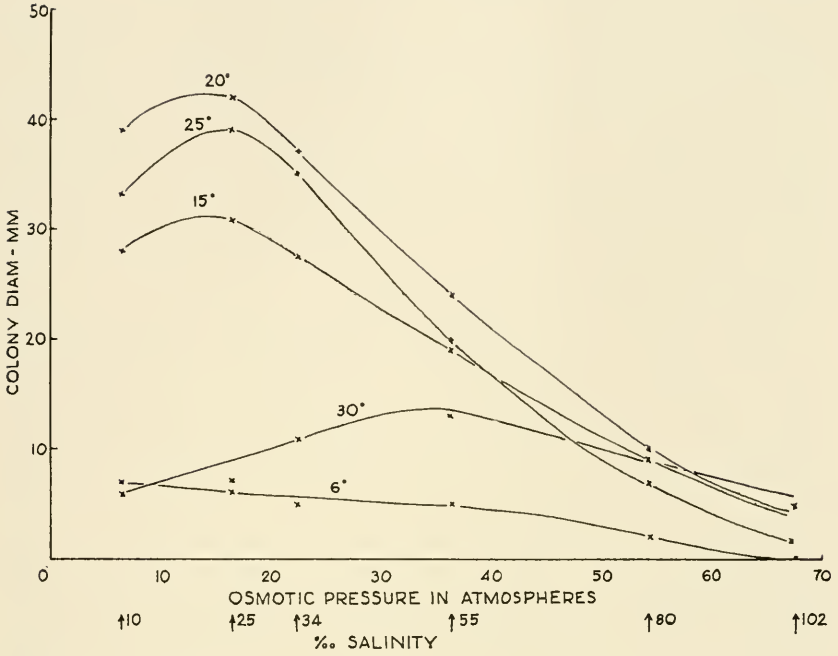

Fig. 5. Growth of *Z. eistla* at different temperatures on subsistence medium plus sea water. No growth at 37 C. Compare with Figure 1.

fact that it first appeared on media of various sea water concentrations. Glucose, glycerol, and erythritol all demonstrate it. The osmotic gradient need not be produced by a metabolically active substance, inasmuch as glycerol is effective as well as glucose. The growth pattern is not dependent on the nutrient level, though the organism must have enough to live on. Raising or lowering the level of glucose, nitrogen source or calcium concentration in the medium did not, in itself, alter the pattern.

Substances which could bring out the *Phoma* pattern had only one feature in common: they all produced osmotic gradients. If the curves for every substance tested are plotted for one temperature, the growth peak appears repeatedly in the same range of osmotic values. For example, at 25 C, the fastest growth for all substances occurred when the osmotic value of the medium lay between about 10 and 25 Atm. At 30 C, the peaks all lay between 30 and 40 Atm except in the case of glycerol which

showed no well-defined peak. At low temperatures, peaks were never sharp, but the curves were generally higher in the lower osmotic ranges. The optimum concentration for best growth at a given temperature varied with the osmotic pressure of the solution regardless of its nutritive or ionic nature, or of its ability to penetrate cytoplasmic membranes. Osmotic pressure thus appears to be the factor responsible for the relation between high temperature and high solute concentration. The osmotic optimum is in turn determined by temperature.

The fact that the relation between salinity and temperature is a function of osmotic pressure adds support to the idea that a sort of biological principle of LeChatelier is operating. This means that if one supplies an organism with sufficient added energy in the form of heat, a balancing force in the form of increased osmotic pressure becomes necessary if growth is to continue. The requirement for higher osmotic values continues with rising temperature until some critical portion of the system breaks down. Moreover, the requirement for increased osmotic pressure with increasing temperature is greater above than below the optimum temperature for growth. In sea water media, between 6 C and 25 C, the optimum osmotic pressure rises only slightly. From 25 C to 30 C, the optimum rises from about 20 Atm to about 40 Atm, an increase of 20 Atm in five degrees. This relation holds generally for other organisms which demonstrate the *Phoma* pattern (7). It must be recognized at the same time that the response to increased heat energy is controlled by genetic factors since, even among those organisms collected in marine habitats, not all show it. Collections of fungi from coastal waters of Delaware, New Jersey, and New York have yielded several *Alternaria,* and other fungi commonly found in the sea, which do not tolerate higher salinities when the temperature is increased.

When *Z. eistla* was cultivated on a subsistence medium and temperature and salinity (as sea water) were varied, the general pattern of the curves was the same as when ample nutrient was supplied, but the optimum temperature for growth dropped from 25 C to 20 C, and growth at 30 C was poor. When the fungus was cultivated on an erythritol medium containing an

abundant energy source, the optimum temperature again dropped to 20 C. *Z. eistla* did not grow at 30 C on erythritol media. Cytoplasmic membranes being practically impermeable to erythritol, there is no tendency for the concentration of this solute to be equalized inside and outside the cells. The medium outside is in effect diluted until the organism apparently finds it as difficult to obtain nutrients as if an insufficient amount were actually present. Glycerol caused a similar if less striking drop in the temperature at which an absolute growth maximum was recorded. It could enter the cells but, being little or not at all metabolized, could disturb the cellular economy by its inert presence. Thus any condition which interfered with the chemical energy supply caused not only an absolute reduction in the growth rate, but shifted the whole temperature curve into a lower range. The same general effect was obtained if sub-optimal osmotic values were attempted, as with a fully nutritious sea-water medium with a value of about 6 Atm. When food was abundant and available, the absolute maximum growth occurred at 25 C; and when the temperature was held at 25 C, the optimum osmotic value was about 17 Atm regardless of the source of the pressure. (This does not include NaCl or erythritol, both of which presented special peculiarities.)

The ecological implications of temperature-salinity relationships in organisms have been considered by several investigators. Kinne (3) discussed the relation between laboratory salinity optima and the distribution of polyps in nature. Gold (2), studying the distribution of lignicolous fungi in an estuary, reported that more of certain types of molds grow in more saline waters during warmer months, and in fresher waters during cooler months. TeStrake (9) investigating the response of *Dictyuchus monosporus* to changes of salinity and temperature in laboratory culture, found that it would grow at salinities and temperatures at which she could not find it in nature, and suggested that a limited nutrient supply may be responsible for its distribution. Experiments with *Z. eistla* on subsistence media indicate that nutrient quantity alone does not determine the general salinity-temperature relation, but affects it only as an overall shift in the data curves.

Some organisms seem from laboratory experiments to be suited to life in the sea, yet because a required type of nutrient is lacking there, they occur instead in habitats which are really sub-optimal for salinity. In a different direction, several *Alternaria* from the sea (25-30 S‰) seem from laboratory data to be essentially terrestrial, yet because dead wood lies in littoral waters, they will live on it and tolerate supra-optimal salinity conditions. A number of hyphomycetous genera have been tested in this laboratory for their ability to grow in salt media, and more than half of them suffer from any increase in salinity at any temperature, even though they tolerate full strength sea water. If they were not cultivated on a variety of media, one might think them natural marine forms.

Organisms which have shown the *Phoma* pattern are euryhaline, or facultative osmophiles, and are not necessarily either terrestrial or marine. At temperatures up to the apparent optimum for the nutrient conditions obtaining, they grow well in solutions supplying 1 to 25 Atm osmotic pressure, i.e. 0 to 30 S‰: but at supraoptimal temperatures, they require ever higher salinities.

SUMMARY

The imperfect fungus, *Zalerion eistla*, from the Atlantic waters of Newfoundland grew better at higher temperatures when supplied with increased sea water content in the medium but not with NaCl. The organism was incubated over a wide range of temperatures on a number of media which were made up with graded concentrations of osmotically active compounds: single salts, as calcium or sodium chloride, metabolizable sugars, and non-metabolizable alcohols, as glycerol and pentaerythritol. The concentrations of the active substances were prepared so as to provide similar osmotic pressures in each series. The interrelation between temperature and salinity appears to be more a matter of osmotic pressure of the surrounding medium than of the type of nutrition. Growth curves from the different runs were similar, whether the osmotically active materials were ionic or not, metabolizable or not, penetrable or not. If the nutrient concentration of the medium was reduced to a subsistence level, the

temperature optimum was lowered, but the general pattern of temperature with relation to osmotic value was not altered.

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Chapter 30

The Importance of Fungi in the Sea

J. Kohlmeyer

INTRODUCTION

The following survey records recent knowledge on marine fungi belonging to the Ascomycetes and Deuteromycetes (Fungi Imperfecti). Lower fungi (Phycomycetes) may be neglected here, as Sparrow (56) has treated them recently in a comprehensive book.

Marine mycology is a rather young field, which has not been commonly known before Barghoorn and Linder's extensive publication in 1944 (3). Marine fungi were also detected and described sporadically since about 1850, but these scattered collections mostly got lost in the abundance of new descriptions of terrestrial species during the past century.

I shall first define the term "marine fungi" as we will use it here. In the present paper "marine" fungi are those that grow and reproduce either submerged in the sea or on the seashore on intertidal substrates (sand, parts of plants, etc.). Spores of fungi that normally develop terrestrially may also be found in the sea, of course. We will not include such species that were isolated out of marine environments, e.g., sea water or tidal-flat muds (16, 54). Even if such collections were made directly in the sea, no direct evidence has proved that these isolated organisms had been active in this habitat. These investigations simply indicate the existence of viable parts of fungi (spores or hyphae) but do not explain where they developed. Of course, we do not consider here those fungi that have been found only in fresh water. Besides "marine fungi" in the above defined sense, some inhabitants of brackish water are treated.

Most of the marine species are very adaptable in their

physiology, and may live in sea water as well as in fresh water (20, 55). The term "marine" for a given species does not mean that it occurs in sea water only. As there are no distinctive separations between many fields of biology, it is nearly impossible to separate clearly marine and aquatic mycology. Hence, sometimes it may be doubtful whether a species should better be called "marine" or "limnic." Gold (20) proposes as another criterion whether a species is marine or not, "the salinity at which optimum growth and reproduction occur and not saline tolerance of that organism."

ADAPTATION TO ENVIRONMENT

Morphological Adaptation

In general, fungal hyphae of the different taxonomic groups look very much alike and any morphological variability can only be expected to occur in the reproductive organs. Among marine Deuteromycetes there are quite a few different shapes of conidia but only a few species show morphological adaptation of the conidia to the water. We know only two examples: *Dinemasporium marinum* Nilsson and *Orbimyces spectabilis* Linder, both provided with spore appendages. It is a surprising fact that spore appendages in this group are so rare, as—on the contrary—Deuteromycetes occurring in fresh water mostly have filamentous spores that often are provided with processes enabling them to float for a long time in water (23, 43, 46). Also, the spores of most marine Ascomycetes possess appendages that assist them to float and to attach and, hence, their absence in marine Fungi Imperfecti is difficult to explain. Most probably, the imperfect fungi of the sea are more closely related to terrestrial forms than marine Ascomycetes are to their respective counterparts of the land. This may be concluded by the fact that species of marine Deuteromycetes predominantly belong to genera that are common on land. The only known exceptions are species of the following monotypic genera which were found only in the sea: *Botryophialophora* Linder, *Cirrenalia* Meyers et Moore, *Cremasteria* Meyers et Moore, *Culcitalna* Meyers et Moore, *Orbimyces* Linder and *Phialophorophoma* Linder.

Many of the ascospores of the marine Ascomycetes are

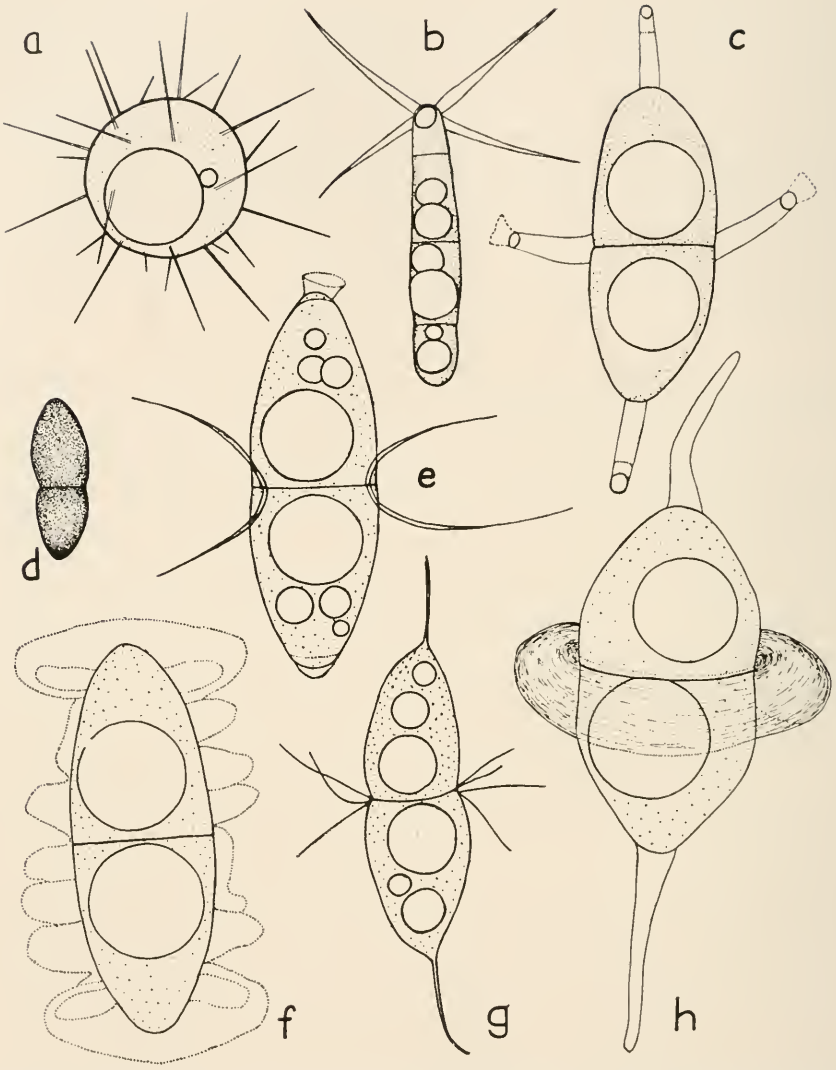

Fig. 1. Ascospores of marine Ascomycetes provided with different-shaped appendages (except 1 d). a) *Amylocarpus encephaloides* Currey, b) *Torpedospora radiata* Meyers, c) *Ceriosporopsis calyptrata* Kohlm., d) *Microthelia maritima* (Linder) Kohlm., e) *Halosphaeria mediosetigera* Cribb et Cribb, f) *Remispora maritima* Linder, g) *Peritrichospora integra* Linder, h) *Halosphaeria torquata* Kohlm. - Magnification approximately 2000 times (figures drawn by Mrs. E. Kohlmeyer).

provided with slime sheaths or appendages effecting prolonged floating in the water and later on causing the firm attachment of the spores to the substrate. These processes are quite differently shaped: there are cilia, tender spines, thorns, slimy caps of different length, tubes or collars (Fig. 1). The appendages are sometimes rather complicated, for instance some of them may be reversed and then form small funnel-shaped caps (*Cerios-poropsis calyptrata* Kohlm. and *Halosphaeria mediosetigera* Cribb et Cribb, see Fig. 1 c and e), or they may consist of fine fibers embedded in a gelatinous mass (*Remispora quadri-remis* (Höhnk) Kohlm. and *R. stellata* Kohlm.). Submicroscopical investigations will certainly show further interesting structures in spore appendages.

Spores of Ascomycetes usually contain one or several big oil drops in each cell and these serve as nutriment for germination and assist the spores to float (Fig. 1). After leaving the fruiting body, the spores are able to germinate and to form long germ tubes within a few hours (31, 32).

Physiological Adaptation

Besides morphological adaptation to life in water, many marine fungi are also physiologically adapted to the sea. Utilization of different nutrients has been studied with pure cultures of only a few species thus far (2, 21, 24, 32, 40, 55). The numerous substrates used in these experiments cannot be mentioned here, as they are of minor importance for the subject of adaptation. The most interesting result of Barghoorn's (2) experiments was the fact that his species were able to grow on media with concentrations of sodium chloride three times as high as sea water. In most cases they developed better in substrates with normal sea water concentrations than they did on media made up with fresh water.

These results were confirmed by Gustafsson and Fries (21) for one species, while the others grew in sea water solutions as well as in media made up with distilled water. Likewise some Deuteromycetes examined by Meyers and Reynolds (40) did not show a difference of growth between substrates with sea-water or distilled water. Mme. Nicot (42) isolated an imperfect

fungus from salty dunes which grew even on media with 20% NaCl. Relationships between salinity and temperature for some marine fungi were investigated by Ritchie (48, 49) and Gold (20).

The results of all nutrition experiments made hitherto seem to be rather contradictory, and an extensive program of physiological experiments with many species of marine fungi would be very important.

Besides an evidently common halotolerance, fungi showed another physiological adaptation to the life in the sea, as Barghoorn (2) found out in his experiments. Five of six species growing on media with different pH preferred values above 7,4. Some species could not even develop below pH 4,7. As sea water generally has values above pH 8 the hitherto examined marine fungi show a distinct adaptation to the alkaline habitat.

SUBSTRATES OF MARINE FUNGI AND THEIR DISINTEGRATION

Fungi as Parasites

While among Phycomycetes a considerable number of species occur parasitic on marine organisms (see the compilation of Mrs. Wilson, 61), parasitic marine Ascomycetes and Deuteromycetes are less known. Algae-inhabiting Ascomycetes may sometimes live in a lichen-similar symbiotic association wherein the fungal component may become a parasite. Sometimes fungal attack of marine algae has been called "gall formation" while other authors speak of "Parasitism." In his host index, Meyers (38) lists, among other plants, twenty-three genera of algae, containing 30 species attacked by Ascomycetes, and during the last years Cribb and Cribb (10, 12, 13), Cribb and Herbert (14), G. Feldmann (17, 18), and Wilson and Knoyle (62) report further parasitic Ascomycetes on algae.

Oudemans (44), Rolland (50), Sutherland (57), J. Feldmann(19), and Cribb and Cribb (10) report algae-inhabiting Deuteromycetes.

There are only a few publications on microscopic examination of attack of fungal hyphae on algal cells. Decay of tissue of calcareous algae by a parasite was investigated thoroughly by

Bauch (4), and Wilson and Knoyle (62) studied mycelia of *Didymosphaeria*-species and their relationships to the hostal tissues.

Higher plants of the sea, of brackish water or of the sea shore are also attacked by fungi. As examples, we will mention the host genera *Juncus, Posidonia, Spartina, and Zostera,* many of which are inhabited by Ascomycetes and Fungi Imperfecti (see the host index of Meyers, 38).

Fungi as Saprophytes

Most marine fungi have been found on dead organic material or even on inorganic substrates (calcareous deposits or sand). It seems to be likely that many parasitic species may also live as facultative saprophytes. For saprophytes in the sea, dead algae as well as dead roots of mangrove-trees, driftwood or driftbark are available as organic substrates.

Algae or higher marine plants washed ashore are mostly attacked by Deuteromycetes (8, 9, 58, 59, 63). Members of the *Moniliales* predominate while the *Sphaeropsidales* are mainly parasitic.

A great part of marine saprophytes hitherto discovered develops on wood or fibers. Thus, these organisms have especially strong cellulolytic abilities. Barghoorn (2) found a heavy decomposition by marine fungi on ropes that were suspended in the sea for 6 months, and pure cultures of Ascomycetes and Deuteromycetes growing on manila twine caused a considerable "loss in tensile strength of the material and an increase in total reducing sugars" (39).

Every piece of wood—even pilings in ports and embankments that are treated with wood preserving chemicals—is superficially softened after being in sea water for a certain time. Therefore it was of economic importance to find out which microorganisms cause wood decomposition in the sea. In natural habitat aerobic bacteria and fungi will certainly attack wood simultaneously. In pure culture-experiments with marine bacteria Kadota (26, 27) has demonstrated the cellulose dissolving ability of these organisms. Pure cultures of marine Ascomycetes and Deuteromycetes grow readily on wood and other cellulose-containing substrates

(2, 25, 30, 32). Cellulolytic activity was proved in other experiments by measuring the increase of reducing sugars.

Attack by fungal hyphae on wood cells can be easily demonstrated by microscopic observation. Considering the type of wood destruction, marine fungi may be called "soft rot fungi" (in the sense of Savory, 51). They decompose mainly the less lignified secondary walls and cause screw-like tunnels within the cell walls (2, 60, 30). Because of their dependence on oxygen, the hyphae mostly grow only a few millimeters deep into the wood. Only in some tropical woods with very large lumina and vessels can fungal hyphae penetrate to the center (6).

Until now the soft-rot type of deterioration was found only on wood that had been decomposed in the sea, thus, possibly different microorganisms (fungi and bacteria) could be involved in the process of softening. But it was proved by the author that pure cultures of marine fungi produce the same screw-like tun-

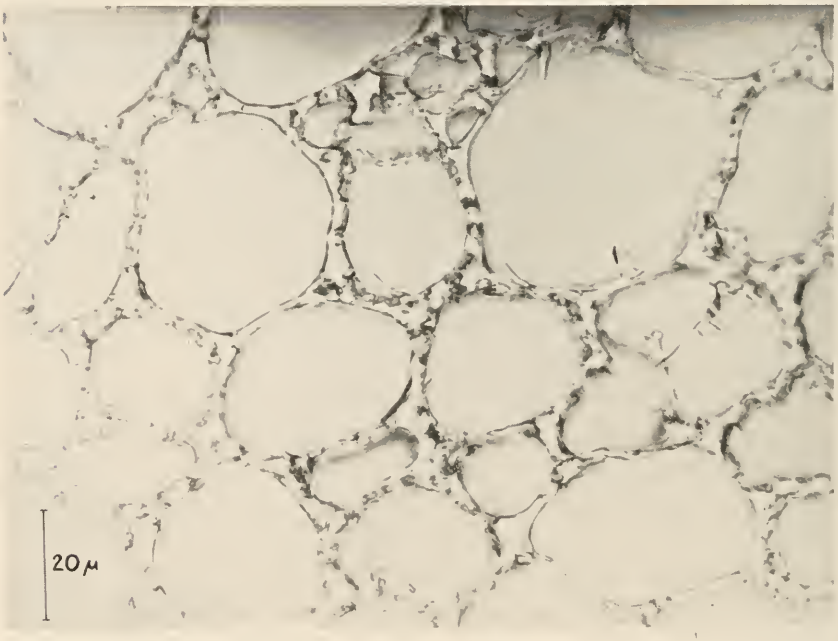

Fig. 2. Balsa wood attacked by a pure culture of *Peritrichospora integra* Linder. In transverse section the tunnels produced by hyphae that are growing in the secondary layer of the cell wall appear as dark holes.

nels within the cell walls as they are found in wood that was infected in the sea. As bacteria attack wood in a different way and do not form these characteristic tunnels, it is evident that "soft rot" decomposition in the sea is caused by marine fungi.

Pure cultures of the following species that were growing on balsa wood caused a distinct decomposition ("soft rot") of the secondary walls of the wood cells (Fig. 2):

Fungi Imperfecti

Cirrenalia macrocephala (Kohlm.) Meyers et Moore
Humicola alopallonella Meyers et Moore
Piricauda pelagica Johnson

Ascomycetes

Ceriosporopsis calyptrata Kohlm.
C. hamata Höhnk
Halosphaeria appendiculata Linder
H. mediosetigera Cribb et Cribb var. *grandispora* Kohlm.
Lulworthia opaca (Linder) Cribb et Cribb
Peritrichospora comata Kohlm.
P. cristata Kohlm.
P. integra Linder
Remispora quadri-remis (Höhnk) Kohlm.[1]

Thus far, only one species of wood inhabiting marine fungi is known to attack ligneous cell walls—unlike the soft rot fungi—without causing tunnels: the Ascomycete *Amylocarpus encephaloides* Currey starts attack in the lumina by dissolving the tertiary walls, thus imitating deterioration of wood by many Basidiomycetes (32, 33).

Direct quantitative determination of wood deterioration by marine fungi has not yet been made. It is known, however, that terrestrial soft rot fungi produce considerable weight losses in wood (15) and cause a reduction of bending strength and toughness, as Armstrong and Savory (1), and Liese and Pechmann (37) found out in mechanical strength tests. Chemical investigations also showed a severe decomposition of cell wall components, mainly of polysaccharide constituents (52). Similar ex-

[1] Pure cultures of these and other marine fungi are maintained in the Botanical Museum in Berlin-Dahlem and in the Centraalbureau voor Schimmelcultures, Baarn/Netherlands.

periments with marine fungi should also be made in order to learn the activity of different species involved in decay of wooden buildings in the sea.

Finally we will mention those organic substrates that have been rarely examined for fungal attack: bark and cones of conifers. This material, however, is normally inhabited by fungi after a longer period of submergence in the sea (32), but it is not clear whether these fungi live on suberin, lignin, and tannin of bark and cones or predominantly on less lignified cellulose fibers embedded in these materials. In all cases microscopic sections of such substrates showed hyphae growing in soft-rot–like tunnels within these fibers.

Besides the above listed organic substances, marine fungi may even attack inorganic material. As examples may be named the Ascomycetes *Peritrichospora integra* Linder and *P. trifurcata* (Höhnk) Kohlm. the perithecia of which develop predominantly on grains of sand, on glass and calcareous deposits of marine animals (22, 32). Another still undescribed Ascomycete (*Ophiobolus* spec.) was found by the author on driftwood from the Puget Sound, Washington, the fruiting bodies of which fungus were formed beneath the empty calcareous tubes of *Bankia*. The necks of these perithecia had grown through the layer of lime to the surface and they protruded as tiny black pipes from the white calcareous case. In sands of different origins Porter and Zebrowski (45) detected sporangia and spores that had developed deeply in fragments of molluscan shells and shells of other marine animals. Probably these fungi are Phycomycetes. Considering all these observations, it seems to be likely that marine fungi take a considerable part in dissolving inorganic substances and transforming them into organic compounds.

RELATIONSHIPS TO MARINE ANIMALS

A possible relationship between wood-destroying animals and soft-rot fungi was discussed recently. Bletchy (7), for instance, pointed out that terrestrial soft-rot fungi facilitate attack on wood by larvae of the common furniture beetle and increase the rate of their development. Within the last years nutrimental relationships also between marine fungi and wood-inhabiting

marine animals have been repeatedly found. Besides the biological importance of this discovery experiments with shipworms and gribbles are of considerable interest, especially for wood protection in sea water.

Shipworms

While adult shipworms are able to digest wood that is free of fungi, the swimming larvae seem not to be able to attack it. They do not settle on fresh wood as was shown in experiments with *Teredo pedicellata* Qutrf. (28). An attraction by wood or other substances to the swimming animals could not be detected, for they encountered the substrate by chance when swimming around. But in tests with alternative possibilities for attachment, wood decayed by fungi was favored above fresh wood samples. When investigating the nutrition of *Teredo*, Lane (36) also postulated a dependence of the animals on marine fungi. The shells of the free swimming larvae are still uncalcified and have no teeth (35), hence—without regard to other facts—mechanical penetration by young animals is favored by the fungal softening of the surface layers of the wood (Fig. 3).

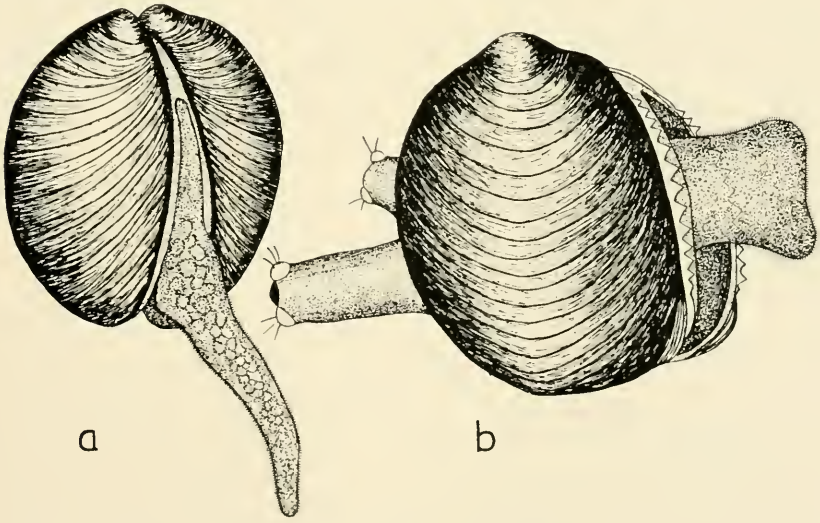

Fig. 3. Larvae of shipworms (*Teredo pedicellata* Qutrf.). a) uncalcified young animal when creeping, b) animal after metamorphosis with calcareous teeth (at right) and a pair of siphons (at left). Magnification approximately 200 times (figures drawn by Mrs. E. Kohlmeyer).

Gribbles

In preliminary publications Schafer and Lane (53) and Becker, Kampf and Kohlmeyer (5) had stated that *Limnoria* species depend on marine fungi in their nutrition. Experiments by Ray (47) as well as by Kohlmeyer, Becker, and Kampf (34) showed that *Limnoria* species may live in wood free from fungi. Thus, the gribble is able to digest uninfested wood. The tests made by the latter authors have demonstrated, however, that lifetime of the gribble *Limnoria tripunctata* Menzies was longer when fed with infested soft wood than on the same substrate without any fungi. In hard wood, duration of life was equal in infested or uninfested material. Reproduction occurred only when fed with wood attacked by marine fungi. Further experiments with other species of *Limnoria* should elucidate whether the positive influence on the gribble exists everywhere.

Finally we will mention that dispersal of wood-inhabiting marine fungi may be favored by wood-eating animals, as conidia and ascospores of some species pass the digestive tract of *Limnoria* without being damaged (29, 34). Animals coming from fungus-inhabited wood migrating to another substrate may carry spores that germinate in the faecal pellets and infest the newly attacked wood. It has not yet been determined whether the viability of spores is influenced in any way by passing the digestive tract of the gribble.

Our knowledge of influence of marine fungi on other sea inhabiting organisms is still fragmentary, but the topics dealt with above show what problems have to be solved and what importance fungi may have for oceanography.

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Chapter 31

Degradation of Lignocellulose Material by Marine Fungi*

Samuel P. Meyers and Ernest S. Reynolds**

An extensive number of papers has appeared in recent years characterizing lignicolous marine fungal populations. In studies of the association of fungi with submerged wood in our laboratory the primary consideration is the determination of the ability of these organisms to attack actively the wood substrate and its constituent parts.

The presence of active cellulolytic systems in various species of lignicolous marine Ascomycetes and Deuteromycetes has been demonstrated (1, 2). Cell-free filtrates from species comprising the major or a significant part of the mycota isolated from submerged wood, incubated on such cellulosic materials as carboxymethlycellulose, powered cellulose, and balsa wood, have produced as much as 3.0 mg reducing sugar per milliliter. Further investigations (3, 4) have indicated the ability of many of these fungi to initiate significant degradation of wood and Abaca cordage.

Current studies in this laboratory include analyses of the dynamics of biological activity and investigations of the physiology of individual taxa, correlated with the rate of infestation and degradation of lignocellulose substrates submerged under natural conditions.

METHODS AND MATERIALS

In studies of infestation of Manila in uniclonal fungal cultures,

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five feet of coiled twine were placed in 500 ml Erlenmeyer flasks, each containing 100 ml of 0.1% yeast extract (Difco) and 0.1% Tris (Trishydroxymethylaminomethane) buffer in sea water. The pH of the medium after autoclaving was 6.5 to 7.0. The same medium without Tris showed a pH of 4.5. In earlier work, the pH of the Manila yeast extract medium was adjusted following autoclaving by addition of pre-calibrated amounts of sterile NaOH. However, a decrease in pH of the solution resulted upon standing, often within twenty-four hours after the addition of the NaOH. A drop in pH also occurred in the flasks containing Tris, although this was in general a slower process, permitting spore germination and initial development of the fungus. Growth usually caused a rise in the pH of the medium.

The Manila used throughout these studies was 100 per cent grade F, non-Davao (Tagaon) twine, supplied by Dr. Bryce Prindle, formerly of the Plymouth Cordage Co., Plymouth, Massachusetts. The effectiveness of Manila cordage as a substrate for growth activities of marine fungi has been discussed in earlier work.

The terms "Manila," "cordage," and "twine" are used interchangeably in this paper and refer to the intact twisted material. The term "fiber" is used to indicate single macroscopic constituent units of the cordage.

Different patterns of infestation of the twine were developed by regulating the arrangement of the material in the culture vessels. In one series of flasks, the cordage was completely submerged, whereas another series was arranged so that only the middle four to six inches of each eighteen to twenty inches length of cordage was actually immersed in the liquid medium. The latter arrangement was used to concentrate maximum fungal growth and reproduction in the central region of the twine, so that this area could be placed between the two grips of the testing machine. This permitted a more definitive evaluation of the effect of the fungus on the cordage itself. Placement of the cordage in the flask whereby only a portion was subject to fungal attack was accomplished by forming the lengths of cordage in each vessel into a tight "U" with the area of the outer ends twisted and wrapped in aluminum foil. Although three to four inches

of the wrapped ends extended beyond the lip of the flask, the foil covering allowed the material to remain sterile after autoclaving and throughout the growth period.

The flasks of the Manila yeast extract medium were sterilized at 121 C for twenty minutes. The sterilization process itself had no measurable effect on the physical properties of the Manila, nor was any deleterious effect noted when the sterilized cordage remained in the yeast extract sea water medium for a period of several months and longer.

The inoculum for the culture vessels was 1 to 2 ml of spore and mycelial suspensions from individual stock cultures, prepared by flooding the slant cultures with sterile sea water.

Inoculated flasks were incubated at 25 C from five days to as long as several months on an intermittent reciprocating shaker set at 60 strokes/min. Flasks were harvested when extensive fungal growth was evident on the twine. Culture vessels were examined for extent of reproduction, cellulolytic activity of the cell-free filtrate, and tensile strength measurements of the infested twine.

Analyses of cellulolytic activity of the cell-free filtrates have been described previously (1, 2). The Nelson-Somogyi method (6) was used to determine the reducing sugars, RS, of the fungal filtrates and the RS produced during the enzymatic tests.

Treatment of the field-exposed cordage for tensile strength properties was similar to the procedure followed with cordage attacked in uniclonal cultures. Uniform periods of drying and conditioning of the twine were maintained throughout the study. Control pieces were tested regularly along with the material exposed in the field to ascertain any possible effect of the drying and conditioning processes on the Manila. Repeated use of such controls has demonstrated that these latter processes do not affect the strength readings or the regularity of the results obtained.

A Dillon Model K Universal Tester has been used in the present work. Use of this instrument has permitted strength determination on small sections of cordage, i.e., six to eighteen inches long. The Scott Tester, used in earlier studies, required a minimum length of five feet of cordage for each test and since

ten breaks were made per test, a total of 50 feet of twine was needed for each examination.

The stationary and moving heads of the Dillon Tester were provided with special 'hata' grips adapted to accept small diameter twine. The fixed measured rate of cross head travel of the instrument was 1.25 inches per minute. A 250 pound dynamometer gauge, with dial divisions of 5 pounds, recorded actual breaking strength of the cordage. Tensile strength is expressed here as the "pull" in lb at the actual breaking point of the Manila.

The average mean breaking strength of the control twine was 102 lb. Strength measurements of 85 pounds or less for the fungal infested cordage were selected to indicate *significant* degradation. Statistical analyses of standard deviation of infested and control cordage have supported these data.

Field Exposure of Manila Cordage

Fifty lengths of sterilized twine, each 24 to 30 inches long, were attached to two separate wooden frames submerged from The Marine Laboratory pier in approximately eight to ten feet of water. The frames were anchored off the bottom so that the twine was held vertically and subject to vigorous tidal action. The individual pieces of Manila were sterilized in separate petri dishes and were attached to the wooden support frame immediately prior to submergence.

Collections were scheduled at intervals of four to five days over a period of approximately two to five months. At each collection, new sterilized sections of twine were placed on the exposure rack. From six to ten pieces of cordage were removed at each collection, one piece of which was transferred immediately to a sterile petri dish for microscopic examination and the subsequent treatment described below.

The cordage selected for microscopic study was examined for fungal growth initially and at later periods during incubation of the twine. Pieces of fiber of the sample were cut with alcohol-sterilized microscissors and transferred to the surface of a sea water medium containing 1.0 per cent glucose and 0.1 per cent yeast extract fortified with an antibacterial mixture. The latter, added to the culture medium prior to inoculation at a final concentration of 1.0 per cent, consisted of chlorotetracycline

·HCL (aureomycin) 10 mg/ml, chloramphenicol 2.0 mg/ml, and streptomycin sulfate 2.0 mg/ml. The sections of fiber, ⅛ to ¼ inches long, selected for the above examination were sampled variously from the entire length of cordage, including material from inner and outer surfaces of single as well as combined twists. Further treatment of certain samples collected included thorough washing of the fiber pieces in successive tubes of sterile sea water.

Uniclonal growth developing on the isolation medium was transferred to stock tubes of sea water agar media containing 1.0 per cent glucose and 0.25 per cent yeast extract.

RESULTS AND DISCUSSION

Cultural Studies

Of the fungi studied previously, two ascomycetous species, *Ceriosporopsis halima* and *Torpedospora radiata*, grown in Manila culture for 78 and 49 days respectively, exhibited greater fungal activity, in terms of loss of tensile strength, than any of the other species examined. Further studies of *T. radiata* and another cellulolytic Ascomycete *Halosphaeriopsis* sp., are discussed below.

The degradation of Manila by these two Ascomycetes is shown in Figures 1 and 2. Both species exhibited significant attack upon the cordage early in their growth period. Development of *T. radiata* and *Halosphaeriopsis* sp. is characterized by copious production of perithecia over the surfaces of the twine. In some tests, especially in the 88 day culture of *T. radiata*, all of the sections of cordage examined broke in the area of maximal perithecial crop. However, in other tests of this species, as well as those of *Halosphaeriopsis* sp., the site of rupture of the cordage was often not in the immediate region of reproduction.

Tensile strength of cordage attacked by *Halosphaeriopsis* sp. for 34 days was nearly equal to that recorded for periods of infestation as long as 85 days. In general, this species exhibits cellulolytic activity and degradative ability much earlier in the growth cycle than the other ascomycetous species examined. Considerable uniformity in fungal attack over the cordage length

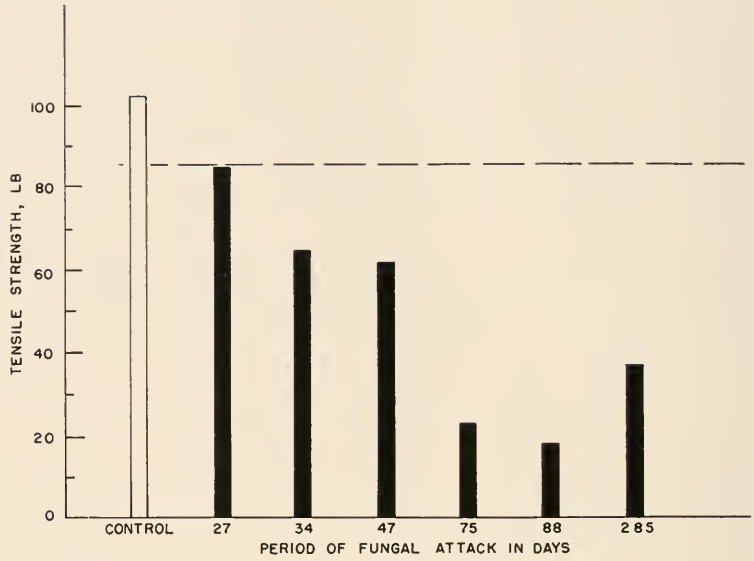

Fig. 1. Degradation of cordage by *Torpedospora radiata.*

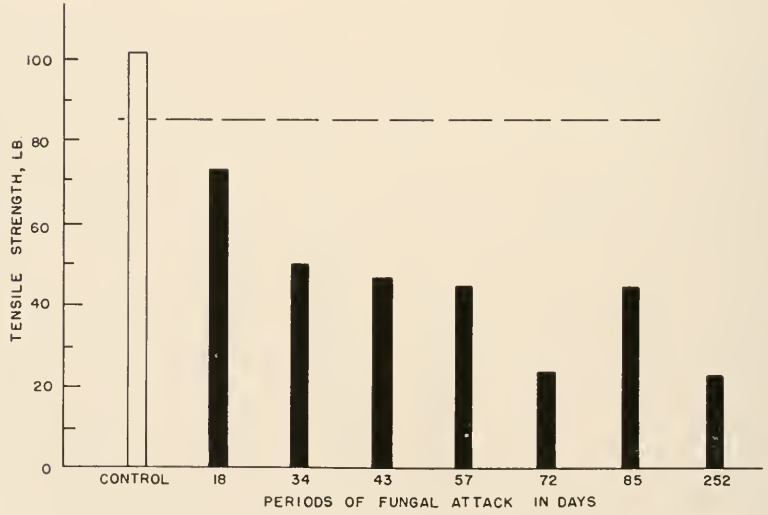

Fig. 2. Degradation of cordage by *Halosphaeriopsis* sp.

was evident by the rather low average standard deviation of these tests of ±8.5. In earlier work using the Scott Tester, and longer cordage units where more irregular fungal infestation and reproduction was apparent, strength tests showed greater standard deviations. Examination of cellulolytic activity of cell-free filtrates from cultures of *Halosphaeriopsis* sp., 24 to 81 days old, gave reducing sugar productions of from 0.155 to 0.308 mg/ml/24 hr. No significant increase in cellulolytic activity was observed with older cultures.

While breakage of the cordage often occurred in areas where reproduction was scant or absent, in general, especially in older cultures of these two species, the correlation between reproduction and immediate site of rupture, noted in earlier work, has been substantiated here. However, it should be noted that in various ascomycetous fungi, particularly in certain species of *Lulworthia,* intensity of reproduction does not correlate necessarily with vigor of degradation of the cordage. It is apparent that differences in growth and cellulolytic activity among the various species of Ascomycetes must be given careful study. These studies as well as further examinations of the fungus/substrate area in the region of maximum reproduction by *T. radiata* and *Halosphaeriopsis* sp. are in progress.

Studies of Species of *Zalerion*

A comparative study has been made of the general cellulolytic activity of a marine deuteromycetous taxon with that exhibited by various marine Ascomycetes. Representatives of the Deuteromycete genus examined, *Zalerion,* are extremely abundant in northern and temperate marine areas, although noticeably absent in warmer oceanic areas. A recent description (5) of the taxon has included studies of cellulolytic activity of the different species on CMC 50 T and Walseth cellulose. The two species *Z. raptor* and *Z. xylestrix* discussed here, have consistently shown high cellulolytic activity on these cellulosic substrates. Further studies of their degradation of cordage and associated enzymatic activity are reported below.

Cell-free filtrates from cultures of *Z. raptor* and *Z. xylestrix* were made initially at three and five days respectively. Each sample, except the three and seven day samples of

Z. raptor taken aseptically, included the filtrate from a total of three to four replicate flasks. Analyses were made of both cellulolytic activity and tensile strength of the cordage. The latter tests consisted of 9 to 12 pieces of cordage. Three pieces of equal length were cut from each five foot loop present in individual flasks.

While both species exhibited slight cellulolytic activity as early as three to five days following inoculation, extensive activity, i.e., above 1.0 mg RS/ml/24 hour, was not noted until after a growth period of approximately twelve days (Table 1). The difference in activity of the two seven day samples of *Z. raptor* may possibly be attributed to different sampling procedures as well as to the different times of inoculation. The seven day sample with over 0.700 mg RS/ml/24 hr was taken from three separate culture vessels whereas the fraction with rather low activity consisted of a small sample taken aseptically from a single flask. The differences in RS production of filtrates collected after twelve days may not be significant. Although the twelve day culture of *Z. raptor* exhibited considerably greater activity than did the thirteen day culture, this may be due in part to the different time of inoculation of the two cultures. Both the seven and twelve day cultures, inoculated a week after the initial set of inoculations, showed greater activity than did cultures of similar age in the earlier test.

Significant reduction in strength of the infested cordage was evident at five and seven days of growth of *Z. xylestrix* and *Z. raptor* respectively (Figs. 3 and 4). Further reduction in strength occurred with increased growth of the fungi. Repeated tests of uninfested cordage gave average readings of from 95 to 105 lb. Considerable differences were noted between the minimal and maximal strength readings of the seven to sixteen day tests of *Z. raptor* and the five to fourteen day tests of *Z. xylestrix*. However, in the 20 day test of *Z. raptor* and the fifteen and eighteen day test of *Z. xylestrix*, considerably less variation between minimal and maximal readings was apparent. Variation between tests occurred frequently as noted by the results of the fourteen day test of *Z. xylestrix*. This particular sample showed

TABLE 1

CELLULOLYTIC ACTIVITY OF ZALERION RAPTOR AND ZALERION XYLESTRIX ON WALSETH CELLULOSE

Fungus	*Age of Culture in Days*	*Activity of Fungal Filtrates mg RS/ml/24 hour*
Z. raptor	3*	0.139
	7*	0.185
	7**	0.765
	12**	1.67
	13	0.937
	16	0.795
	20	1.05
Z. xylestrix	5	0.138
	12	1.25
	12	1.11
	14	1.31
	15	1.15
	18	1.12

* Samples taken aseptically from culture vessel.
** Flasks inoculated one week after initial experiment.

greater cellulolytic activity (Table 1) than the other fungal filtrates of this species.

Field Studies

Measurements of breaking strength of cordage exposed at The Marine Laboratory pier are plotted in Figure 5.

In view of the complexity of biological and physical factors affecting the submerged cordage, the correlation between decrease in cordage strength with increased exposure is noteworthy. Reduction in strength was not evident prior to ten to twelve days following submergence. Although frames C and D were submerged approximately 30 to 40 feet apart, considerably less variability in the relationship of increased degradation with length of exposure was noted with the cordage on frame D.

Fungal infestation of the twine occurred within five days following submergence, with considerable variation in the intensity of attack along the entire length of the cordage. Variability in infestation has been observed between individual fibers

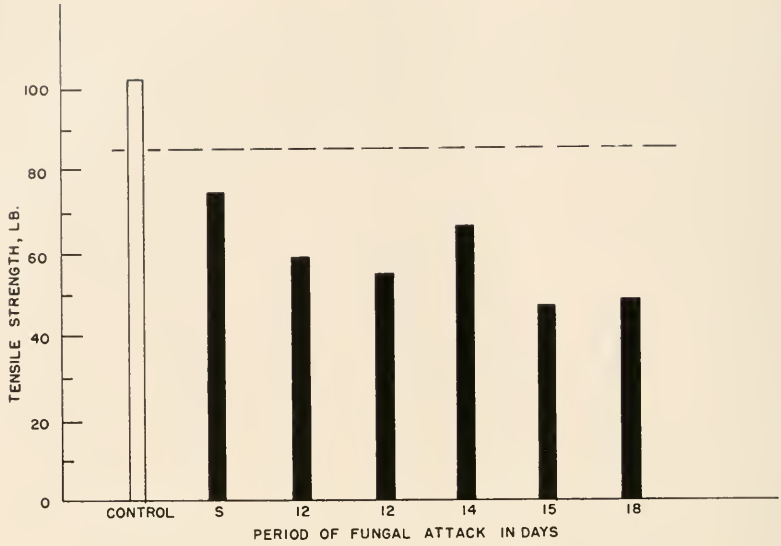

Fig. 3. Degradation of cordage by *Zalerion xylestrix.*

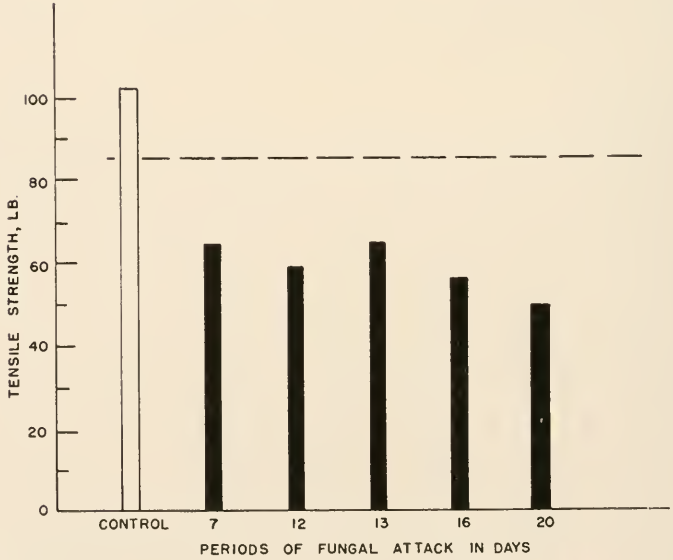

Fig 4. Degradation of cordage by *Zalerion raptor*

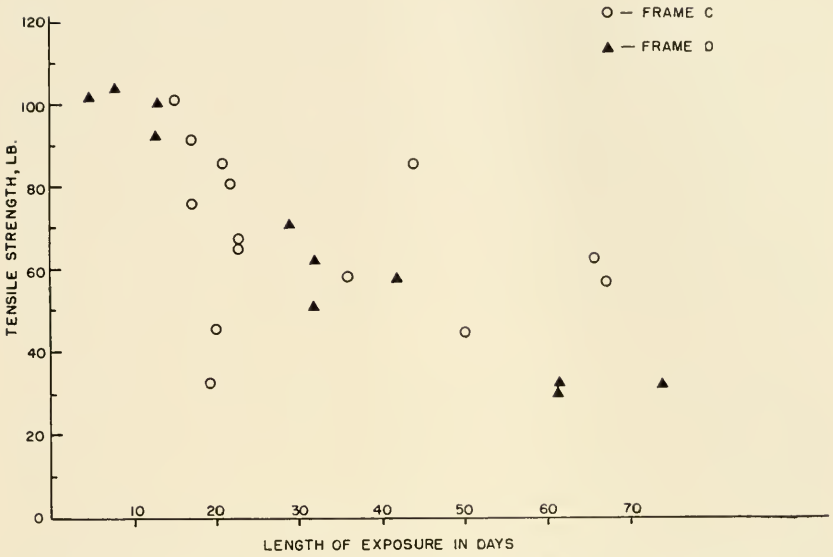

Fig. 5. Degradation of cordage exposed at Marine Laboratory pier.

of single twists as well as among closely adjacent fibers. More extensive and uniform fungal infestation of the cordage occurred with increased submergence.

In the characterization of the fungi present on the cordage, attempts have been made to differentiate between fungi actually penetrating the fibers and those present on the surface of the cordage or in the detritus on the exposed material. The predominant group of fungi isolated from the infested fibers were various ascomycetous species, including representatives of *Lulworthia, Peritrichospora,* and *Torpedospora.* At least three to four species of *Lulworthia* colonized the exposed cordage within the initial 10 days of exposure. As many as ten to twelve different deuteromycetous taxa have been isolated during the early period of submergence. With longer exposure the total number of different types of fungi on the cordage decreases. After approximately one month, the majority of the fungi isolated were Ascomycetes, particularly *Lulworthia,* which was often the only Ascomycete genus collected.

Species of *Aspergillus, Penicillium, Fusarium, Phoma* and

Pestalotia are common inhabitants of the Manila in the initial week of the exposure period and the frequency suggests that they are perhaps indigenous in the sea water environment. However, removal of the extraneous material from the surfaces of the cordage through several washings in sterile sea water also has reduced considerably the frequency of development of the various deuteromycetous fungi. This evidence, although preliminary, suggests that the latter group may be active largely on the surfaces of the submerged cordage. It is possible that several of these species function in the decomposition of the benthonic material on the fiber surfaces.

Strength determinations were made of Manila attacked in uniclonal culture by various isolates of *Lulworthia* collected from the cordage exposed at The Marine Laboratory pier. Although vigorous mycelial growth was apparent in the culture flasks after the termination of the test at twenty-four days, the majority of the eight isolates examined did not cause significant degradation of the cordage (Fig. 6). The average tensile strength varied from 73 to 92 pounds, the latter being well within the range of the control cordage. In cultural studies with *Lulworthia floridana,*

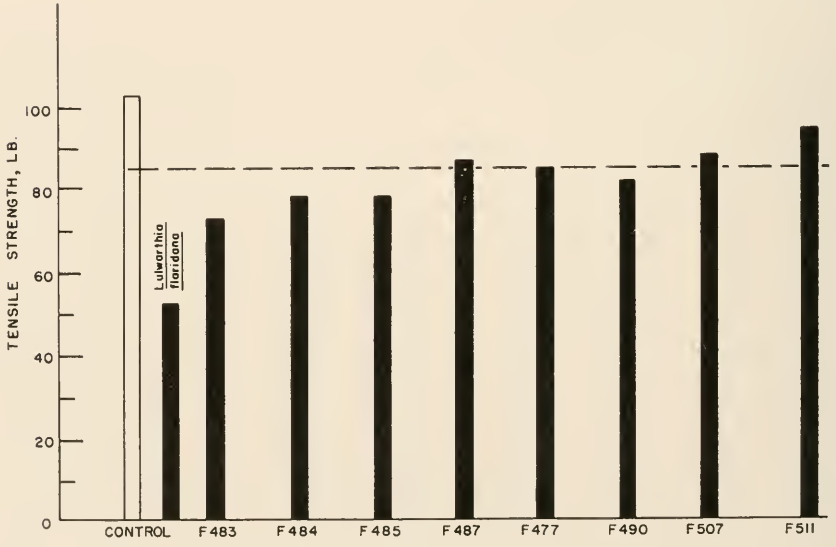

Fig. 6. Degradation of cordage by *Lulworthia* sp.

significant reduction in cordage strength by the fungus was demonstrated after a growth period of 40 days.

Considerable variablity in the intensity of reproduction on the Manila is evident. In some instances rupture of the cordage occurred in these areas, whereas in various tests no correlation between maximal reproduction and area of break was indicated. It is possible that in older cultures of *Lulworthia,* as has been observed earlier in such cultures of *T. radiata* and *Halosphaeriopsis* sp., more vigorous degradation of the lignocellulose substrate occurs in the region of fungal reproduction. Studies along these lines are in progress.

Because of the variabilities of fungal infestation in uniclonal growth, we have not been able to show an absolute correlation between the nature and intensity of fungal attack and the actual decrease in the breaking strength of the cordage. Furthermore, the role of the fungal biota in the degradation of cordage exposed in the marine environment cannot be estimated with accuracy. Obviously, other organisms as well as complex environmental factors are active in the degradative process. However, it is obvious that the specificity of fungal attack is an extremely important consideration in any evaluation of the contribution of fungi to other biological processes. The laboratory studies discussed in this paper and the frequency of isolation of cellulolytic species from submerged cordage is presumptive evidence for the participation of marine fungi in degradative processes under natural conditions. Subsequent critical examinations of microbial decomposition of submerged wood, and its multiphasic processes, must consider the diverse activities of wood-inhabiting marine fungi in this phenomenon.

ACKNOWLEDGMENT

We gratefully acknowledge the enthusiastic participation by Miss Katherine Kamp in numerous technical aspects of this study. The invaluable suggestions of Dr. Bryce Prindle in preparation of the testing procedures are appreciated.

SUMMARY

Exposure tests of Manila (Abaca) in Biscayne Bay, Florida,

over a period of five months, have demonstrated significant loss in cordage strength correlated with length of exposure of the material. Infestation of the cordage by the ascomycetous genus, *Lulworthia,* is evident but degradation was absent. Whereas, pure culture studies of other selected marine species, including *Zalerion raptor* and *Zalerion xylestrix,* and two Ascomycetes, *Torpedospora radiata* and *Halosphaeriopsis* sp., show significant production of cellulolytic activity and degradation of the cordage at various periods early in the growth of the organisms. An association between intensity of degradation and development of reproductive processes is suggested. Variations in reponse of the different isolates demonstrates the need for recognition of specificity of activity for accurate interpretation of the contribution of fungi to marine biology.

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Chapter 32

Yeasts in Marine Environments* † **

J. W. Fell *and* N. van Uden

Considerable research has been devoted to the bacterial flora of the oceans (21). However, the knowledge of marine occurring yeasts has been limited and only within recent years have there been extensive studies on this subject. In a series of papers first appearing in 1952, Russian microbiologists reported the quantitative distribution of yeasts in the Black and Okhotsk Seas, the Pacific Ocean and the Arctic (8, 10, 12, 15, 16). Yeasts were found in littoral zones and in open ocean waters as far as 60 miles from shore. Populations were most dense in the inshore locales and decreased progressively with distance from shore. Similarly, the number of yeasts per unit volume decreased with depth although viable cells were reported from 4,000 meters. This quantitative decrease with depth was not always consistent, as increases were observed at variable depths that were often in the range of thousands of cells per liter. It may be concluded from the Russian studies that yeasts have a wide distribution in marine waters and probably represent more than transitory or incidental forms.

To be reported here, is a summation of observations on the distribution, ecology, and taxonomy of yeasts isolated from the subtropical Atlantic near Miami, Florida and the warm temperate Pacific adjacent to La Jolla, California. Primary emphasis was

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examination of yeasts from waters and sediment as well as associated plants and animals.

METHODS

Sampling stations in the Atlantic were located in Biscayne Bay, an estuary along the SE coast of Florida, and selected locales in the Gulf Stream and the vicinity of Bimini, the Bahamas. Pacific collection sites were coastal areas near La Jolla, California. Water samples from open ocean areas were obtained with van Doren samplers. Sediments were collected either with a shallow water coring device (6), or a gravity corer in the deep sea.

The most successful method for the isolation and enumeration of yeasts from water samples was membrane filtration. In this technique a known volume of water was filtered and the membrane placed on an isolation medium (2% glucose, 1% peptone, 0.5% yeast extract and 2% agar in filtered sea water). This medium was made selective for yeasts by adjusting the pH to 4.5 with lactic acid or by addition of an antibiotic mixture (final concentration of 10 mg% chlortetracycline HCl, 2 mg % chloramphenicol and 2 mg % streptomycin sulfate.) Solid materials (algae, sediments and intestinal samples) were surveyed by incubation in flasks containing sterile sea water, 0.5 per cent glucose, and the antibiotic mixture. The samples were agitated for two to four days and aliquots streaked on isolation medium. Quantitative, as well as qualitative, results were obtained by the addition of a known quantity of the sample to 2 to 4 volumes of sterile sea water and then agitated for approximately 50 minutes. This procedure was followed either by direct plating or membrane filtration of a known volume of the washings. Streaked plates and filter membranes were incubated for two to three days at temperatures of 18-20 C for Pacific samples and 25 C for those from the Atlantic. Individual colonies were selected for further study on the basis of their microscopic and macroscopic morphology. Identification procedures were those advocated by Wickerham (20), Lodder and Kreger van Rij (13), and van Uden and Farinha (19). The liquid assimilation tests were kept in continuous agitation by means of a rotary shaker (2) or roller drum apparatus. This technique assured optimal aeration of the culture,

dilution of inhibitory metabolites, and continous bathing of the developing cells with nutrients. The prolonged time required for positive assimilatory reactions in stationary cultures was thereby reduced to less than five days, and the development of latent reactions was accelerated.

OBSERVATIONS AND CONCLUSIONS

Examination of waters and sediments from Florida coastal regions and adjacent off-shore areas demonstrated that yeasts were present with rare exception in all sample areas; however, distinctive qualitative and quantitative differences were observed. Environmental factors were found to be of major importance in determining the character of the resident yeast population. Population densities may be contingent upon the availability and concentration of organic material required for the growth and reproduction of the indigenous yeast flora. Sea water may normally contain tens or hundreds of yeast cells per liter, however, in regions of grass and algal beds there may be 5,000 to 6,000 viable yeast cells per liter of water. Determination of the yeast flora of marine vegetation (e.g., species of *Thalassia, Penicillus, Udotea, Sargassum, Laurencia,* etc.) by direct culture and surface washing techniques indicated that yeasts are relatively sparse on these plants and appear to be incidental transients. Allen and Dawson (1) reported that certain algal forms elaborate antibacterial substances and it is reasonable to assume that yeasts may also be influenced by such organic compounds. Our observations suggest that the organic constituents present in algae and grasses normally become accessible to the yeasts only after death and dissolution of the plants. The complex algal polysaccharides are probably reduced to simpler molecules by bacteria and actinomycetes before they serve as energy sources for most yeasts. Indirect evidence for this was the failure of yeasts (*Candida parapsilosis, C. tropicalis, C. albicans, Cryptococcus albidus,* and *Rhodotorula macilaginosa*) to grow either in sterile extracts of fresh algae (*Batophora oerstedi, Udotea flabellum, Penicillus captitatus, Laurenia poitei*) or in extracts supplemented with all growth requirements except a carbon source. Decomposing giant kelp from California waters was found to have viable cell

counts of *Metschnikowiella zobellii* (18) that ranged from 500 to 39,000 cells per wet gram of algae. In contrast, the closely related *M. krissii* has not been found in the algal heaps but was frequent in the surrounding waters. Apparently the products of microbial decomposition on the surface of the kelp are not utilized by *M. krissii.*

In open ocean areas, dense yeast populations have been associated with planktonic zones rich in organic matter (11). Other regions suitable for yeast growth and reproduction are current boundaries where contiguous water masses of opposing densities result in the accumulation of organic matter. Kriss (8, 9) observed that at such interfaces the rate of reproduction and total population of microorganisms far exceeds that occurring within the adjacent water masses. Similar data were obtained in our work by means of a series of vertical casts taken to depths of 500 meters in the Gulf Stream near Cat Cay, Bahamas (Figure 1). Oceanographic data obtained by our laboratory indicated that the 340 meter peak with approximately 2300 cells per liter coincides with an intrusion of Atlantic water into the Gulf Stream, while the 500 meter peak with 2000 cells per liter indicates a nutrient maximum at this depth. Although these studies are in a preliminary phase, it would appear that quantitative differences in yeast population could be of practical value in the location of current boundaries.

Terrestrial run-off is a major factor that influences the character of the yeast population of coastal areas. A comparison of yeast species collected from waters and sediment of Biscayne Bay, an area subjected to terrestrial pollution, and the relatively uncontaminated Gulf Stream (Table 1 and Table 2) suggests two points. First, the predominate species found in offshore regions are highly oxidative. *C. parapsilosis,* normally an actively fermenting species, customarily fails to ferment both glucose and galactose upon initial isolation from the open ocean and attains this capacity only after continued maintenance in culture. In contrast, estuarian strains of this species do not require a period of adaptation to manifest fermentative abilities. This tendency toward reliance on oxidative dissimilation may be due to the low organic content of offshore waters. Second, it is to be noted

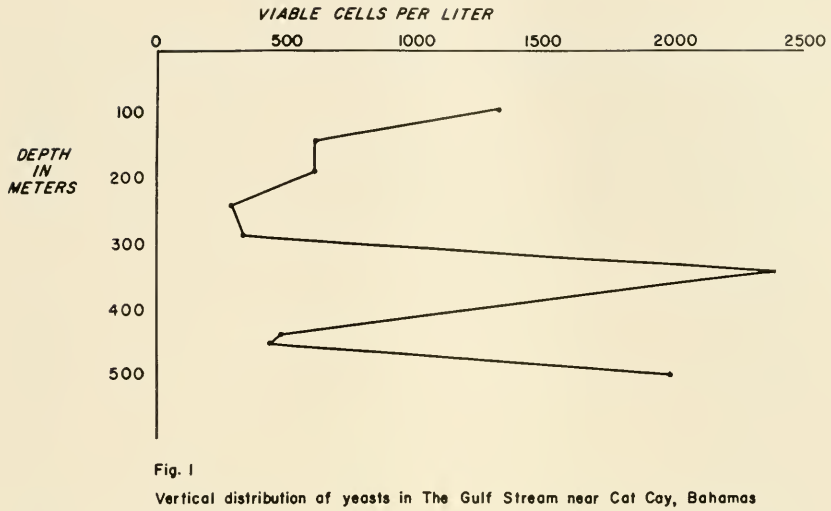

Fig. 1. Vertical distribution of yeasts in The Gulf Stream near Cat Cay, Bahamas.

that some isolates from estuarian waters (i.e., *C. tropicalis, C. parapsilosis, C. krusei, C. guilliermondii,* and *T. cutaneum*) are often found associated with terrestrial substrates such as soils, vegetation and may be cultured from man (4) and other warm blooded animals (17). This suggests that these species constitute pollution organisms when found in the sea. In evidence of this, these species were obtained in highest quantity (to 5,000 cells per liter) in the vicinity of areas of heaviest pollution such as the mouth of the Miami River, a zone of admixture of estuarian and river waters. In contrast, species abundant in sea water and not associated with the gastro-intestinal tract of animals, as *Rhodotorula mucilaginosa* and *R. glutinis,* failed to show quantitative increase in pollution areas. It should also be noted that *C. tropicalis, C. krusei,* and *T. cutaneum* were not obtained in pollution-free areas of the Pacific (unpublished data of van Uden and Castelo Branco) or the Gulf Stream. As a further index to the effects of contamination, a semi-enclosed artificial lagoon used for bathing was periodically examined for yeasts known to be associated with human intestinal contents, skin, and body orificies. Total yeast counts ranged from 0 to 320 viable cells per liter

TABLE 1

RELATIVE INCIDENCE OF YEASTS IN SEA WATER

Estuarian Waters (Biscayne Bay, Fla. unpublished data of Buck and van Uden)		*Open Ocean waters (Gulf Stream near Bimini, Bahamas)*		*Pacific Coastal Waters (near La Jolla, Calif., unpublished data of Van Uden and Castelo Branco)*	
Candida krusei	14%	"Black yeasts"	52%	*Metschnikowiella zobellii*	21%
C. tropicalis	13%	*Candida parapsilosis*	34%	*Cryptococcus albidus*	14%
Rhodotorula mucilaginosa	13%	*Rhodotorula glutinis*	8%	*Rhodotorula glutinis*	14%
R. glutinis	11%	*R. mucilaginosa*	2%	"Black yeasts"	14%
Candida guilliermondii	10%	*Candida guilliermondii*	2%	*Cryptococcus laurentii*	9%
C. parapsilosis	6%	Species with an incidence of less than 2%	2%	*Metscknikowiella krissii*	9%
R. graminis	5%			*Cryptococcus terreus*	3%
Trichosporon cutaneum	3%	*Cryptococcus laurentii*		*Candida guilliermondii*	3%
*Species with an incidence of less than 2%	25%	*Rhodotorula aurantiaca*		*Species with an incidence of less than 2%	14%
		Debaryomyces kloeckeri			
		Candida tenuis			

* To be reported in a future communication

TABLE 2

YEASTS COLLECTED FROM MARINE SEDIMENTS

Estuarian (Biscayne Bay, Florida)	*Deep Sea*
Candida albicans	GULF STREAM (near Cat Cay, Bahamas)
C. boidini	*Candida curvata*
C. intermedia	*C. guilliermondii*
C. guilliermondii	*C. parapsilosis*
C. melinii	*C. tenuis*
C. parapsilosis	*Cryptococcus albidus*
C. tropicalis	*C. neoformans var uniguttulatus*
Cryptococcus albidus	*Torulopsis famata*
C. laurentii	
Rhodotorula glutinis	
R. graminis	TONGUE OF THE OCEAN (near High Cay, Andros)
R. minuta	*Cryptococcus diffluens*
R. mucilaginosa	*C. laurentii*
Rhodotorula sp.	*Rhodotorula glutinis*
Trichosporon cutaneum	*R. mucilaginosa*
Debaryomyces kloeckeri	*Torulopsis famata*
Saccharomyces aestuarii	*Debaryomyces kloeckeri*
S. fructuum	

and were at a maximum following periods of heavy bathing activity. Lowest counts were obtained during periods of inclement cool weather. Unequivocal evidence of human contamination was the repeated isolation of the recognized pathogenic form, *C. albicans*. Laboratory studies showed that strains of *C. albicans* and *C. stellatoidea* isolated from human clinical material were able to survive in sea water for more than fourteen days.

These findings suggest that certain yeasts, which are normally associated with terrestrial sources, are probably pollution organisms when occurring in marine locales. Considerably more extensive and detailed yeast speciation studies of both polluted and unpolluted sea water areas are mandatory before more definite conclusions can be drawn. Novozhilova (15) identified 525 cultures from the Black and Okhotsk Seas and the Pacific Ocean, however, she limited herself to the identification of yeasts which do not form pseudo-mycelium. In such a study, *Candida* or *Trichosporon* species could have been confounded with *Torulopsis* or not identified. Bhat and Kachwalla (3) found that *C. tropicalis* represented one-third of the isolates obtained from water samples collected two to six miles off the coast of Bombay. If the degree of pollution in that area of the Indian Ocean were ignored there still remains the possibility that the existence of *C. tropicalis* may be dependent on the water temperature rather than the factor of pollution per se. Biscayne Bay and the Indian Ocean off Bombay are warm, sub-tropical waters, as compared with the warm, temperate zone of the Pacific near La Jolla, California. These considerations, however, fail to account for the absence of this organism in the warm waters of the Gulf Stream.

The depth within the sediment of an active yeast population is probably limited by the availability of oxygen for growth processes (Table 2). Yeasts were confined to the upper 2 cms of a sediment core obtained in the Gulf Stream at a water depth of 540 meters (5). However, in the Biscayne Bay estuary yeasts were found at sediment depths of 9 cm. The presence of yeasts at slightly greater depths within estuarian sediments is undoubtedly due to the considerable wave agitation and rapid settling of sediment that is characteristic of estuarian and other shallow water areas.

In an analysis of the species distribution in the various areas studied (Table 1), C. *parapsilosis* was found to be the most ubiquitous yeast in Florida waters, it was common in the waters and sediments of the estuary and was the most abundant offshore form. *C. tropicalis* and *R. mucilaginosa* were the most numerous estuarian yeasts. In distinct contrast, however, none of these species were isolated from Pacific waters. *Metschnikowiella zobellii and M. krissii,* described by van Uden and Castelo Branco (18), were predominate among the yeasts isolated near La Jolla, California. Yeasts with a similar morphology have been observed as parasites in the fresh-water crustacean *Daphnia magna* by Metschnikoff (14) and in larvae of the peratogonid fly *Dasyhelea obscura* by *Keilin* (7) but were not cultured for further study. The minimum number of viable cells for *M. zobellii* ranged from 20 to 580 cells per liter of sea water and for *M. krissii* from 10 to 570 cells per liter. High counts of *M. zobellii* were obtained from fish intestines and giant kelp. These findings suggest that *M. zobellii* and *M. krissii* are well adapted to marine environments.

Since yeasts appear to be relatively common in the sea, they are undoubtedly ingested during the normal feeding activities of fish. Examination of a variety of genera of fish from Biscayne Bay and Bahamian coastal waters revealed that yeasts appear to be restricted both quantitatively and qualitatively in the intestinal tract of most fish. Although a wide variety of species of yeasts were isolated (Table 3), the intestinal yeast flora of any particular fish may be a reflection only of recent habitats and feeding habits. In contrast to the scarcity of yeasts in the intestinal tract of fish from Atlantic waters, two species of fish, (*Atherinopis affinis littoralis* and *Trachurus symmetricus*) from the Pacific consistently maintained higher numbers of yeasts in the intestinal contents than were observed in the surrounding water. The predominate species was *M. zobellii* with cell numbers ranging from 25 to 5,700 cells per ml or gut contents. Counts for other yeast species inhabiting the intestines ranged from 5 to 380 cells per ml. These results suggest that some yeast species appear capable of growth in the digestive tract of certain marine fish.

TABLE 3

YEASTS ISOLATED FROM SPECIES OF MARINE FISH

BISCAYNE BAY, FLORIDA
Candida guilliermondii
C. parapsilosis
Rhodotorula mucilaginosa
Rhodotorula sp.
Trichosporon cutaneum
Debaryomyces kloeckeri
Debaryomyces minuta
Hanseniaspora valbyensis
Hansenula anomala
BIMINI, BAHAMAS
Candida parapsilosis
C. tropicalis
Rhodotorula glutinis
R. minuta
Trichosporon cutaneum

Many of the yeasts that have been encountered in the sea probably constitute transients (e.g., *C. tropicalis, C. krusei,* and *T. cutaneum*) that depend on the proximity of land for their presence and even survival. Alternatively, they may be of cosmopolitan distribution (e.g., *C. parapsilosis, C. guilliermondii, Cryptococcus spp.* and *Rhodotorula spp*), inhabiting terrestrial, estuarian, and open ocean environments. Yeasts isolated from estuarian and true marine locales have been found to be indistinguishable from recognized terrestrial forms. The fact that tests have not indicated any major metabolic or physiological differences between yeasts isolated from terrestrial and marine environs raises the question as to what constitutes a marine yeast. For a bacterium to be considered as a true marine form demands that in primary culture it demonstrate optimal growth in media prepared with sea water, and conversely that growth fails in fresh water. This criterion has been successfully employed in delineating most marine bacteria. In our experience, no yeast marine species by applying this criterion, as all yeasts encountered yet isolated from the marine situation can be declared a true grow equally well in either medium. Regardless of composition it

is most likely that any single isolation medium will fail to support growth or insure reproduction of all yeasts contained in a marine sample. Evidence for this was the occasional yeast isolated in this study that died out after two to three transfers on a battery of media prepared with sea water.

Contrariwise, a number of marine isolates that grow in restricted fashion on initial isolation attained physiologic vigor and demonstrated excellent growth in subsequent transfer. This is exemplified by certain off-shore isolates of *C. parapsilosis* which, as previously discussed, required a period of adaptation on organically rich media before they were able to evidence fermentation. All yeasts isolated from the sea were tested for the ability to utilize for growth, carbohydrates known to occur within algal cells. These strains did not demonstrate any significant metabolic variations from terrestrial counterparts when grown on such sugars as fucose, dulcitol, mannitol and galactose.

In this study no yeast encountered has shown unequivocal dependence on factors or conditions distinctive for the marine environment, nor has any isolate fulfilled the basic requirement deemed necessary to be termed a true marine form. The one possible exception are species of *Metschnikowiella*. They appear to be well adapted to life in the sea and perhaps are closer to the concept of a valid marine yeast than any other species yet encountered. With the information currently available, it would appear desirable to define a marine yeast merely as any yeast that is a consistent component of the oceanic microflora with demonstrated ability to survive and reproduce in this environment.

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SUMMARY

1. At least 30 different species of yeasts have been isolated from the marine environments studied. The distribution of species as well as their numbers and metabolic characteristics are governed by existing environmental conditions.

2. In regions of high organic matter, such as grass and algal beds, high concentrations of plankton, and current boundaries, a quantitative increase in the yeast population has been observed.

3. Due to low organic content in the open ocean, the resident yeasts rely mainly on oxidative metabolic processes.

4. The exterior surfaces of living marine algae do not appear to sustain significant yeast populations, apparently the native algal polysaccharides become available only after prior degradation by other micro-organisms.

5. Within the intestinal tract of certain marine fish, particular yeast species (e.g., *Metschnikowiella zobellii*) exist in greater concentration than in the surrounding water, whereas other yeast species occur as incidental transients.

6. Yeasts occur in marine sediments but appear to be limited to the upper surface layers.

7. With rare exception, the yeasts encountered in this study represent terrestrial species of transitory status in the marine situation or adaptive forms which survive in both environments. The *Metschnikowiella* species are well adapted to life in the sea and more closely conform to the concept of a true marine yeast than any other form encountered.

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Chapter 33

On the Physiology of the Photoautotrophic Purple Bacteria from Lake Beloye

L. K. Osnitskaya

The photosynthesizing purple sulfur bacteria are capable of utilizing light energy for the assimilation and utilization of carbonic acid owing to the presence of a cellular pigmental complex.

Under natural conditions these bacteria commonly dwell where there is light, and usually develop at some depths beneath the cover of the green algae.

The direct dependence of the development of purple sulfur bacteria on the photosynthetic process indicates that the optimum conditions for photosynthesis are also most favorable for the development of the bacteria. Thus light is one of the basic factors determining the development of purple bacteria.

French (2, 3) has shown that in purple bacteria (Athiorhodaceae) it is possible to observe a relationship between photosynthesis and the energy of incident light, which may be graphically expressed as a sigmoidal curve.

A similar tendency of light curves was observed in purple sulfur bacteria (Thiorhodaceae) by Wassink and his co-workers (12). Later on Morita (7) confirmed the results obtained by French (3) on other representatives of the nonsulfur bacteria. Clayton (1) investigated the influence of light on phototaxis in purple nonsulfur bacteria.

Though the above-mentioned investigators have shown the influence of the light intensity upon the assimilation of CO_2 by purple bacteria, they have not dealt with a most essential problem, i.e. the need of various species of purple bacteria for light energy. Besides, the above-cited investigations were carried out as short–term experiments with resting cultures, i.e., cell suspensions washed off the nutrient medium by centrifugation and

transferred to artificial conditions of the Warburg apparatus for manometric determinations. Whereas from the physiological viewpoint the true regularities in the development of the organism may be observed only under conditions which meet the requirements for the natural development of the organism.

In this connection, it is appropriate to mention the works of Maximova (4, 5, 6) who was the first to investigate the effect of varied intensity of light on the nonsulfur purple bacteria *Rhodopseudomonas palustris* in normal environmental conditions exposed to light and utilizing ready-made organic compounds. It was found that the light energy requirements of *Rh. palustris* under conditions photoheterotrophic nutrition are rather low; comparatively low intensities cause light inhibition of the culture.

It was of great interest for us to investigate the influence of light intensity upon the development of typical photosynthetic purple sulfur bacteria developing at the expense of photoassimilation of carbonic acid, and to establish their requirements for light energy.

The enrichment culture of the photosynthetic purple sulfur bacteria was obtained from silt samples from Lake Beloye. Following special cleaning treatment we isolated a pure culture of bacteria which was identified as *Chromatium vinosum* representative of the Thiorhodaceae, according to morphological and physiological properties (8).

To provide the optimum environment for the development of the culture under laboratory conditions, we chose the following method of cultivation. A sterile medium of composition K_2HPO_4-0.5%, $MgCl_2$ - 0.02%, NH_4Cl - 0.1%, NaCl - 0.5%, $Na_2S \cdot 9H_2O$ - 0.02%, $NaHCO_3$-0.5% per liter of distilled water of pH 7.4-7.5 was inoculated with bacteria and poured into sterile, hermetically sealed glass bottles or bottles with ground stoppers. The culture was grown in a thermostat at 27-29° C which was exposed to incandescent light.

Under such conditions the culture developed efficiently and a day later a visible growth was noted; in two to three days we observed profuse accumulation of the bacterial mass with a purple-red color. The bacteria did not grow in darkness, though they re-

tained their viability. On being exposed to light, they reproduced intensely.

The photosynthetic activity of the isolated bacteria, i.e., their capacity to assimilate carbonic acid under photoautotrophic development was established experimentally. We carried out quantitative estimations of assimilated carbon dioxide, the bacterial cell carbon content, the oxidized hydrogen sulfide, the amount of SO_4 and the number of bacterial cells in the course of the development of the culture. The methods of analysis were given earlier (8). Table 1 shows that the bacteria developed sucessfully in a medium where carbon dioxide acted as the sole source of carbon. The bacterial development and accumulation of biomass in terms of cell carbon, proceeded in accordance with the consumption of carbon dioxide and the oxidation of hydrogen sulfide to sulphuric acid.

TABLE 1

THE CONSUMPTION OF CARBON DIOXIDE IN THE COURSE OF PHOTOAUTOTROPHIC DEVELOPMENT OF *Chromatium vinosum*

Duration of Experiment (Days)	*Number of Bacteria (10^6/ml)*	*Consumed CO_2, mMoles*	*Oxidized H_2S, mMoles*	*Cell Carbon (mgr/ml)*
3	49.5	7.3	4.53	9.4
8	100.9	14.3	5.48	13.0

Thus, the bacteria we deal with are actually typical photoautotrophic specimens which, according to the classical definitions of microbiology, are incapable of utilizing ready-made organic compounds as nutrients. However, our further investigations have shown that when exposed to light the culture is capable of consuming some organic compounds in the absence of carbon dioxide. A detailed description of the methods is given in (9, 10, 11).

The results of the investigation of the development of *Chr. vinosum* on media, where the only sources of carbon were acetic acid, propionic acid and a mixture of propionic and acetic acids, are shown in Table 2.

The development of *Chr. vinosum* in an acetic medium results in the consumption of 8-9 mMoles of acetic acid in the

TABLE 2

THE CONSUMPTION BY *Chr. vinosum* OF ORGANIC ACIDS AS THE SOLE CARBON SOURCE IN THE MEDIUM

Carbon Source in the Medium	*Growth (Days)*	*Biomass mgr/100 ml*	*Consumed Acids Acetic, mMoles*	*Propionic, mMoles*	*Cell Carbon (mgr/ml)*
I.					
CH_3COONa	8	28	7.8	—	15.24
CH_3COONa	12	30	9.0	—	15.6
II.					
CH_3CH_2COONa	10	34	—	4.6	14.64
CH_3CH_2COONa	12	39	—	5.8	16.32
III.					
CH_3CH_2COONa + CH_3COONa	10	49	4.8	2.1	18.84
CH_3CH_2COONa + CH_3COONa	17	57	7.5	2.1	22.8

course of eight to twelve days; it should be noted that the bulk of the carbon consumed from the acetic acid was used for building the material of the bacterial cells, as seen from the corresponding increase of the cell carbon content. The culture was able to incorporate the propionic acid by constructive metabolism; the consumption of the propionic acid in the course of ten to twelve days amounted to 4.5-6 m moles. Judging by the considerable increase of the bacterial cell carbon for the same period of time, one may assume that the carbon of the propionic acid is in the main involved in the building up of bacterial cells.

In the development of *Chr. vinosum* in a medium containing acetic and propionic acids (both of them are regarded as sources of carbon for the bacteria), the acetic acid is consumed more intensely than propionic.

The data presented enables us to conclude that the purple sulfur bacteria *Chr. vinosum,* though an autotrophic organism by nature, is capable of switching over to photoheterotrophic nutrition (entirely or partially) under certain environmental conditions; the change of nutrients does not impair its development. The task of our further study was to investigate the influence of the intensity of light upon photoautotrophic development.

The experiments on the effect of varied light intensities were

performed in a luminostat designed so that its various compartments received different amounts of light energy. This was attained by regulating both the power of the lamps and the distance of the lamp from the culture. The light exposure was continuous. A layer of running water (3.5 cm) between the experimental vessels and the lamps absorbed the heat radiation of the latter.

The measurement of light intensity was carried out with a thermal column calibrated for conversion of light energy to erg/cm² sec.; and with the aid of the Goriayev chamber we estimated the cell yield; the inoculum was always carried out with similar amounts of homogenous material.

Table 3, containing data regarding the dynamics of the culture development in the course of ten days under conditions of varied intensities (from 1.4×10^3 erg/cm² sec. to 6.28×10^3 erg/cm² sec.) shows that at comparatively low intensities of light ($-1.4 - 3.4 \times 10^3$ erg/cm² sec.) the bacteria showed marked development; however, at the very lowest intensities (-1.4×10^3 erg/cm² sec.) the development on the third day was very slow (only 5.95 million cells per ml) as against 18.3 million cells per ml when the intensity is 6.28×10^3 erg/cm² sec.

TABLE 3

THE DYNAMICS OF DEVELOPMENT OF *Chr. vinosum* AT EXPOSURE INTENSITIES FROM 1.4 TO 6.28x10³ ERG/CM² SEC.

Nos.	*Light Intensity (Thousands of erg/cm² Sec.)*	*Number of Bacteria (10^6/ml)*			
		After 3 Days	*After 5 Days*	*After 7 Days*	*After 10 Days*
1	1.4	5.95	36	69	84
2	2.24	13.0	39.5	76.5	89.6
3	3.4	15.8	39.2	80.0	89.7
4	5.7	16.9	41.0	84.5	89.9
5	6.28	18.3	43.0	89.7	92.7

The difference in the yield between specimens growing under different conditions of light exposure has been observed throughout the entire experiment; however, if the cell yield of

the three-day cultures exposed to light with an intensity of 1.4 $\times$ 10^3 erg/cm^2 sec. is three times less than for cultures exposed to 6.28 $\times$ 10^3 erg/cm^2 sec., in this case on the seventh and later on the tenth day, this difference will amount to only 10 to 15 per cent.

In experiments with higher intensities (ranging from 5.77 $\times$ 10^3 erg/cm^2 sec. to 13.6 $\times$ 10^3 erg/cm^2 sec.) in which the dynamics of development were observed daily, a great change in the development of cultures was noted in one day; on the second day the cultures exposed to intensities ranging from 9.2 and 13.5 $\times$ 10^3 erg/cm^2 sec. exceeded by four to five times the cultures which had received about 5.77 $\times$ 10^3 erg/cm^2 sec.; but on the third day and especially on the fourth day the divergence between the cultures considerably diminished (Table 4).

TABLE 4

THE DYNAMICS OF DEVELOPMENT OF *Chr. vinosum* AT EXPOSURE INTENSITIES FROM 5.77 TO 13.5x10^3 ERG/CM^2 SEC.

Nos.	*Light Intensity (Thousands of erg/cm^2/sec.)*	*Number of Bacteria (10^6/ml) After 1 Days*	*After 2 Days*	*After 3 Days*	*After 4 Days*
1	5.77	1.4	11	58.4	73.1
2	6.93	2.72	29	61.3	75.0
3	7.92	2.07	38.6	66.1	75.0
4	9.2	2.27	49.6	68.2	74.2
5	13.5	3.32	55.9	69.0	80.0

Table 5 presents data from experiments carried out under the intensity of physiological radiation amounting to 7.35, 11.75 and 23.3 $\times$ 10^3 erg/cm^2 sec. The table shows that *Chromatium vinosum* developed favorably even at exposure intensity of 23.3 $\times$ 10^3 erg/cm^2 sec. The bacteria developed successfully also under exposure of 32 $\times$ 10^3 erg/cm^2 sec. On this basis one may assume that the threshold of luminous inhibition of the purple sulfur bacterium *Chr. vinosum* is beyond the tested intensities of physiological radiation.

Therefore, on the basis of the obtained data it is believed that

TABLE 5

THE DYNAMICS OF DEVELOPMENT OF *Chr. vinosum* AT EXPOSURE INTENSITIES FROM 7.35 TO 23.3x10³ ERG/CM² SEC.

Nos.	*Light Intensity (Thousands of erg/cm²sec.)*	*Number of Bacteria (10^6/ml) Experiment No. 2 2 days*	*Experiment No. 2 8 Days*
1	7.35	5.75	52.0
2	11.75	21.6	66.5
3	23.3	22.92	71.5

the purple sulfur bacterium *Chr. vinosum* is capable of photosynthesizing within a broad range of light intensities.

Though the development of the culture is also observed at low light intensity (1.4 thousand erg/cm² sec.), the insignificant and slow increase of the cell numbers shows that this light intensity is inadequate to ensure the development of these bacteria at the expense of photosynthesis.

Taking into consideration the above-mentioned ability of the exposed purple sulfur bacterium *Chr. vinosum* to utilize, under light exposure, some organic compounds whose assimilation requires a smaller quantity of light energy, it is possible to imagine that in natural habitats and under insufficiently favourable lighting conditions (the shadiness of the environment, profuse development of phytoplankton etc.) *Chr. vinosum* develops partly at the expense of photoheterotrophic nutrition. The high intensity of light stimulates a more rapid development of the culture and has the most pronounced effect during the early periods of the culture growth, i.e., during the second and third day. At later periods, i.e., on the fifth to tenth day, the effect of light intensity upon the development of the culture is not so distinct.

It is possible to assume that during the period of intense cell reproduction the photoautotrophic bacteria (which have no possible way for building cell material other than CO_2 photoassimilation) have particular need for light energy to realize this process. At later stages of the culture development the effect of light intensity is less pronounced owing to the various biochemical processes occurring in the medium. Possibly, this attenuation of the development of the bacterial culture may be ex-

plained by a more rapid exhaustion of the nutritive substrate at high light intensities.

CONCLUSIONS

Summing up the presented material, we come to the following conclusions:

1. The purple sulfur bacterium *Chr. vinosum* isolated from Lake Beloye is a typical representative of the photosynthetic microorganisms developing under light at the expense of carbon dioxide as the sole carbon source.

2. However, the bacteria may follow a photoheterotrophic path of development making use of carbon contained in some organic compounds (acetic acid, propionic acid, etc.) for building the organic material of their cells.

3. The culture is capable of developing by photoassimilation within the ranges of investigated intensities of physiological radiation (from 1.4×10^3 erg/cm^2 sec. to 32×10^3 erg/cm^2 sec.).

4. When the intensity of the incident light is weak (under $3\text{-}5 \times 10^3$ erg/cm^2 sec.), the bacteria develop and reproduce very slowly.

5. Light intensities of $5\text{-}6 \times 10^3$ erg/cm^2 sec. and higher are sufficient for normal bacterial development; higher light intensities cause strongly pronounced initial acceleration in the development of cultures and higher yields than under comparatively weaker exposure; however, subsequently the cultures developed and grew equally well irrespective of the radiation rate.

6. The highest dependence of the development of the culture on radiation intensity is observed in young cultures during the first two to three days of growth.

7. The obtained data are also of ecological interest as they show the possibility for these bacteria to reproduce in natural environments under various conditions of light exposure.

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Chapter 34

Bacterial Viruses in the Sea*

R. Spencer

There have been a number of investigations over the years concerning the occurrence in the sea of bacterial viruses, or phages, active against human intestinal bacteria of the family *Enterobacteriaceae*. The earlier work has been reviewed by ZoBell (19) and more recent investigations have been carried out by Guelin (9, 10). It will suffice to state here, however, that although phages active against a variety of enterobacteria can readily be demonstrated in polluted estuarine and coastal waters, their presence has not been demonstrated in waters remote from terrestrial contamination. Attempts to isolate phages from sea water against *marine* bacteria have, however, been successful in some cases. ZoBell (19) and Carlucci and Pramer (6) were successful in isolating from sea water taken close to the coast, and Smith and Krueger (16) from harbour mud, phages which were active against marine bacteria, and although ZoBell reported that he was unable to demonstrate the presence of phages in water collected beyond the littoral zone, such phages have subsequently been isolated by Kriss and Rukina (11) and by myself (17, 18).

Kriss and Rukina (11) examined a variety of samples of water from different stations and different depths in the Black Sea, together with samples of mud, for the presence of phages active against bacteria previously isolated from the Black Sea, and succeeded in isolating phages active against bacteria classified as *Micrococcus albus, M. candidus, Sarcina nivea, S. flava, Mycobacterium hyalinum, M. album, Pseudobacterium biforme, Bacterium qualis, Bacillus subtilis, B. angulans, B. goniosporus,*

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B. disciformus and B. mesentericus (12, and Kriss, *personal communication*).

I reported in 1955 (17) the isolation of a phage from a sample of sea water collected in the North Sea some ten miles off the coast of Scotland, which was active against strains of *Photobacterium phosphoreum*, and more recently (18) the isolation from the same area of six phages active against several species of bacteria previously isolated from sea water.

It must thus be concluded that phages do exist in sea water remote from terrestrial contamination, and that they may readily be isolated by recourse to suitable techniques. As, however, attempts to isolate such phages by various workers have not always been successful, the methods used with success by myself, and the experiments which led up to them, will be given in detail.

METHODS FOR THE ISOLATION OF PHAGES FROM SEA WATER

It was expected that if phages were present in sea water, they would be so in only low concentrations and that it would thus be necessary to examine large volumes of sea water against a variety of strains of bacteria. Also, as many marine bacteria have cultural requirements of, for example, temperature and salinity, different from those of terrestrial bacteria, it was considered necessary to pay careful attention to the cultural conditions of temperature and salinity in the isolation methods. Finally, it was thought that phages found in a particular area of sea were most likely to be active against bacterial strains which were themselves present in that area, and consequently that bacterial strains should be isolated from the area to be examined for use in the subsequent phage isolation attempts.

An enrichment or *indirect* method was first developed and was as follows. To each of a series of sterile bottles were added 100 ml of a quadruple strength sea water-based nutrient broth containing 4 per cent peptone and 4 per cent Lab Lemco, 300 ml of aseptically collected sea water and 8 ml of broth culture of four different bacterial strains. In each experiment, 6 liters of sea water were examined, against 40 bacterial strains. Half the inoculated bottles were incubated at 20 C for two to three days

and half for seven days at round 0 C. After incubation, a sample from each bottle was tested for the presence of phage active against each of the inoculated bacterial strains by means of lysis of the appropriate strain in a soft agar layer. Firstly, however, it was necessary to remove completely the large numbers of bacteria present in the sample without affecting unduly any phage which may have been present, and consideration was given to Fredericq's (8) chloroform technique for this purpose. A preliminary examination of Fredericq's technique, however, showed it to be unsatisfactory in the present circumstances, as the chloroform which dissolved in the medium inhibited the bacterial growth in the soft agar layer, and it was not practicable to aerate all the samples as recommended by Adams (2). Consequently, the usefulness of two other organic solvents, toluene and carbon tetrachloride, which are much less soluble in water than chloroform, (0.057 ml/100g at 16 C for toluene and 0.077g/100g at 15-25 C for carbon tetrachloride, as compared with 0.822g/100g at 20 C for chloroform, 15), was investigated. Under the experimental conditions used, these two substances were found to be more satisfactory than chloroform, in that bacterial growth in the soft agar layers was not inhibited and there tended to be less effect on the marine phages isolated (Table 1) although it was subsequently found that two out of the seven marine phages isolated, namely *P/SW31* and *P/SW34*, were inactivated by all three solvents. Carbon tetrachloride was selected in preference to toluene because of its slightly greater bactericidal effect during the shaking stage and because, as it is heavier than water, sampling of the aqueous phase was facilitated. The final method for the removal of bacteria was as follows. The supernatant was decanted from the bottle and centrifuged to remove most of the bacteria. A 50 ml amount was placed in a bottle and 0.5 ml of carbon tetrachloride added. This was shaken for one minute, allowed to stand for five minutes, and the bacteria-free aqueous layer removed for phage assay by the soft agar layer method given below.

Where phages were isolated by the indirect method, there was the possibility that they arose not from the sea water but because one of the four bacterial cultures added was lysogenic.

TABLE 1

THE EFFECT ON PHAGE TITRE OF SHAKING SUSPENSIONS OF VARIOUS PHAGES WITH CERTAIN ORGANIC SOLVENTS (10 ML. OF PHAGE SUSPENSION WERE SHAKEN WITH 0.1 ML. OF SOLVENT FOR ONE MINUTE FOLLOWED BY FIVE MINUTES STANDING BEFORE SAMPLING).

Phage	*Suspending Medium*	*Phage Titre After Shaking as Percentage of Initial Titre*		
		Solvent		
		Toluene	*Carbon Tetrachloride*	*Chloroform*
NCTC 7814[1]	Tap water broth	111(2)	93(2)	116(1)
NCTC 8400[2]	Tap water broth	103(2)	105(3)	106(1)
ATCC 11986[3]	Tap water broth	98(4)	95(4)	77(2)
P/14	Sea water broth	76(4)	75(4)	85(2)
P/L/34	Sea water broth	105(1)	101(1)	—
P/SW1/a	Sea water broth	91(2)	87(2)	91(1)
P/SW1/b	Sea water broth	95(1)	88(1)	83(1)
P/SW31	Sea water broth	0(2)	0(2)	0(2)
P/SW34	Sea water broth	0(2)	45(2)	0(2)
P/SW38	Sea water broth	91(1)	82(1)	76(1)

The figures in parenthesis show the number of replicate experiments of which the titre given is the mean.

[1] anti-*Staphylococcus aureus* phage

[2] anti-*Escherichia coli* phage

[3] anti-*Bacillus mycoides* phage

Where a phage was isolated, the four cultures were tested against one another for lysogenicity; with negative results.

The aim of the *direct* method for phage isolation was not only to isolate phages, but also to measure their concentrations against any particular host organisms, and consequently the standard soft agar layer method for phage assay was adapted to this purpose. To avoid interference by colonies developing from bacteria in the sea water, it was necessary to remove completely all such bacteria without affecting the phage concentration. Cellulose ester membrane filters were used for this purpose as it was found that virtually complete recovery of certain terrestrial and marine phages suspended in sea water was possible, provided more than 200 ml of the suspension were passed through the filter, Table 2.

In the direct method, sea water was passed through a cellu-

TABLE 2

THE EFFECT OF VOLUME FILTERED ON THE PERCENTAGE RECOVERY IN THE FILTRATE OF VARIOUS PHAGES SUSPENDED IN SEA WATER AND FILTERED THROUGH CELLULOSE ESTER MEMBRANE FILTERS

	Volume Filtered ml.			
Phage	20	50	100	200
	Percentage Recovery of Phage			
NCTC 7814	77	73	82	100
NCTC 8400	45	60	86	103
ATCC 11986	44	56	96	100
P/14	60	90	96	95
P/L/34	36	34	46	103
P/SW38	81	80	88	89

lose ester membrane filter to remove bacteria and 10 ml samples were mixed with 10 ml of a sea water based nutrient agar (peptone 1%, Lab Lemco, 1%, agar 1.5%), melted and cooled to 44 C, together with 2 ml of a young culture of the appropriate bacterial strain, and immediately layered onto the surface of an agar plate of the same composition. After a period of incubation to allow bacterial multiplication to take place, the presence of any phage active against that particular bacterial strain was manifested by the appearance of plaques in the depths of the soft agar. Normally, 600 ml amounts of sea water at a time were examined against a total of 40 bacterial cultures with incubation at both 0 C and 20 C.

A collection of luminous bacteria isolated from the intestines of fish were used in the earlier part of the work, but results with these strains were poor, only one phage being isolated. In the later stages, 40 strains were isolated from sea water and results with these strains were much better, six phages being isolated. Of the seven phages, four were detected by the parallel direct and indirect methods at both 20 C and 0 C. Three of these phages were present in a concentration of one to five particles/10 ml of sea water, and one in a concentration of approximately 100 particles/10 ml. The remaining three phages were detected by the indirect method only where they must have been present in a concentration of at least one particle/300 ml. Table 3 gives

details of the phages isolated. In all, six liters of sea water were examined by the direct method and almost 40 liters by the indirect method.

TABLE 3

DETAILS OF THE MARINE PHAGES ISOLATED

Phage	*Host Organism*	*Method(s) of Detection*	*Titre in Sea Water Sample*
P/14	*Photobacterium phosphoreum*	early indirect at 20 C	1 or more particles per 300 ml
P/L/34	*Cytophaga* sp.	early indirect at 20 C	
P/SW1/a	*Flavobacterium* sp.	direct and indirect, O C and 20 C	1-5 particles per 10 ml
P/SW1/b			
P/SW31	*Pseudomonas* sp.		100 particles per 10 ml
P/SW34	*Pseudomonas* sp.		
P/SW38	*Pseudomonas* sp.	indirect at 20 C	

THE CHARACTERISTICS OF MARINE PHAGES

Although phages active against marine bacteria have now been shown to be present in sea water, virtually nothing would appear to be known of the ecology of such phages; their distribution in the seas, in space or in time, their interrelationship with bacterial populations; nor their characteristics. A few figures for their concentration in one area over a limited period of time have been given, together with a few details of certain characteristics of one marine phage (17). These few details do, however, raise the interesting possibility that some marine phages may have certain characteristics quite different from terrestrial bacteriophages and that these characteristics may be a reflection of their environment. Two characteristics which tend to differentiate marine bacteria from terrestrial bacteria are response to temperature and response to the ionic concentration of their environment, and consequently the remainder of this paper will be concerned with recent work on the various facets of these two characteristics of the marine phages isolated by myself.

TABLE 4

THERMAL INACTIVATION OF VARIOUS MARINE PHAGES SUSPENDED IN SEA WATER BROTH

Phage	*Temperature of Exposure*	*Time of Exposure Minutes*									
	C	0	½	1	1½	2	5	10	15	30	60
P/14	50	***	***	***	***	***	**	†	†	–	–
	55	***	***	†	–	–	–	–	–	–	–
	60	***	–	–	–	–	–	–	–	–	–
P/L/34	55	***	***	***	***	***	***	***	***	***	*
	60	***	***	***	***	***	***	***	***	***	†
	65	***	***	***	***	***	*	†	–	–	–
P/SW1/a	55	***	***	***	***	***	***	***	***	***	*
	60	***	***	***	***	***	**	–	–	–	–
	65	***	***	***	**	†	–	–	–	–	–
P/SW1/b	55	***	***	***	***	***	*	–	–	–	–
	60	***	***	***	**	*	–	–	–	–	–
	65	***	†	–	–	–	–	–	–	–	–
P/SW31	55	***	***	***	***	***	***	***	***	***	**
	60	***	***	***	***	***	***	***	***	***	–
	65	***	***	***	***	†	†	–	–	–	–
P/SW38	55)										
	60)	No inactivation evident									
	65)										

*** confluent lysis
** confluent plaques
* many isolated plaques
† few isolated plaques
– no lysis

THE EFFECT OF TEMPERATURE

Thermal Inactivation

The resistance to thermal inactivation of six of the marine phages was examined by exposing the phages, at a titre of 10^6 particles/ml, suspended in sea water broth, to temperatures of 45, 50, 55, 60 and 65 C, for periods up to one hr. A loopful of each preparation was withdrawn at intervals and tested for lytic activity on seeded agar plates. The results are given in Table 4. In no case was inactivation evident within one hour's

exposure at 45 C, and in only one case, *P/14*, at 50 C. With phages *P/L/34*, *P/SW1/a*, *P/SW1/b*, and *P/SW31*, inactivation became evident after an hour's exposure at 55 C and was virtually complete within five minutes at 65 C. Phage *P/SW38*, however, showed no signs of inactivation after one hour's exposure at 65 C.

The resistance to thermal inactivation of the least resistant phage, *P/14*, was investigated in more detail. Samples of the stock preparation were diluted ten-fold in sea water peptone water or other media, and standard drops of these preparations were placed by means of a dropping pipette onto a waxed surface. These drops were then taken up by capillarity in fine capillary tubes. The lower ends of the tubes were heat sealed and the tubes placed in water baths at various temperatures. Tubes were withdrawn after various periods of time and the contents expelled into 1 ml amounts of sea water broth at room temperature, 20-22 C. Plaque counts were then made on these samples. Figure 1 shows the progress of thermal inactivation at 40, 45 and 50 C of *P/14* in sea water peptone water and it is evident that considerable thermal inactivation was taking place at 45 C although it was not detectable by the previous cruder method. A similar slight degree of inactivation at 40 C to that in sea water peptone water also took place in sea water broth while inactivation in tap water broth or peptone water was slightly more rapid. Although this point has not yet been examined, it is probable that a more detailed examination of the other phages by this method would similarly show thermal inactivation at somewhat lower temperature than shown in Table 3.

The literature on the thermal inactivation of phages can not be reviewed here but it would appear that, in general, terrestrial phages suspended in organic media such as broth are not appreciably inactivated within one hour at temperatures below 60 C. Five of the six marine phages, however, showed complete or marked inactivation within one hour at 55 C and were probably undergoing inactivation at 50 C, certainly so in the case of *P/14*, and it would thus appear that the marine phages examined are, in general, more heat sensitive than terrestrial bacteriophages. Phage *P/SW38* may, however, possess the more extreme degree of thermal stability exhibited by many of the phages active

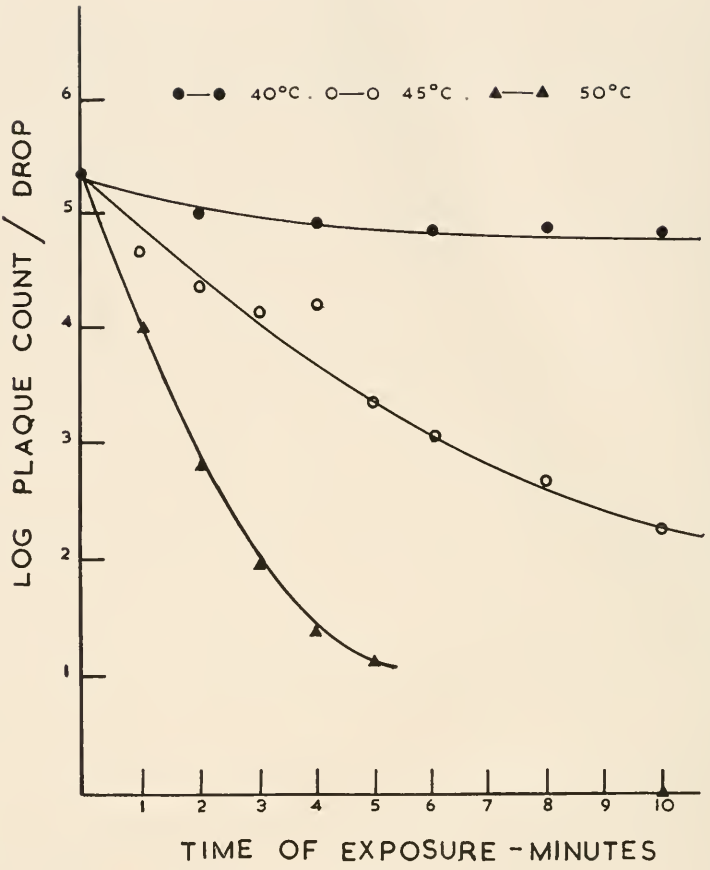

Fig. 1. The thermal inactivation of phage P/14 suspended in sea water peptone water, at 40, 45 and 50 C.

against the lactic streptococci. More detailed comparative investigations with marine phages and representative terrestrial phages will certainly be required before it can be concluded that a fundamental difference exists in sensitivity to thermal inactivation.

Burnet and McKie (5) studied the influence of the cation balance of the suspending medium on the thermal inactivation of various phages and showed that while both mono-and divalent cations increased heat sensitivity when used separately, when combined in a suitable proportion they exerted a "balanced

salt action." Phages survived heating in balanced salt solutions as well as they did in broth. This work has, by-and-large, been confirmed by Adams (1) and Amos (3) although Adams found, in contradistinction to Burnet and McKie (5), that an increase in the cation concentration of the suspending medium resulted in an increase in the thermal stability of one phage, T5. In view of the comparatively high salt content of the habitat of marine phages, the influence of the cationic composition of the suspending medium on the thermal inactivation of *P/14* at 40 C when suspended in sea water, distilled water, and in various NaCl concentrations was investigated with the results shown in Figure 2. It can be seen that inactivation in sea water was very slight, and

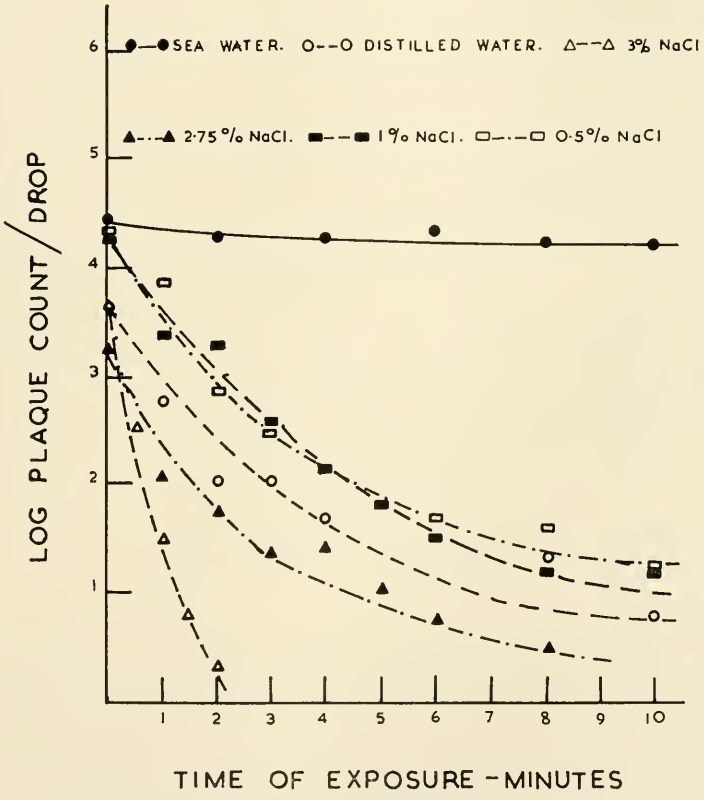

Fig. 2. The thermal inactivation at 40 C of phage P/14 suspended in various inorganic media (1).

similar to that in sea water peptone water, Figure 1. Inactivation in all the NaCl solutions was, however, much more rapid. This does not appear to have been due to a positive action of the NaCl, at least up to a concentration of 2.75 per cent, approximately that in sea water, as the rates of inactivation were similar to that in distilled water. A more likely cause is the presence in sea water of some factor which reduces the effect of sodium cloride on thermal inactivation. The results shown in Figure 3 indicate that the magnesium ion, present in sea water as $MgCl_2$ at a concentration of 0.495 per cent, serves this purpose, and that

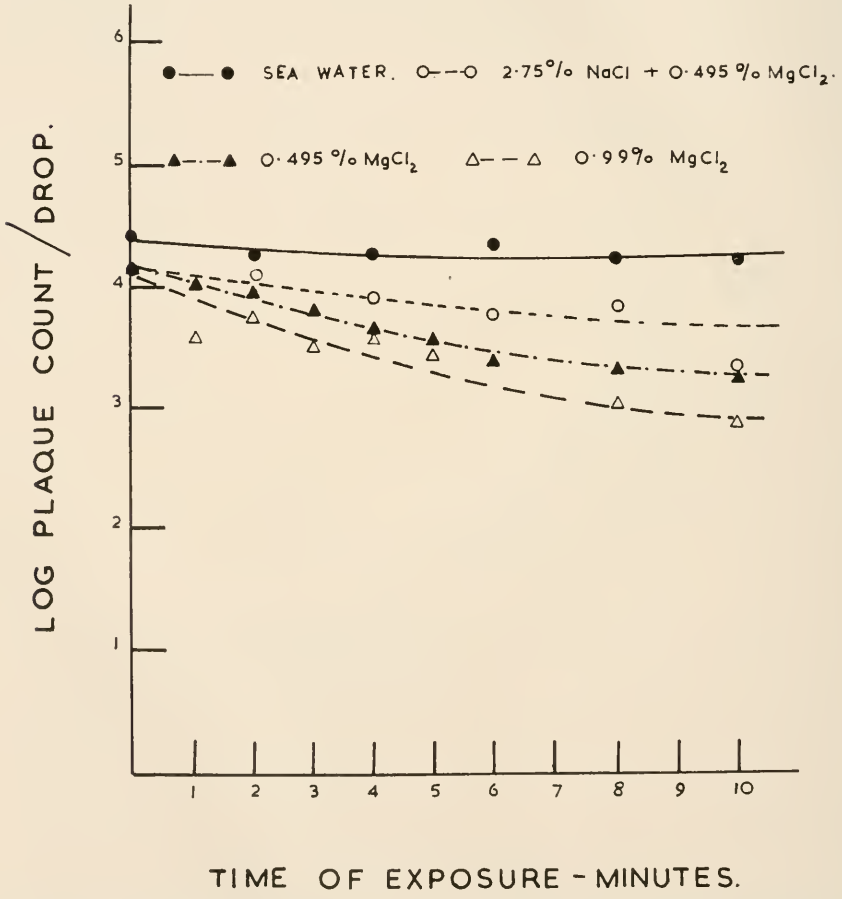

Fig. 3. The thermal inactivation at 40 C of phage P/14 suspended in various inorganic media (11).

the effect of sea water in minimizing thermal inactivation is probably a "balanced salt" effect.

Effect of Incubation Temperature on the Ability to Lyse the Host Organism

The bacteria against which the phages were isolated were all psychrophilic, that is, were capable of growing at temperatures around 0° C and consequently the ability of the phages to cause lysis at such a low temperature was investigated. Sea water Lemco agar plates, surface seeded with the appropriate bacterial strain, together with samples of the phage preparations, were placed in a refrigerator fitted with a recording thermometer and running at 1 C ± 1 C. After some hours had elapsed and the inoculated plates and phage preparations had reached the ambient temperature of the refrigerator, drops of the phage preparation were placed on the surface of the cultures. The plates were then left in the refrigerator for up to twenty-one days for bacterial growth to occur. In all cases, lysis developed. All the phages would thus appear to be capable of multiplication at the low temperatures of their environment. The only other report of a phage able to multiply at such low temperatures would appear to be that of Elder and Tanner (7).

Several of the phages were unable to cause lysis, under the experimental conditions used, at temperatures such as 30 or 37 C at which the host bacterial strain grew well. The phenomenon was most marked, however, with *P/14* which would not cause lysis at 22 C, although it would at 21 C. The host strain would grow at temperatures up to 25 to 28 C.

THE EFFECT OF SALINITY

Survival in Sea Water

Because of the sparse bacterial population of the open seas, it is probable that marine phages would need to be capable of prolonged survival in sea water at temperatures between 0 and 20 C to ensure eventual contact with a sensitive bacterial cell. This ability was investigated by adding phage preparations to 2.5 liter amounts of sea water, previously membrane filtered to remove bacteria which would interfere with the subsequent phage

assay. These samples were then stored either in the dark at 0 C or in day light at room temperature, 20-22 C, and the phage concentration was assayed after various periods up to one month. The anti-coli phage, *NCTC 8400,* was included.

The anti-coli phage was completely inactivated within eleven days at 0 C and within seven days at 20 C, whereas the marine phages were not inactivated within one month, although some inactivation was becoming evident at the end of this period, particularly at the higher temperature in day light.

The Effect of the Ionic Environment on the Ability to Lyse the Host Organism

The phages were isolated by the use of sea water-based media and it was soon observed that three of the phages, *P/14, P/L/34* and *P/SW1/b* would not always grow on tap-water-based media containing 0.5% NaCl. Some preliminary experiments have been carried out on this phenomenon.

Phage *P/14.* It was shown that the requirement for sea water based media was not purely osmotic in that the addition to a basal salt-free tap water medium of a variety of salts to a molar concentration equivalent to that of NaCl in the sea water medium did not effect lysis. However, the addition of 0.1 M $MgCl_2$ allowed lysis to take place at a minimum concentration of 0.4 M NaCl, while 0.2 M $MgCl_2$ allowed lysis to take place with 0.1 M NaCl, although not in its virtual absence. It would thus appear that although both NaCl and $MgCl_2$ are required for lysis, there is no requirement for a fixed proportion; to a large extent, but not entirely so, these salts can replace one another. It has not yet been determined whether other salts can replace NaCl or$MgCl_2$ except that as the phage is citrate insensitive, Ca^{++} is not required for lysis.

Phage *P/L/34.* Unlike *P/14,* phage *P/L/34* would give lysis on the tap water media on some occasions but not others. It was again shown that the partial requirement for a sea water based medium was not osmotic, at is could not always be satisfied by the addition of salts to the basal salt-free tap water medium. It was found, however, that although tap water medium containing 0.5% NaCl would not always support lysis, the basal

medium without the addition of NaCl would. It was also found that if the bacterial cells used to inoculate the surface of the agar medium were not washed free from salts, lysis could on occasions take place up to a NaCl concentration of 0.4 M. The addition of $MgCl_2$ in 0.1 or 0.2 M concentration would uniformly allow lysis to take place up to a NaCl concentration of at least 0.6 M. These preliminary results are interpreted as indicating that once again both NaCl and $MgCl_2$ are required for lysis but that a balance is required. If the balance is destroyed by the presence of too much NaCl, lysis will not take place. Conversely, if the concentrations of the two salts are balanced, lysis will take place even at very low total concentrations of salts. It has also been shown that $MgSO_4$ and $CaCl_2$ can replace $MgCl_2$, and that KCl and Na_2SO_4 can destroy the balance. It would thus appear that this balance is essentially a monovalent/divalent cation balance.

Phage *P/SW1/b.* It should be noted firstly that phage *P/SW1/a,* isolated against the same host organism, causes lysis quite well on the tap water media. The requirement of *P/SW1/b* for a sea water based medium has not yet been investigated in any detail but the nature of the requirements appear to differ from those of *P/14* and *P/L/34.*

The requirement of many phages for cations for adsorption to and thus lysis of the host bacterium is well known (2). Ca^{++} in particular is frequently required but univalent cations such as Na^+ may also be necessary. The concentrations of the cations required, for example 0.01 M for univalent and 0.001 M for divalent cations for phage T1 (14) or 0.014-0.06 M for monovalent cations and approximately a tenth of these values for divalent cations for adsorption of a *Shigella dysenteriae* phage (4), were, however, much lower than those required for *P/14.* The apparent requirement of *P/L/34* for a cationic balance, even at high cation concentrations, does not seem to have been met with in the case of terrestrial phages. It has been shown that certain marine bacteria have a positive requirement for Na^+ (13), which again has not been met with in the case of terrestrial bacteria, and it may be that with both bacteria and phages, an

increased requirement for cations may be a reflection of a marine environment.

SUMMARY

There can be no doubt that phages active against marine bacteria do exist in sea water. Such phages may readily be isolated, provided that due attention is paid to such factors as the choice of host bacterial strains, media and temperature of incubation, and that a sufficiently large volume of sea water is examined. The quantitative occurrence in sea water of phages active against particular bacterial strains may even be determined. Little is known, however, of the ecology of such phages, nor their characteristics, although indications have been obtained that they may differ from terrestrial bacteria in respect to their temperature relations, such as thermal inactivation and range of temperature for multiplication, and ionic relations, such as ability to survive in sea water and special requirements of monovalent and divalent cations, particularly Na^+ and Mg^{++}, for lysis.

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Chapter 35

Studies on a Marine Parasitic Ciliate as a Potential Virus Vector*

Liselotte Moewus

INTRODUCTION

There has been much speculation on the role of viruses in the causation of diseases of fish. Indirect evidence and some direct evidence has been evolved. In general it may be said that the present state of knowledge of virology of the aquatic world is very limited. Epidemic outbreaks at seasonal intervals have been observed in ponds of fish hatcheries and in aquaria, that is, in environments with relatively small bodies of water and high population densities. Transmission experiments with crude extracts of the diseased organs or of skin lesions have been successful in some cases. Ultracentrifugates and bacteria-free filtrates have been found to be infective in other instances (9, 10). Electron microscopic studies have revealed the particulate nature of the carp pox virus and of the Bauchfellwassersucht of trout (9). By cytological investigations, the presence of inclusion bodies in tumor cells of several species of fish has been demonstrated (9). Nothing is known about viruses in a typical natural marine environment. This is not surprising because it is rather difficult to observe sick fish in the ocean.

The present report details work with a parasitic marine ciliate discovered by the author in the course of studies with Dr. M. Michael Sigel while developing a methodology for marine virology and in a search for viruses in marine animals. Among the animals employed we encountered several seahorses which carried tumorlike nodules on their bodies. When one tumor was cut up and pieces of tissue were placed on plasma clot in culture tubes and incubated at 20 C, in three out of four cultures

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a ciliated protozoan was found to be present after two days. The ciliates fed on the provided tissues and developed into small populations. The cultures proved to be bacteria free. After transfer into monolayer cultures of fish cells it could be observed that the parasites devoured the fish cells within a few hours and responded to this meal with further increase in numbers. They grew as well on monolayer cultures of mammalian cell lines which were obtained in Eagle's Tissue culture medium, supplemented with 10 per cent human serum. It was decided to study this parasitic ciliate, as such an organism and similar parasites of marine animals may conceivably play an important role in the transmission of viruses among animals in the sea.

MATERIALS AND METHODS

Carnivorous Feeding Method

A. *Passages of Stock Cultures.* Tissue cultures of the human cancer cell strain KB grown in milk dilution bottles in 10 ml of Eagle's medium (with 10% calf serum) have been used for weekly passages of two clonal strains of the ciliate. One day before the actual transfer was made the ciliate culture and the fresh KB feeder culture were checked for sterility. To the KB culture 0.5 ml of ciliate suspension was added. The cultures were incubated at 20 C in a refrigerated incubator. To the date of writing, the clones have been passed twenty-four times in this fashion.

B. *Quantitative Growth Experiments with Carnivorously Feeding Ciliates.* Kimble screw cap tubes were seeded with 50,000 KB cells in one ml of Eagle's medium (with 10% calf serum) and incubated at 37 C until a confluent monolayer of cells had developed. These feeder tubes were changed from growth medium to maintenance medium which contained 5 per cent calf serum. A counted number of ciliates contained in 0.1 ml of maintenance were then added to a set of KB tubes maintained in 0.9 ml of medium and to a set of tubes with an equal amount of medium alone. These experimental tubes were incubated at 20 C. At chosen time intervals, sister cultures of both kinds were fixed with one drop of 10 per cent formalin. The culture

fluids from several tubes of one kind were pooled and samples were counted in a hemocytometer. From young populations, ten samples were counted and from heavier ones, four samples were counted. When fish tissue culture cells were used in place of KB cells as food source, the cultures had to be washed before inoculation because they were grown in Eagle's medium with increased NaCl concentrations. In order to find a comparative basis for the quantitation of food cells of different origin we have started to carry out protein determinations of a control set of feeder cultures at the beginning of the growth experiment. This method, however, needs further elaboration.

Saprozooic Feeding Method

Two factors have been under investigation in the saprozooic feeding system. The influence of temperature on the growth rate and final yields has been studied with cultures grown at 20 C, 29 C and 37 C. Adaptation to media of various salinities was also tested. The array of free amino acids and vitamins present in Eagle's medium did not support growth of our strains. Therefore Melnick's medium, designed for primary monkey kidney cells, was chosen. It consists of Hank's balanced salt solution supplemented with 10 per cent lactalbumin and 5 per cent calf serum and an addition of penicillin (200 units/ml) and streptomycin (0.10 mg/ml). Before each growth experiment the ciliates were preadapted to the media or temperatures for one week. Fifty thousand animals were introduced into 50 ml aliquots of media. At chosen time intervals, 0.5 ml samples were removed from two sister cultures and counts were made. Depending on the densities of the populations, four to ten counts were carried out.

Virus Studies

Since no suitable laboratory strain of a marine fish virus was at hand at this stage of our investigations we had to make use of mammalian viruses for our explorative and methodological studies. The ease of growing and of measuring infectivity titres of poliovirus in KB—which cells serve as standardized food source —suggested its usefulness for our purpose. Moreover, polio strain Mahoney was found to have a remarkable temperature stability at a range from 20 C to 30 C provided the experimental periods

were not extended beyond one week of incubation. It was also found that its infectivity withstood osmotic pressures in the range of 320 mOsM to 1245 mOsM.

A. *Exposure to Virus of Carnivorously Feeding Ciliates.* Well developed monolayer cultures were infected with poliovirus for a period long enough to allow virus adsorption and multiplication, but not gross lysis of cells. This part of the experiment was carried out at 37 C. After all supernate virus had been carefully washed out, the infected cultures were divided in three groups. One set was frozen at minus 20° C at this point of the experiment in order to find out in a later titration the actual amount of virus present at 0 time. The second set was incubated at 30 C and 35 C in order to follow up the production of virus under the experimental conditions. To the third set a known number of ciliates were added. The same numbers of ciliates were suspended in comparable volumes of maintenance medium and also incubated. The two latter groups of cultures represented a comparison of ciliates in feeding and in starving condition. Supporting experiments had shown that the presence of poliovirus in feeder cells did not influence the growth rate of the ciliates. The ciliate inoculum had to be large to guarantee a fast destruction of KB cells so that they would not be able to produce considerable amounts of virus during the experiment. At chosen time intervals sister cultures of the three experimental sets of tubes were removed from the incubator. The cilates were counted and the specimens were frozen at minus 20 C for a later virus assay.

B. In similarily designed experiments, tissue cultures of grunt fin cells (*Haemulon flavolineatum*) were used as food supply. In this case we know that the feeder cells are not able to produce any virus above the offered seed dosage which is suspended in Eagle's maintenance medium. We compared in this system the effects on poliovirus of vigorously feeding ciliates and of starving ciliates.

EXPERIMENTAL RESULTS

Life Cycle Studies

Twenty clones were isolated in mammalian feeder cultures of the KB line. In fifteen clones a sexual reaction of the selfing

type occurred regularly two to three days after transfer to a fresh feeder culture. Upon re-isolation of mating pairs it was found that the conjugants do not separate but die. Thus selfing causes a drastic drop in cell numbers in the population. The survivors vary greatly in size, from 15 μ to 40 μ in length. When subclones were established from the smallest and largest individuals, they exhibited the same pattern of life cycle: the feeding animals were 45 μ long and after selfing had occurred the population consisted of diverse sizes and was remarkably thinned out. Among the survivors the small individuals predominated. This morphological variability was not found in five out of the original twenty isolates. During twenty-two passages no sexual reaction was observed. The cells were large, 45 μ to 50 μ, throughout the cultural life cycle. When a certain density was reached in a population the cells became cannibalistic. Phenol red incorporated in the media indicated that the cannibalistic clones shifted the pH from 7.4 to 6.8 within a seven day culture period. This pH change did not take place in the sexual strains. Clone T5 was chosen as representative of the selfer strains, clone T16 for the cannibalistic form.

Taxonomic Studies

Taxonomic studies were carried out with material stained with the Lwoff-Chatton silver line method. The number of cilia meridians was found to be 10-13. The buccal apparatus is of typical tetrahymenal character. A roundish macronucleus and one spherical micronucleus were observed. At the posterior end one caudal cilia can be seen. There is generally one contractile vacuole and vacuole pore. These morphological features place the form in the genus *Tetrahymena*. Our strains represent probably the macrostome and the microstome form of one and the same species. Facultative parasitism is known for these holotrichous ciliates (2).

Growth Experiments

A typical growth experiment of the carnivorously feeding T16 strain is represented in Figure 1. While in maintenance medium alone no increase is observed, the cell numbers rise seven-fold above the input within 45 hours in the KB fed ciliate

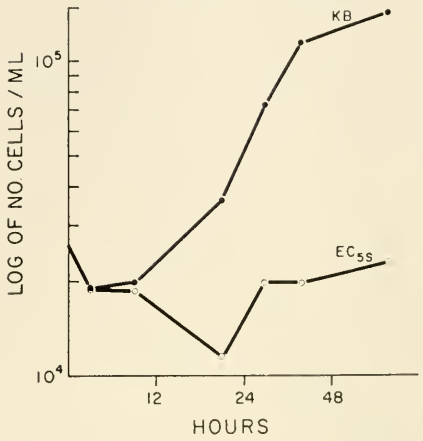

Fig. 1. Growth of clone T16 in Eagle's maintenance medium and in tissue cultures of the human cell line KB.

cultures. Figure 2 shows the reproducibility of such growth experiments. T16 strain was given grunt monolayer cultures as food in two independent experiments. In both cases the growth rates are comparable while the final yields depend on the number of fish cells offered. For comparison of the growth rates of parasitic and saprozooic animals, growth obtained in Melnick's mammalian tissue culture medium is indicated by the third curve.

The effect of temperature on ciliate production in Melnick's medium, prepared with Hank's salt solution, was measured over twelve successive passages. The data obtained are represented in Table 1. T5 was better fit to grow saprozoically than the cannibalistic strain T16.

Table 2 compares growth of both strains in the saprozooic feeding system in media of different salinities and at different temperatures. Final yields of 50 ml standard cultures were determined after seven days in twelve successive passages. Both strains exhibited better growth in the low salinity media. The effect of temperature was clearly expressed in the low salinity media but is insignificant in the seawater media, in which the difference in cell production of both strains was barely recognizable. Microscopial observations revealed that in T5 a weak selfing took place occasionally in Melnick's seawater cultures.

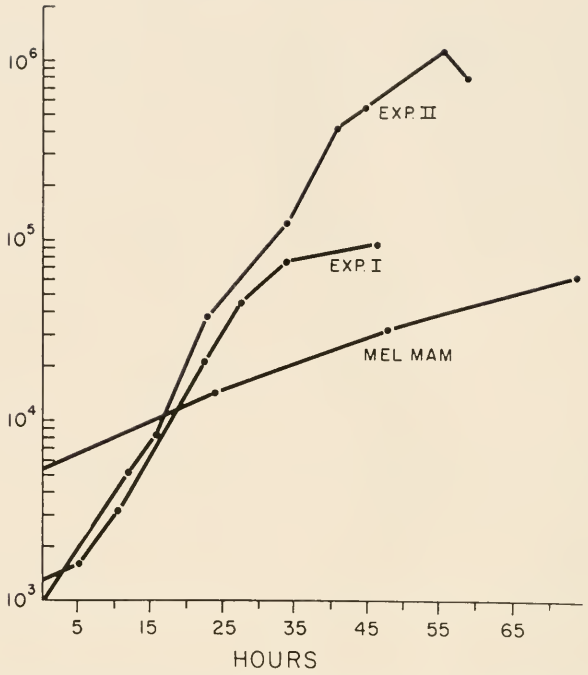

Fig. 2. Growth of clone T16 in tissue cultures of marine fish fin cells versus growth in Melnick's mammalian tissue culture medium.

Melnick's mammalian medium seemed to be hypotonic to T16, the cells looked swollen and rounded, while in seawater cultures they have a clearly outlined sigmoid elongated shape.

Virus Experiments

When we combine in one cultural system three biological systems—ciliate, food cell and virus—we have to expect that they act upon each other and react with each other. A careful study has to be made as to balancing out all experimental factors such as temperature, duration of incubation, salinities of the media, physiological condition of the ciliate and the food cell, etc. Several supporting experiments preceded the actual infection experiments.

It was found that poliovirus strain Mahoney is temperature stabile within certain limits. Inactivation took place in samples suspended in Eagle's maintenance medium over a period of 11

TABLE 1

EFFECT OF TEMPERATURE ON FINAL CELL YIELDS OF STRAIN T5 AND T16 (50ML CULTURES OF MELNICK'S MEDIUM, SPEED LEVEL 1,000 CIL/ML, SEVEN DAYS CULTURE PERIOD)

	T16		*T5*	
No. of Passages	*20 C*	*29 C*	*20 C*	*29 C*
1.	136,300	110,300	53,000	40,000
2.	140,300	68,700	46,000	26,000
3.	182,750	81,725	53,000	86,000
4.	193,000	137,700	60,300	61,700
5.	197,700	200,300	42,700	62,300
6.	198,700	167,000	64,000	52,300
7.	138,400	131,200	76,500	51,000
8.	130,500	113,500	51,750	34,500
9.	114,500	66,750	75,600	25,600
10.	159,500	101,500	101,500	32,750
11.	167,250	112,000	113,000	36,750
12.	184,500	77,250	58,750	28,500

TABLE 2

AVERAGES OF FINAL YIELDS OF TWELVE SUCCESSIVE PASSAGES OF STRAINS T5 AND T16 IN MEDIA OF DIFFERENT SALINITIES (50 ML CULTURES, SEED LEVEL 1,000 CIL/ML, SEVEN DAYS CULTURE PERIOD)

	T5		*T16*	
	20 C	*29 C*	*20 C*	*29 C*
Melnick's Mammalian medium (320 mOsM)	161,950cil/ml	122,527cil/ml	66,425cil/ml	44,783cil/ml
Melnick's Seawater medium (983 mOsM)	46,958	45,333	31,463	29,587

days in the following order: at 23 C—50 per cent loss, at 30 C—90 per cent loss and at 37 C—99 per cent loss. This information helped to find the proper dosages to be used and to determine the duration and incubation temperatures of future experiments.

The results of incubation of poliovirus suspended in several media of various salinities are tabulated in Table 3. There was no significant difference in effect of the various media on virus

at 0 time or on virus survival after seven days of incubation. Even the medium containing 100 per cent seawater, while showing what appears to be a decrease in infectivity of 1 log at time 0, failed to exert any consistent effect upon incubation.

TABLE 3

INFECTIVITY OF POLIO STRAIN MAHONEY RECOVERED AFTER INCUBATION MEDIA OF VARIOUS SALINITIES

Media	*Log TCD/ml at 0 time*	*After 7 Days of Incubation*	
	Seed Virus 10^5 TCD/cm	*20°*	*29°*
Eagle's maintenance	5.33	4.5	4.3
Eagle's growth	5.00	5.0	4.3
Melnick's mammalian	5.33	5.0	4.5
Melnick's in 50% seawater	4.50	5.0	4.33
Melnick's in 100% seawater	4.00	4.5	4.33

A very interesting fact evolved when poliovirus was given to grunt fin cell monolayer cultures. No cytopathogenic changes could be observed. When, however, the supernates were screened for the presence of active virus, it was found that the infectivity was lost within a shorter span of time than could be accounted for by mere loss through incubation at 20 C. This "factor" released by fish cells into the medium lost its effect gradually with duration of incubation. The nature and specificity of this interaction is unknown and is the subject of further studies by our group.

A very important support for our hypothesis that parasitic ciliates act as vectors between fish seems to be the fact that ciliates were not killed if exposed to poliovirus either in the carnivorous or in the saprozooic feeding system. As long as the chosen experimental set-up (nutrient supply and temperature) guaranteed multiplication, we found comparable cell production in the presence and in the absence of virus. The infectivity titres suffered some loss in long extended experiments due to the incubation effect but not in short term experiments. Unfortunately ciliates did not stand up well to centrifugation. It was, therefore, difficult to separate them from the culture fluid. We have, however, some evidence that virus was associated with the

ciliate cells and was not confined to the supernates. Further work has to be done to prove this fact beyond doubt.

Table 4 gives one example of an experiment where infected KB cells have served as food. Four logs of virus were present at 0 time as found in the KB controls. A heavy inoculum of ciliates was used to insure a fast destruction of KB cells in order to prevent virus production beyond the level present at time 0 (time when ciliates are introduced). One sees that the feeding and multiplying ciliates did not destroy the virus seed at 30 C. There was, as would be expected, less ciliate growth at the higher temperature, which on the other hand favors polio multiplication. We are not prepared at the present time to state that the rise of titre in the 35 C cultures was due to true virus production carried out by the ciliates. A few undestroyed KB cells could have been responsible for this newly formed virus.

TABLE 4

EXPOSURE OF CILIATES TO POLIO INFECTED MAMMALIAN FOOD CELLS (KB LINE)

	0 time		*After 24 Hours of Incubation at*			
			30°C		35°C	
	Cil/ml	Log TCD/ml	Cil/ml	Log TCD/ml	Cil/ml	Log TCD/ml
Polio contained and produced in KB cells		4.0		7.7		7.5
Ciliates incubated in EC_5S (Starving)	170,000	No virus offered	141,000		105,000	
Cilates + Polio infected food cells (Feeding)	170,000	4.0	440,000	4.3	200,000	5.7

To eliminate any doubt about the nature of newly formed virus, we made use of a feeder cell which cannot support poliovirus production. Such an experiment is shown in table 5. We started with a lower inoculum allowing for a 48 hour experimental time. This resulted in an 80 fold increase of the feeding population. Here we encountered the drop of infectivity caused by fish

cell culture fluid. This inhibition seemed to be removed after 48 hours. The original seed was recovered. The reason for the large decrease in the amount of poliovirus in the presence of starving ciliates remains to be determined.

TABLE 5

EXPOSURE OF FEEDING AND STARVING CILIATES (T16) TO POLIO VIRUS (FISH TISSUE CULTURES AS FOOD)

	0 Hrs.		*48 Hrs. at 20° C*	
	Cil/ml	*Log TCD/ml*	*Cil/ml*	*Log TCD/ml*
Polio (MAC) in EC_5S		5.16		4.5
Ciliate in EC5S+ MAC (Starving)	1,000	5.16	3,400	2.88
Ciliate in EC_5S+ MAC (Feeding)	1,000	3.5	79,166	5.0

DISCUSSION

It is a well established fact that some viruses pass through invertebrate or arthropod hosts without loss of infectivity. Virus transfer between fish, if carried out by a vector, calls for a facultative parasite. Such an organism should be able to live periodically independent in seawater, either in a motile phase or in the form of cysts. Thus far we have not found encystation in our two strains of ciliates. We have, however, shown that they can employ two feeding systems, the carnivorous way of life representing the parasitic phase and the saprozooic way of life representing

TABLE 6

OSMOTIC PRESSURE OF NATURAL AND EXPERIMENTAL ENVIRONMENT

	MOsM
Seawater	1245
Hank's BSS	290
Grunt Serum	410
Human Serum	310
Melnick's monkey kidney medium	320
Melnick's seawater medium	983

the free living phase. Growth experiments indicate that the efficient replication requires the carnivorous mode of feeding. The strains are better adapted to the lower salinities found in mammalian tissue culture medium and in the fish body fluids. Table 6 gives a comparison of the osmolarities of several environments the parasites are exposed to in our experiments.

As is known from tissue culture research, a slight hypotonicity guarantees best growth results. This rule is also valid for fish tissue culture (1) although L. Grützer obtained good results with isotonic medium (6). In our case, media prepared with various proportions of seawater did not give better growth than media designed for mammalian cells. It has been reported that fresh water ciliates lose their pulsating vacuoles and decrease in body size if gradually adapted to seawater (3, 7). In all media used in our investigation, pulsative vacuoles were found to be active. Cell sizes were larger in Melnick's seawater medium than in the low salinity medium.

A recent report (11) describes the successful isolation of a genuine fish virus by means of tissue cultures of appropriate fish cells. The discovered virus causes pancreatic necrosis in trout. It is planned to use this virus for similar experiments as described in Part 3 of this publication.*

Tetrahymena and several other ciliates have been exposed to influenza virus by Groupé and co-workers (4, 5). He emphasized the fact that the infectivity titres were reduced by a factor present both in live and in killed *Tetrahymena* cells, while hemagglutinin was destroyed only by actively growing but not by starving cells.

Our experiments indicate that poliovirus is not destroyed or inactivated by feeding ciliates but a profound and apparently irreversible inactivation of virus occurs in the presence of starving ciliates kept in Eagle's medium.

SUMMARY

In our search for viruses in marine fish an accidental discovery was made. A holotrichous hymenostome ciliate (*Tetrahy-*

* We thank Dr. Kenneth Wolf, U. S. Fish and Wildlife Service, Kearneysville, West Virginia, for sending his material to us.

mena species ?) was found associated with skin tumors of a seahorse. Two types of clones were established from the original population; one showed selfing and polymorphism, the other one was non-sexual and had different physiological properties. Axenic media and solid food were used in quantitative growth experiments. Endurance to raised temperatures and to various osmotic pressures were studied. Virus (polio strain Mahoney) was offered via ingestion of infected food cells. The infectivity of the virus was recovered from cells and culture supernates. From the listed data, the conclusion was drawn that parasitic ciliates may be considered as potential vectors of fish viruses.

ACKNOWLEDGMENTS

We thank Mr. William Clem and Mr. John Perchalski for providing fish tissue cultures for our studies. The Chatton-Lwoff silver line straining of our material has been carried out by Margaret Dyssart, Department of Zoology, University of Illinois, to whom we are most grateful. Osmolarity determinations have been done in the Department of Physiology, University of Miami School of Medicine. We thank Dr. W. Glen Moss for this assistance. The aquarist of the Miami Seaquarium, Mr. Edward Nichols, has given us courteous and most helpful cooperation.

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Part 4
Heterotrophy in Marine Microbiology

Chapter 36

Distribution and Ecology of *Azotobacter* in the Black Sea

L. N. Pshenin

The study of the productivity of a sea includes the quantitative calculation of organisms and the revealing of the dominant forms playing an important role in those or other processes of the cycle of substances (10). Nitrogen fixation in a sea, one of the most important processes, has been studied little from the quantitative and qualitative points of view. The aim of the present investigation (1955-1960) was to characterize the distribution and ecology (in their principal features) of *Azotobacter* and *Clostridium* in different regions of the Black Sea. In this article the materials on *Azotobacter* are mainly examined. At the same time nitrogen-fixing ability was studied in some other forms of microorganisms which usually developed in the elective (for *Azotobacter*) media inoculated by sea water.

MATERIALS AND METHODS

Our observations for the distribution of *Azotobacter* were carried out at 90 stations in different regions of the Black Sea, and in the west half of the sea in February 1956 they were carried out synchronously with the observations for the distribution of phytoplankton (3) at the same stations and depths. In shallow-water and in the surface layer, samples of the water were collected by a sampler supplied with sterile flasks; but in the deep-water regions 10 to 2000 m, they were collected by a bathometer. One hundred sixty samples of water taken at 30 stations were examined. Samples of the sediment were collected by a bottomgrab or by a corer at 44 stations. Thallus (*Phyllophora*) was collected from industrial trawls at 16 stations in the Zernov Phyllophora field. Coastal algae (*Ulva*, *Cistoseira* and *Enteromorpha*) were also examined. The number of bacteria was

TABLE 1

QUANTITATIVE DISTRIBUTION OF AZOTOBACTER IN WATER OF THE BLACK SEA

Depth in Meters	Central Deep-Water Regions: Section Evpatoria- Bosporus February, 1956			Central Deep-Water Regions: Section Yalta - Batumi August, 1956			The Region of Phyllophora Field: July 1956			Coastal Sites: April 1956		Coastal Sites: July August 1955
	Distance From Coast in Miles											
	110	77.5	28	71	70	80	15	20	24	4	0.1	0.6
	Number of Cells and Microcolonies of Azotobacter in 1 l. of Water											
0	< 9	2380	230	>2380	23	2380	>2380	2380	>23800	< 9		
5							2380	95	>23800		2380	23800
10	< 9	23	< 9					<90	2380	< 9		230
25	94	< 9	< 9	23	2380	23		230	>23800	< 9		
50	< 9	< 9	< 9	< 9	>2380	23				< 9		
75	23	< 9	23							< 9		
100	23	9	23	< 9	2380	2380				23		
150	< 9	< 9	23	230	23	230						
200	23	9	< 9	230	>2380	2380						
250	< 9	23			2380	230						
300	< 9	< 9	23	9	23	230						
500	9	23	23	< 9	23	>2380						
1000				23	23	2380						
1500				230	23	>2380						
2000					>2380	23						

determined by the method of titres after careful and repeated microscopy of the inoculations. The Fjodorov liquid medium was used for aerobes and Vinogradsky medium for anaerobes; both contained 50 per cent sea water. The water was inoculated in the following quantities. 100, 10, 1 and 0.1 ml., the sediment and thallus of algae—10; 1; 0.1 and 0.01 g. The method of titres does not give any possibility of estimating the absolute numbers of cells of heterotrophic bacteria (4), though it gives the possibility of characterizing the number of cells and microcolonies. The washings from algae were used for a direct microscopic count of the general number of the bacteria. From fresh thallus *Phyllophora* the impresses of the growth of bacteria in natural conditions were made on slides. From water and sediment on elective media, about 50 strains of *Azotobacter,* 30 strains of *Clostridium* and more than 100 strains of some other microorganisms have been isolated. Nitrogen fixing ability of the strains was determined by the micromethod of Kjeldahl.

RESULTS

It follows from Table 1, that *Azotobacter*, found in all the regions over the whole water column "O_2-and H_2S zones" of the Black Sea, is distributed microzonally. This is explained, obviously, as in the case of other heterotrophic bacteria (4) by the microzonal distribution of organic matter in water. The number of *Azotobacter* in water depends upon the season of a year. The comparative analysis of the distribution of *Azotobacter* and phytoplankton in water of the deep-water regions of the Black Sea has shown that in winter, in 10,000 cells of phytoplankton only few individuals occur (on the surface horizon, tens) and in summer hundreds to thousands and at a depth of 175-200 m tens of thousands of cells and microcolonies of *Azotobacter.* Between the general quantity of phytoplankton and the quantity of *Azotobacter* there is no direct dependence. Probably most of the vegetating species of phytoplankton are not connected by symbiotic relationships with *Azotobacter.* However, a direct dependence is displayed between the content of *Azotobacter* in sea water and the content of large forms of phytoplankton. The number of the latter in winter 1956 was considerably less than in

summer seasons. The maximum number of large forms of phytoplankton in summer reaches 6000 cells in 1 ml of water. Their biomass forms about 70 per cent of the biomass of all summer forms of phytoplankton mainly at the expense of warm- water species: *Rhizosolenia calcar-avis* and *Ceratium tripos* (6). Besides, more abundant nourishment of zooplankton in summer in the open sea leads to the increase of the content of the remains of poorly digested vegetable food in sea water (1, 2, 5). The maximum numbers of *Azotobacter* in sea water in summer often reach more than 2380 cells and microcolonies per 1 liter. The comparison of the data on the quantitative distribution of *Azotobacter* and phytoplankton gives an opportunity to suppose that moribund cells of large forms of phytoplankton and large pieces of indigested vegetable food of zooplankton are the main source of carbon for *Azotobacter* in sea water. If one proceeds from this premise, then the vertical distribution of *Azotobacter* in water of the deep-water regions of the sea at different seasons is explained rather easily. Vegetable detritus settling at different speeds can carry *Azotobacter* to deeper layers of water and to the bottom. Apparently the occurrence of *Azotobacter* all over the water column (including the "H_2S-zone") is due to this reason. There as the observations show, it can keep its vitality.

The microscopy of the 640 initial cultures (series of flasks with the Fiodorov medium, inoculated with different volumes of sea water of each sample) has shown that from 160 samples of the water, *Azotobacter* has been found in 122 samples, and microorganisms different from *Azotobacter* and *Clostridium* (more often nonsporeforming rods and Spirilla) in 148 samples. The latter two forms prevailed over *Azotobacter* quantitatively both in natural conditions in sea water, according to the data of the method of titres, and in the *Azotobacter* medium inoculated with sea water (the results of microscopy of the inoculations). They were isolated in cultures much easier than *Azotobacter*. One is left with the impression that in sea water, *Azotobacter* has serious competitors. This is confirmed by the fact that in most of the inoculations containing *Azotobacter* (83 samples of water from 122) cells of unhealthy or dead *Azotobacter* in appearance predominated, while in the same inoculations, nonsporeforming rods

and *Spirillum* always had a healthy appearance. *Clostridium* has not been found in the water column of the deep-water regions. In the region of the Zernov Phyllophora field and in the coastal sites it has been found in lesser numbers than *Azotobacter*.

The number of *Azotobacter* in muds is larger than in water. It is seen from Table 2 that over 70 per cent of samples of the sediment taken in coastal regions of the Black Sea contained more than 2,380 to 23,800 cells and microcolonies of *Azotobacter* in 1 kg. In 30 per cent of the samples of the sediment, *Azotobacter* has not been found. At the same time, in summer, i.e., in the period of the maximum development of *Azotobacter* in water, its greatest numbers were only more than 230 to 2380 per liter, and they were found in smaller numbers of samples (50%) in comparison with the muds. In the region of the Phyllophora field and in coastal sites of the sea, strong wind disturbances mixing the sediments sharply increased the content of *Azotobacter*, and especially, *Clostridium* in water. After a durable calm, these regions of the sea differ little from the deep-water regions in the quantity of *Azotobacter* in water. In the coastal muds in zone of H_2S, *Azotobacter* occurs as often in the muds as in the "O_2 zone." As the experiments showed, natural concentrations of H_2S had no lethal influence on *Azotobacter*. There are some reasons to expect that in the coastal muds containing H_2S, *Azotobacter* can find some conditions for its development and fixation of nitrogen, a method to increase the content of molybdenum and natural radioactive elements, the presence of radiogenous oxygen and some available sources of carbon (2, 7, 9).

The microscopy of wash water and impresses from thallus on slides showed that on the surface of *Phyllophora* there exists a rich microbe population in the form of microcolonies, pseudozooglea or diffuse accumulations consisting of cells morphologically identical to *Azotobacter* and to some other microorganisms. The general number of bacteria obtained by the method of direct microscopic count and their biomass per 1 kg. of thallus was on the average, 439 billions of cells or 105.4 mg. The numbers of *Azotobacter* obtained by the method of titres varied from 2380 to 23,800 cells and microcolonies per kg of fresh thallus. *Clostridium* occurs on *Phyllophora* more seldom

TABLE 2

QUANTITATIVE DISTRIBUTION OF *Azotobacter* IN THE SEDIMENTS OF THE BLACK SEA

Regions of the Sea	*Zones**	*Depth in Meters*	*Distance from Coast in Miles*	*Number of Stations*	*Number of Cells and Microcolonies of Azotobacter in 1 kg of Damp Mud*
North-east	O_2	8 to 26	0.5 to 29	9	2380 to >23800
Sea coast of the Crimea	O_2	14 to 100	0.16 to 12	7	2380 to >23800
	O_2	88 to 150	4 to 11.5	5	< 960
	H_2S	200	14.5	1	> 23800
Sea coast of the Caucasus	O_2	10 to 80	0.25 to 3	8	2380 to 23800
	O_2	24; 30	0.4; 1	2	< 960
	H_2S	260 to 1000	3 to 5.5	4	2380 to 23800
	H_2S	300; 338	3; 4.5	2	< 960
The region of Cape Caliacre	O_2	85; 96	12; 17.5	2	< 960
By the section of Yalta-Batumi	H_2S	2100 to 2160	82.5 to 95	3	< 960
	H_2S	1730	55.5	1	23800

* O_2—"oxygen zone" of the Black Sea.
H_2S—"hydrogen sulphide zone."

than *Azotobacter* and in lesser numbers. Between the epiphytic *Azotobacter and Phyllophora* there exist metabiotic and, perhaps, symbiotic relationships (8). *Azotobacter* and *Clostridium* have been found also on *Cistoseira, Ulva* and *Enteromorpha* inhabiting the bays of the Crimea.

The results of Laboratory experiments (Table 3) showed that the productivity of nitrogen fixation by the strains of *Azotobacter* using glucose varied from 1.5 to 10.7 mg N per gram of glucose, making up on the average 6.5 mg N/g glucose. The strains of *Spirillum* had higher levels of nitrogen fixation (from 4.2 to 16.1 mg N/g glucose) and on the average it reached 8.7 mg N per 1 g. The activity of nonsporeforming pigmented rods is

similar to that of *Azotobacter*. The other microorganisms showed considerably lower nitrogen fixing ability (Table 3).

TABLE 3

NITROGEN-FIXING ABILITY OF MICROORGANISMS ISOLATED FROM WATER AND SEDIMENTS OF DIFFERENT REGIONS OF THE BLACK SEA*

Organisms	*Area of Isolation*	*Depth in Meters*	*Number of Investigated Strains*	*Quantity of Fixated N in mg per g of glucose*	
				In Each Strain Taken Separately	*Average*
Azotobacter chroococcum	Water	0	2	7.7—9.4	
	Water	15	1	5.0	
	Water	250	1	5.5	
	Mud	22	1	1.5	
	Mud	335	1	2.8	
Az. vinelandii	Water	0	1	4.7	6.5
	Mud	19	1	6.1	
	Mud	30	1	5.9	
	Mud	80	1	6.6	
	Mud	1730	1	7.9	
	Phyllophora	22	1	10.3	
Az. nigricans	Mud	100	1	10.7	
Clostridium	Water	0:10	3	0.8—1.2	
	Mud	8 to 2160	11	0.7—3.5	1.6
Spirillum	Water	0:200	5	4.2—16.1	8.7
Nonsporeforming rods with yellow pigment	Water	0:200:2000	3	0.4—8.6	5.7
nonpigmented	Water	100:150	2	1.3—3.6	2.5
Torulopsis	Water	100:150	2	0.9—1.1	1.0
Rhodotorula	Water	35:50	2	0.1—0.4	0.25

* The regions of the investigation are shown in Tables 1 and 2.

Thus the results of the field and laboratory observations give the reasons to believe that *Azotobacter* and *Clostridium* have an essential importance in the process of nitrogen fixation in the muds of the Black Sea, and *Azotobacter* has the same importance on the surface of macrophytes too. However, in water of the open sea, especially in the deep-water regions, the leading role in nitrogen fixation belongs, apparently, to some other organisms. The participation of *Clostridium* in this process in water of the deep-water regions of the open sea probably falls off completely.

SUMMARY

Azotobacter found in all the investigated regions all over the water column ("O_2 and H_2S zones") of the Black Sea, are distributed microzonally. The number of *Azotobacter* in water depends upon the season of a year. In the open sea the numbers of cells and microcolonies of *Azotobacter*, per 10,000 cells of phytoplankton, were hundreds and thousands times more in summer than in winter. A direct dependence is displayed between the content of *Azotobacter* in sea water and that of the large forms of phytoplankton, but not between the former and the general quantity of phytoplankton. Apparently, the main source of carbon for *Azotobacter* in sea water are the moribund cells of large forms of phytoplankton and particles of indigested vegetable food of zooplankton, usually more abundant in summer. The presence of *Azotobacter* in water of the "H_2S- zone" is caused, probably, by the sedimentation of vegetable detritus carrying down *Azotobacter*. Besides *Azotobacter*, in shallow waters *Clostridium* has also been found, though it has not been found in water of the deep-water regions. In muds and on *Phyllophora* the number of *Azotobacter* is greater than in water. Muds from different depths always contained *Clostridium* in greater numbers than *Azotobacter*. Wind disturbances on the sediments influence the number of *Azotobacter* and *Clostridium* in shallow waters. In water of all the regions of the Black Sea, nitrogen fixing nonsporeforming rods and Spirilla have been found. They dominated quantitatively over *Azotobacter*. The average level of the productivity of nitrogen fixation by *Azotobacter* was lower than that of *Spirillum*, and it was approximately the same as in nonsporeforming pigmented rods. Some other microorganisms (nonpigmented nonsporeforming rods, *Clostridium* and yeast) have shown lower nitrogen fixing ability. In the muds of the Black Sea *Azotobacter* and *Clostridium* apparently play a considerable role in the process of nitrogen fixation and *Azotobacter*,—on the surface of macrophytes too. However, in sea water the leading role belongs, apparently, to some other organisms, and the role of *Clostridium* in the deep-water regions probably falls off completely.

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Chapter 37

Metabolic Pathways of Bacterial Nitrification*

M. I. H. Aleem and Alvin Nason

INTRODUCTION

After Pasteur's suggestion was confirmed by Schloesing and Muntz some 85 years ago that nitrification is brought about by living microorganisms, the process was shown to occur in two steps—first, the oxidation of ammonia to nitrite, followed by the oxidation of nitrite to nitrate (37). The organisms catalyzing each reaction were subsequently isolated in pure culture and named *Nitrosomonas* and *Nitrobacter*. The former converts ammonia to nitrite, and the latter oxidizes nitrite to nitrate. The discovery that they possessed the unique ability to grow and persist indefinitely in media devoid of organic substances led to the concept of chemoautotrophy in microbiology (38). A quarter of a century later, Otto Meyerhof described their growth characteristics and the effects of various organic and inorganic compounds on the primary oxidation reactions brought about by these chemoautotrophs (32).

Until a few years ago little was known about the intermediary metabolism or the mechanisms of utilization of the energy liberated from the oxidations of NH_3 and NO^-_2. Somewhat earlier several determinations and calculations had established that the energy released is used with an efficiency about 5 to 10 per cent by *Nitrosomonas* and *Nitrobacter* (10, 32). The investigations

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of Dr. H. Lees and his co-workers during the last ten years (9, 11, 12, 22, 28) have indicated in part the probable pathways of ammonia and nitrite oxidation by intact cells of *Nitrosomonas* and *Nitrobacter* respectively. Their studies have contributed to our understanding of the biochemistry and mechanisms involved in the primary oxidation reactions by these nitrifying organisms. More recently Aleem and Alexander (1-3, 8) demonstrated for the first time an active nitrite oxidizing system at the cell-free level. Aleem and Nason (4-7, 33, *) subsequently purified and characterized the nitrite oxidizing system from *Nitrobacter* as a cytochrome-containing electron transport particle (designated as nitrite oxidase) which catalyzes the enzymatic transfer of electrons from nitrite to molecular oxygen via cytochromes *c*- and a_1-like components. They showed that nitrite oxidation in the presence of partially purified nitrite oxidase particles was accompanied by phosphate esterification resulting in the formation of ATP. Almost simultaneously Delwiche (31) also reported the coupling of ATP formation to nitrite oxidation using crude cell-free preparations of *Nitrobacter*. Soon afterwards Nicholas and Jones (34) obtained a partially purified hydroxylamine-oxidizing system from *Nitrosomonas*. Very recently Delwiche *et al.*, has reported the oxidation of hydroxylamine and concomitant phosphate esterification by crude, cell-free extracts of *Nitrosomonas* (15). According to several recent reports (19, 20, 30), certain heterotrophic nitrifiers also appear to play a limited part in the nitrification process. There is no evidence at this time that the oxidation of ammonia or nitrite is accompanied by phosphate esterification in these organisms. Full attention, therefore, will be devoted in this review to the metabolic studies that have been conducted on the chemoautotrophs, *Nitrosomonas* and *Nitrobacter*.

AMMONIA OXIDATION BY NITROSOMONAS SPS.

Nitrosomonas cells derive their energy for growth and cell synthesis from the aerobic oxidation of ammonia to nitrite (10, 21) as indicated in the over-all equation:

$$NH_4^+ + 1.5O_2 \rightarrow 2H^+ + NO_2^- + H_2O + 66.5 \text{ K.Cal.}$$

* unpublished data.

The transformation of ammonia to nitrite has been shown to be stoichiometric with the optimal reaction taking place between pH 7.5 to 8.8 and at a substrate concentration of 3 to 5 $\times$ 10^{-2} M (16, 26, 32). According to Hofman and Lees (22) the efficiency with which the organism uses energy released during the above reaction in order to assimilate CO_2 falls off as the concentrations of the reaction product (NO_2^-) and cell carbon increase in the culture. This apparent decrease in free energy efficiency has been attributed to an expenditure of energy in order to offset the tendency of the nitrite accumulating in the growth medium to enter the cell. According to Engel and Alexander (16), however, the resulting increase in nitrite concentration has no apparent effect on substrate oxidation and cell proliferation indicating that during the active period of cell growth, the free energy efficiency of *Nitrosomonas* remains unchanged.

Some 35 years ago, Kluyver and Donker (27) hypothesized that the oxidation of ammonia takes place in three steps, each consisting of two-electron changes: ammonia $\rightarrow$ hydroxylamine $\rightarrow$ hyponitrite $\rightarrow$ nitrite. The stoichiometric formation of nitrite in the course of ammonia oxidation indicates that there is very little, if any, accumulation of the intermediates during the reaction (23). The use of suitable inhibitors which act selectively on particular steps in the above suggested series of reactions has proven to be a useful means of accumulating intermediates during the reaction sequence. The first experimental evidence pointing to hydroxylamine as an intermediate of ammonia oxidation was demonstrated by Hofman and Lees (23) and has since been confirmed by other workers (16-18, 24, 25, 34, 35). In the presence of 3 $\times$ 10^{-3} M hydrazine, the oxidation of ammonia by *Nitrosomonas* cells leads to the accumulation of hydroxylamine in the medium by apparently inhibiting the further metabolism of hydroxylamine to nitrite. The observed effect of 10^{-5}M thiourea or allylthiourea in depressing the oxidation of ammonia but not of hydroxylamine by *Nitrosomonas* also implicates hydroxylamine as an intermediate in the nitrification process (21, 23). Moreover, *Nitrosomonas* cells, upon aging under refrigerated, frozen or lyophilized conditions, lose their ammonia oxidizing ability at a faster rate than that of hydroxylamine oxidation (9,

18). This has been ascribed to some modification in the cell membrane whereby the ammonia oxidizing portion of the reaction sequence is more adversely affected than the hydroxylamine oxidizing system which remains intact (29).

Nitrosomonas sps. have always been regarded as strict chemoautotrophs metabolizing only the inorganic ammonium salts. According to a recent report by Ruban (36), these bacteria also possess specific deaminases which enable them to utilize the amino groups of several purine derivatives such as guanine, uric acid and allantoin. The amino acid alanine, however, was not metabolized unless it was pretreated with *Pseudomonas* autolyzates for its deamination.

Thus far ammonia oxidation at the cell-free level has not been accomplished in spite of innumerable efforts by various workers (*, 24, 25). Although Imshenetskii and his school (24, 25) claim to have demonstrated the oxidation of ammonia and hydroxylamine by *Nitrosomonas* autolysates, their data hardly support a positive conclusion. They prepared antolysates by shaking *Nitrosomonas* cells with glass powder for twenty-four hours at 40° C, and performed their experiments at 37° C with incubation periods ranging as high as five days under conditions which favored the complete denaturation of the metabolizing enzymes. No precautions were indicated to eliminate unbroken cells or contamination by other microorganisms. The transformation of ammonia to nitrite taken as an average of fifteen experiments, gave inconsistent results and insignificant amounts of nitrite (in the order of 0.54 ppm. NO_2^--N per 5 days). According to the recent studies of Anderson (9), cell-free *Nitrosomonas* extracts failed to oxidize ammonia because its oxidation to hydroxylamine is an endergonic reaction (Δ_F = 4.7 K.Cal.) which presumably requires energy-rich phosphate for its activation. The procedures for preparing cell-free extracts might have disrupted the energy coupling mechanisms. It would be worthwhile to determine whether ammonia oxidation is catalysed by cell-free *Nitrosomonas* extracts in the presence of added or generated nucleoside tri-phosphates.

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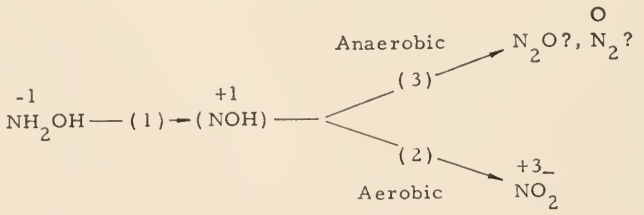
* unpublished data.

Engel and Alexander (17, 18) observed a slow disappearance of hydroxylamine at the cell-free level with no apparent accumulation of nitrite for periods as high as six hours. They suggested that hydroxylamine activating enzymes may be catalyzing the reaction with the formation of an "unidentified" intermediate whose further conversion does not occur due to the lability of the necessary enzyme. Nicholas and Jones (34) have since reported the conversion of hydroxylamine to nitrite by cell free extracts of *Nitrosomonas europea* within relatively short periods of twenty minutes if added mammalian cytochrome *c* or phenazine methosulfate was used as an electron acceptor. They achieved a 40 fold purification of the hydroxylamine enzyme by means of ammonium sulfate and DEAE cellulose fractionation techniques. Although hydroxylamine completely disappeared from the system, nitrite formation ranged from 40 to 73 per cent of the calculated values based on O_2-uptake measurements using cell-free extracts and suitable electron acceptors. Their results suggest a possible intermediate, as yet not identified, between hydroxylamine and nitrite. Delwiche *et al.*, recently reported the results of preliminary experiments with crude cell-free preparations of *Nitrosomonas* indicating that the formation of high-energy phosphate bonds as adenosine triphosphate (ATP) may accompany hydroxylamine oxidation (15). Anderson (9), ruled out the possibility of hyponitrous acid as an intermediate because of the inability of the hydroxylamine activating enzymes to oxidize the added compound. *Nitrosomonas* extracts, however, in presence of hydroxylamine, decolorized methylene blue anaerobically with an output of gas; but under aerobic conditions methylene blue increased NO_2^- formation although in quantities which were significantly smaller than the corresponding O_2 uptake (9). In the light of the above observation, Lees (29) sugggests the following pathway of hydroxylamine oxidation:



Reaction (1) can take place using methlene blue as the hydrogen acceptor with the presumption that the hypothetical nitroxyl (NOH) is the unstable and unidentified intermediate at the oxidation state of plus 1 for the nitrogen atom. Reaction (2) required O_2 and is completely inhibited by cyanide whereas reaction (3) is ascribed to the unstability of (NOH). With the exception of hydroxylamine, the intermediates during the enzymatic conversion of ammonia to nitrite by *Nitrosomonas* are not known.

Although *in vivo* ammonia oxidation is highly sensitive to various metal binding agents, there is no information regarding the participation of any metal in the ammonium or hydroxylamine metabolism of these bacteria. Similarly there is virtually no information about the electron transport system and the energy coupling mechanisms (other than the recent preliminary reports of Nicholas and Jones (34), and Delwiche (15) cited above) as well as assimilatory processes in these micro-organisms.

NITRITE OXIDATION BY NITROBACTER

Nitrobacter sp. as strict chemoautotrophs derive their entire energy for growth and metabolism from nitrite oxidation (with an energy efficiency of 5 to 8%) according to the equation (10):

$$NO_2^- + \tfrac{1}{2}\ O_2 \rightarrow NO_3^- + 17.5\ \text{K.Cal.}$$

Optimal nitrite oxidation by intact cells occurs at pH 8.0 and at a substrate concentration of 3 to 5 $\times$ 10^{-2} M at 28-30° C (2). The optimal nitrite concentration for the *Nitrobacter* strain used by Lees and Simpson (28) was somewhat lower, namely, 4 $\times$ 10^{-3} M. The bacterium requires 5 ppm of each magnesium and phosphorus, and 7 μg of iron per liter for the optimal growth as studied in a modified growth medium devoid of calcium carbonate particles (2). Although a slight stimulation in growth was observed with traces of molybdenum and zinc (1 μg/L), the results were variable. Neither vitamins, amino acids nor any other organic confactors are required for its normal growth (2). Zavarzin (39) also reported that although nitrite oxidation was stimulated by molybdenum or tungstate in the presence of iron, it was somewhat depressed when both molybdenum and tungstate

are present. The accumulation of the end product, NO_3^-, does not interfere with the nitrification process (2). Nitrite oxidation, however, is markedly inhibited at alkaline pH by the initial substrate of nitrification, namely ammonia, presumably because of the shift in the ammonium-ammonia equilibrium towards ammonia (2). These studies suggest the reason for nitrite accumulation in soils of alkaline reaction following heavy applications of ammonium fertilizers (3). In striking contrast to extreme sensitivity of intact *Nitrobacter* cells to low levels of ammonia, is the fact that the cell-free nitrite oxidizing system is unaffected under similar conditions (2, **).

The recent studies of Aleem and Alexander (1), and Aleem and Nason (4, 5, 6, **) have yielded considerable information about the biochemistry of nitrite oxidation. Aleem and Alexander (1) initially obtained an active cell-free nitrite oxidizing system by sonic disintegration of *Nitrobacter* cells. The nitrite oxidizing activity, which resides solely in the red particulate fraction, was responsible for the stoichiometric conversion of nitrite to nitrate as measured by nitrite disappearance, nitrate formation and O_2-uptake (Figs. 1 and 2). The enzyme possesses a relatively high Michaelis constant for nitrite (3.3×10^{-2} M) and is uninhibited even by concentrations as high as 8×10^{-2}M nitrite. The relatively high substrate concentration necessary for optimal activity of the cell-free nitrite oxidizing system is in striking contrast to the low substrate-optimum (8×10^{-3} M) for the intact cells (1, 2, 28). Indophenol, ferricyanide and menadione are enzymatically reduced accepting electrons either from NO_2^- or DPNH* in the presence of purified nitrite oxidase particles.**

According to Aleem and Alexander (1), either ferric or ferrous ion stimulated nitrite oxidation by the cell-free extracts while copper was found to be inhibitory. Subsequently a specific iron requirement for nitrite oxidation was shown (4) by the unique

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* The following abbreviations are used: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide respectively; TPN TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; FMN and FAD, flavin mononucleotide and flavin adenine dinucleotide respectively.

** unpublished data.

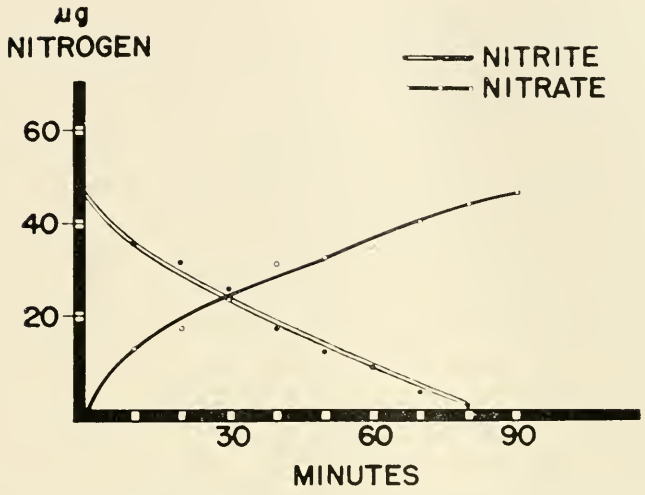

Fig. 1. Nitrogen balance in *Nitrobacter agilis* extracts. Reaction vessel contained extract nitrogen, 0.28 mg; K_2HPO_4, 100 μmoles; and 5 μmoles of $FeCl_3$ and nitrite in 5.0 ml.

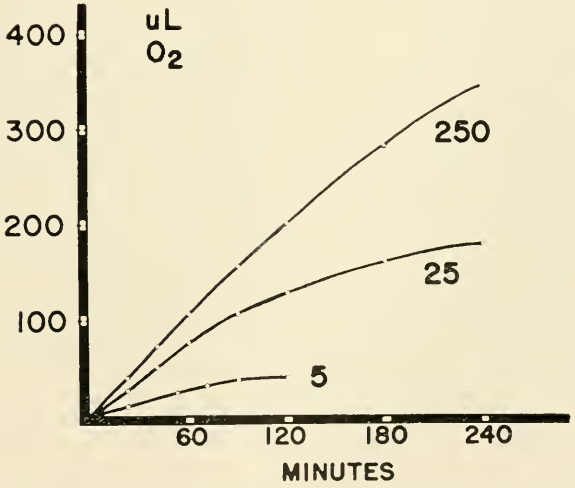

Fig. 2. Utilization of O_2 by *Nitrobacter agilis* extracts in the presence of various concentrations of nitrite. The Warburg vessel contained nitrite, 50 μmoles of K_2HPO_4, 5 μmoles of $FeSO_4$ and sufficient extract to give 0.28 mg. total nitrogen. The final volume was 3.0 ml. nitrite supplied at concentrations of 5, 25, and 250 μmoles per flask.

ability of ferric or ferrous ions to restore the completely inactivated nitrite oxidase resulting from dialysis of the enzyme against cyanide solution. No other metal could either replace iron or stimulate nitrite oxidation by the purified *Nitrobacter* particles (4). Zavarin (39) had previously found a stimulation of growth by iron, molybdenum or tungstate and implicated their role in the nitrification process. He postulated a direct participation of magnesium and flavins in nitrite oxidation, and reported that 10^{-3} M iodoacetic acid or sodium tungstate failed to inhibit nitrite oxidation by intact cells; nor was the process apparently affected by specific SH group inhibitors. In the studies of Aleem and Nason (4), it has thus far not been possible to observe a flavin component spectrophotometrically or to show an effect of added FAD, FMN or riboflavin on the nitrite oxidase system. In addition, nitrite oxidase activity was completely suppressed by 10^{-3} parachlormercuribenzoate and fully restored by the addition of 10^{-3} M cysteine or glutathione. The nitrite oxidase system has also been found to be highly sensitive to cynide, azide and other metal binding agents as well as to relatively high concentrations of certain respiratory chain inhibitors, e.g., antimycin A and heptyl-4 hydroxyquinoline-N oxide (5, * *).

. . . *Nitrobacter* like many other aerobic micro-organisms possesses cytochromes which have been implicated to be directly concerned with nitrite oxidation. Lees and Simpson (28) observed that *Nitrobacter* cells treated with nitrite in presence of chlorate showed a gradual fading of the cytochrome bands, especially at 551 mμ, and a corresponding decrease in O_2-uptake. Cyanate inhibited nitrite oxidation and apparently accounted for the failure of the 551 band to appear upon addition of nitrite or dithionite. These observations led Lees and Simpson to suggest that the cytochrome with absorption maximum at 551 mμ is intimately concerned with nitrite oxidation. Zavarzin (39) confirmed the results of Lees and Simpson and also implicated flavins in nitrite oxidation.

Aleem and Nason (4) using a split-beam spectrophotometer*

* We are indebted to Drs. B. Chance and R. Estabrook for some of these measurements.

** unpublished data.

or a Cary spetrophotometer, found that intact *Nitrobacter* cells or various cell-free fractions possessing nitrite oxidase activity showed upon addition of nitrite, absorption maxima in the 550 and 520 mμ regions (representative of the alpha and beta peaks of a cytochrome component of the *c* type) and in the 585-590 mμ and 438 mμ regions (indicative of the alpha and gamma peaks of a cytochrome *a* type, probably a_1) (Fig. 3). The cell-free fraction, 144,000 x g supernatant which lacked nitrite oxidase activity gave smaller absorption maxima at 550 mμ and negligible peak at 585-590 mμ. The results suggested that nitrite oxidation cannot take place if cytochrome oxidase (cyt. a_1-like component) is absent and implicated both cytochrome *c*- (551) and *a*- (589)-like components in the action of nitrite oxidase. Addition of $FeSO_4$ to the particulate enzyme inactivated by cyanide dialysis resulted in the appearance of reduced cytochrome peaks, probably due to the non-enzymatic reduction of cytochromes by iron. Ferric ions in the absence or presence of nitrite, oddly enough, failed to restore the steady state absorption spectra of the cyanide inactivated nitrite oxidase. These observations in general strongly suggest that biological nitrite oxidation occurs in a series of enzymatic steps involving the sequential transfer of electrons from nitrite to molecular oxygen via cytochromes *c*- and a_1-like components (4). It may be that iron is acting between NO_2^- and cytochrome *c* but the exact role of the metal is not yet clear.

Cell-free extracts of *Nitrobacter* possess an active NO_2^--cytochrome *c* reductase* which catalyzes the transfer of electrons from nitrite to mammalian cytochrome *c*. Presumably its natural electron acceptor is the cytochrome c-like component of *Nitrobacter*. The further transport of electrons from cytochrome *c* to O_2 is catalyzed by the cytochrome oxidase present in the extracts.* The pathway of electron transport involved in nitrite oxidation may be represented by the following scheme:

$$NO_2^- \xrightarrow[\text{reductase}]{\text{Nitrite-cytochrome } \underline{c}} \text{cytochrome } \underline{c} \xrightarrow{\text{Cytochrome oxidase}} \text{cyt. } \underline{a_1} \longrightarrow O_2$$

* unpublished data.

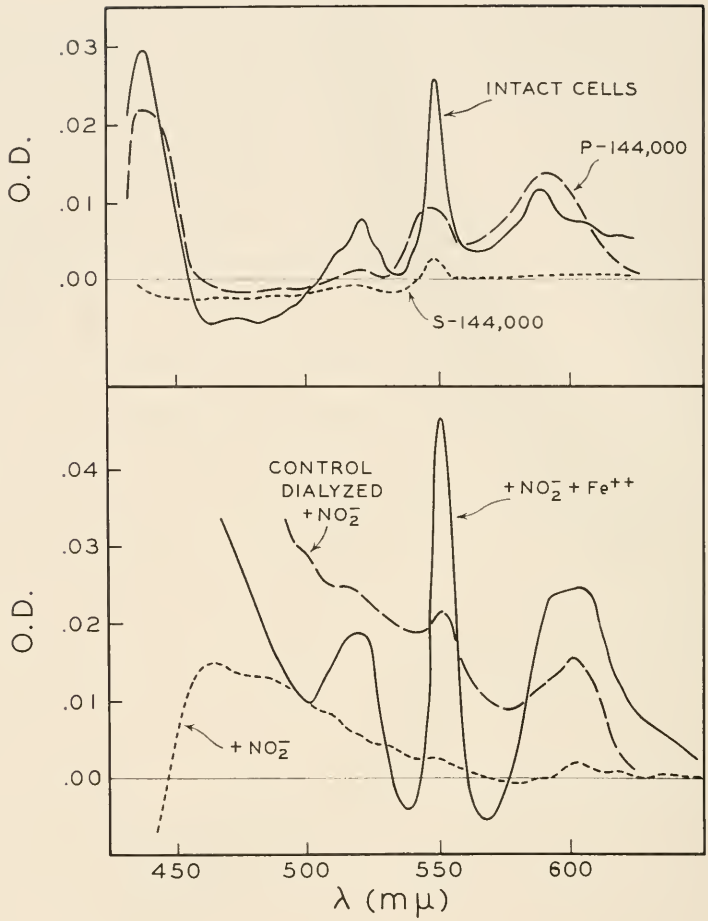

Fig. 3. (*Above*) Steady state-oxidized difference spectra of intact *Nitrobacter* cells and cell-free fractions P-144,000 and S-144,000 upon addition of KNO_2. (*Below*) Steady state-oxidized difference spectra of cyanide dialyzed and control dialyzed fractions P-144,000 upon addition of KNO_2 and $FeSO_4$. The ($+NO_2^-$) and $+ NO_2^- + Fe^{++}$) curves are for cyanide-dialyzed fraction P-144,000.

Phosphate esterification has been shown to be specifically coupled to the enzymatic oxidation of nitrite by the cytochrome-containing electron transport particle which catalyzes the formation of high energy phosphate bonds in the form of ATP (6). Phosphorylation does not occur in the absence of the oxidizable

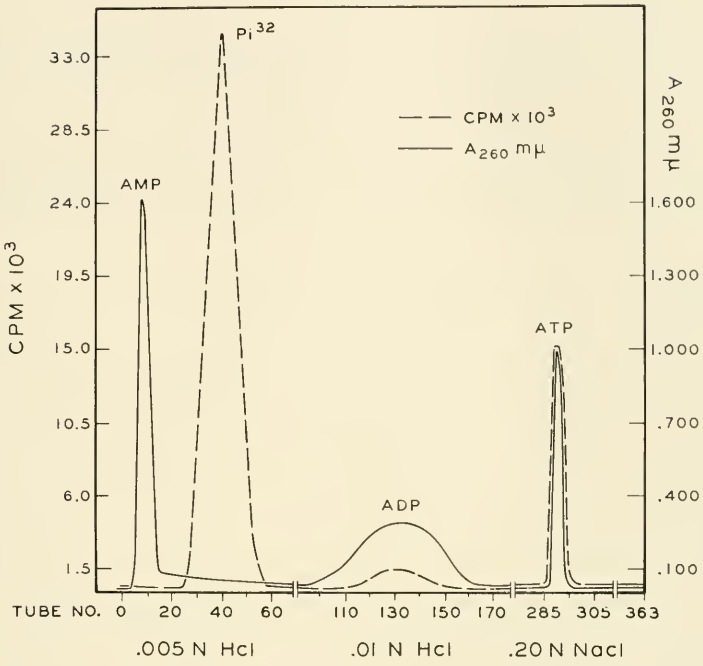

Fig. 4. Demonstration of ATP formation concomitant to nitrite oxidation by partially purified nitrite oxidase, using column chromatographic procedure.

substrate, nitrite, a suitable phosphate acceptor (ADP), or a hexokinase trapping system when catalytic quantities of ADP are used (Table 1). That ATP is the phosphorylation product, was confirmed by the separation of ADP and ATP from a complete nitrite oxidase reaction mixture (containing substrate quantities of ADP and no hexokinase system) by established ion exchange chromatography (Fig. 4), and paper chromatography procedures (6). Most of the P^{32} was incorporated into the ATP fraction and the remainder in the ADP. Nitrite oxidase particles possess ATP-ase, myokinase, and ATP-ADP exchange activities but did not exhibit any ATP-P^{32} exchange reaction.* According to the studies of the authors, the phosphorylation was not uncoupled by 2,4,dinitrophenol, L-thyroxin, dicumarol, etc. (6). The recent preliminary report by Malavolta *et al.* (31) also indi-

* unpublished data.

TABLE 1

FACTORS AFFECTING OXIDATIVE PHOSPHORYLATION BY PARTICULATE NITRITE OXIDASE* FROM N.AGILIS

	O_2-uptake μL per hr†	P^{32}-uptake per hr, ‡ cpm	P/O (corrected)**
	Exp. I		
Complete	146	1,640	0.14
-Nitrite	7	454	—
-Hexokinase, -Glucose	154	413	0.03
-Mg^{++}	159	794	0.06
-Nitrite, -Mg^{++}	6	58	0.00
	Exp. II		
Complete	162	412	0.03
-Nitrite	12	82	—
-ADP	169	44	0.00
-Mg^{++}	141	274	0.01
-Nitrite, -Mg^{++}	1	50	0.00
Exp. III (Mg^{++} omitted from reaction mixture)			
Complete	174	985	0.07
-Nitrite	1	58	—
-Hexokinase, -Glucose	182	99	0.004
-Nitrite, -Hexokinase, -Glucose	0	52	0.00

* Resuspended pellet collected from 27,000 x g-supernatant by centrifugation at 95,000 x g. The complete reaction mixture contained 10 micromoles phosphate, 25 micromoles nitrite, 0.2 micromoles ADP (PABST Co.), 15 micromoles $MgCl_2$; 0.5 mgs hexokinase (type III Sigma Co.,), 50 micromoles glucose, 0.5 ml nitrite oxidase corresponding to 0.6-1.5 mg protein, P^{32} (phosphatope oral, without preservative, without carrier - E. R. Squibb and Sons, New York) approximately 3 x 10^5 cpm per Warburg flask.

The final volume was made up to 3.2 ml with O.1M Tris pH 7.5. The central well contained filter paper and 0.2 ml of 10 per cent NaOH. After a ten minute preincubation period at 30 C, the reaction was started by tipping in the enzyme from the side arm and O_2-uptake determined at ten minute intervals for one hour.

† These values represent the consumption of O_2 by the total volume of reaction mixture (3.2 ml) contained in the Warburg flask.

‡ These values represent the actual counts per min measured in the organic phosphate fraction in an aliquot equivalent to 1/48 of the volume of the total reaction mixture (3.2 ml) contained in the Warburg flask.

** P/O ratios are corrected for by subtracting the endogenous O_2 and P^{32} uptakes obtained in the minus nitrite reaction mixture.

Exp. I contained 6.7 x 10^5 cpm of initially added P^{32} orthophosphate per Warburg flask. Exp. II contained 5.2 x 10^5 cpm and Exp. III contained 4.3 x 10^5 cpm.

cates the formation of ATP during nitrite oxidation. Studies are now in progress to determine the sites of phosphorylation and the exact site of involvement of iron in the electron transport chain.

BIOLOGICAL FORMATION OF NITRITE AND NITRATE BY HETEROTROPHS

A number of gram negative bacilli have been reported to form very small quantities of nitrite from ammonia (20). However, none of the soil isolates including bacteria, actinomycetes and fungi are known to produce nitrite exceeding 0.5 ppm NO_2-N, (19) suggesting that heterotrophic ammonia oxidation, if it is coupled to phosphate esterification, is not the main energy yielding reaction in those organisms. Nevertheless, in view of the prevalence of these organisms in natural environments, it seems possible that they may play a substantial role in the nitrogen economy of nature. Several heterotrophs belonging to the genera *Alcaligenes, Corynebacterium,* and *Nocardia* which do not produce nitrite from ammonia, can utilize oximes, ethylurethane and hydroxylamine to yield nitrite. The transformation is inhibited by hydrazine and is unaffected by thiourea or allylthourea (14, 30). These results suggest that the nitrification of pyruvic oxime and hydroxylamine takes place along a pathway which probably resembles the chemosynthetic hydroxylamine oxidation by *Nitrosomonas.* The second step of nitrification, namely the formation of nitrate, is apparently included in the metabolism of *Streptomyces nitrificans* and *Aspergillus flavus.* The latter is able to produce substantial amounts of nitrate in the order of 50-60 ppm NO_3-N from complex nitrogenous materials such as casein, peptone and yeast extract (19) presumably by the ammonification of these substrates and its subsequent conversion to nitrate. All the enzymatic steps involved in the transformation of ammonia to nitrate are possibly contained in a single organism like *Aspergillus flavus,* whereas two distinct chemoautotrophs *Nitrosomonas* and

* unpublished data

Nitrobacter are successively involved in the sequential oxidation of ammonia to nitrite, and nitrite to nitrate. Our knowledge of the pathways and mechanisms of heterotrophic nitrite and nitrate formation from ammonia or other nitrogenous compounds is sadly lacking. A comparative study of chemoautotrophic and heterotrophic nitrification in terms of electron transport systems, energy coupling mechanisms and the possible intermediates should prove to be highly significant.

CONCLUDING REMARKS

Bacterial nitrification brought about by the chemoautotrophs *Nitrosomonas* and *Nitrobacter* sps. is an energy yielding enzymatic process. It involves biological oxidation-reduction reactions in highly organized steps which are carried out by specific enzymes with specific affinities for their substrates. Both ammonia and nitrite oxidations are apparently mediated by cytochrome systems (4, 27, *).

During the biological transformation of ammonia, the latter is first converted to hydroxylamine and then to nitrite, presumably via an unidentified and unstable intermediate at the nitrogen oxidation level (+1) represented by (NOH). This perhaps further dissociates to N_2O and N_2. Recently Cresswell and Hewitt (13) reported the oxidation of hydroxylamine by a peroxidase system of the marrow plant resulting in the formation of nitrite and unidentified gaseous products. To date there is no evidence which would indicate that a similar system is responsible for the oxidation of hydroxylamine by *Nitromonas*.

Thus far the biological oxidation of nitrite by molecular oxygen has been most clearly characterized. Nitrite is first activated by the nitrite-cytochrome *c* reductase accounting for the flow of electrons from NO_2^- to cytochrome *c* causing the oxidation of the former and the reduction of the latter. The further transfer of electrons from cytochromes *c* to *a*, and finally to O_2 is catalyzed by cytochrome oxidase causing oxidation of cytochrome *c*, reduction of *a* and finally oxidation of *a* and reduction of O_2. These oxidation and reduction processes taking place in

* unpublished data

the electron transport chain are coupled with phosphate esterification although their exact sites have not yet been determined. It may be assumed tentatively that phosphate esterification occurs in that part of the electron transport scheme involving the action of cytochrome oxidase in view of the high positive potential of the NO_2/NO_3 system (+0.54 volts at pH 7.0) (9a).

Finally, the question as to how *Nitrosomonas* and *Nitrobacter* cells obtain their reducing power, which is presumably also derived from ammonia and nitrite oxidation, respectively, is still untouched. Assuming that electrons provided by ammonia and nitrite ultimately reach the level of pyridine nucleotides or succinate, then in view of the high positive potential of these substrates (viz., ammonia and nitrite) energy must be provided to make possible the transfer of their electrons "uphill." Investigation of such systems should prove to be of great importance.

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Chapter 38

Proteolysis and Nitrate Reduction in Sea Water

Jean Brisou and Huguette Vargues

The nitrogen cycle takes place in the marine environment as well as on land. Bacteria play important roles in this cycle and are chiefly responsible for the processes of ammonification, nitrosation, nitration, denitrification and proteolysis. A great number of investigations have been already made in this field. It is known that the fixation of nitrogen is performed in the sea by *Azotobacteria,* which are active in aerobic conditions and by *Clostridia* of the *pasteurianum* type, which are active in anaerobic conditions. Ammonification occurs as well in oxybiosis as in anoxybiosis, and nitratation is performed by *Nitrobacter.* Photeolysis and the first step of denitrification, i.e., the reduction of nitrate to nitrite, are performed by a great variety of aerobic and anaerobic bacterial species living in sea water. The transition areas near the beaches and in the estuarine muds at shallow depths receive continuously and abundantly materials from land drainage.

Our work is concerned with proteolysis and nitrate reduction in sea water and especially with the systematics of the main aerobic genera and families responsible for the reactions.

It is clear that these bacteria are not the only active ones and that the anaerobes play also an important role. However, our investigations have been intentionally restricted to the study of the aerobic Gram negative species which are active in the considered processes.

TECHNIQUES

All the bacteria studied here (131 strains) were isolated from sea water, estuarine muds, shell-fish or fishes. Some originated from a salt lake in the Sahara, where NaCl concentration

is 3.3 Gm (%) and equivalent to NaCl concentration in sea water.

The isolations have been made on a medium prepared with natural sea water, flesh of fishes or shellfish, 1 per cent lactose, 0.05% 2-4-diphenyl tetrazolium, and bromthymol blue; pH adjusted to 7.3. The cultures have been incubated at 25C for twenty-four hours. This routine technique provides a rapid isolation of several different species from a single inoculated specimen (sea water, mud, intestinal tract of fishes, etc.).

After isolation and purification, the strains have been identified by our routine techniques (sugar fermentations, protein metabolism, organic acid production, etc.) and finally all the strains were tested for agglutination with *Achromobacter* and *Erwinia* anti-sera. The identifications have been made according to the French classifications of Magrou and Prevot (8) and Brisou (1).

Proteolysis has been tested at 22 C and pH 7.3 with: (1) 15% gelatin stab and with: (2) 1% peptone water added with a small piece of gelatin according to the technique of Kohn.

Nitrate reduction was tested in peptone water containing 1% potassium nitrate, and nitrite formation was characterized by the Griess reaction after 48 hours of incubation at 25 C.

For the halophilic bacteria, the media were prepared with natural sea water.

RESULTS

For a total of 131 strains investigated, 77 are proteolytic and 77 nitrate reducing; 51 strains have both these activities; 26 strains are inactive.

The separation of these strains in the different genera is as follows:

Vibrios

All the 13 strains of *Vibrio* isolated are strictly halophilic. Origins: salt lake of Sahara and Atlantic Oceans (Cap Ferret beach).

Proteolytic:	8	(61%)
Nitrate reducing:	7	(53%)
Both activities:	4	(30%)

Pseudomonodaceae

a. Chromogenic *Pseudomonas* producing fluorescent pigments. Origins: muds, fishes and shellfish. Number of strains: 15.

Proteolytic:	8	(54%)
Nitrate reducing:	12	(79%)
Both activties:	7	(46%)

b. *Achromobactereae* (non-pigmented, motile) Origins: muds, sea water (Atlantic Ocean, Mediterranean), fishes and shellfish. Number of strains: 43.

Proteolytic:	25	(59%)
Nitrate reducing:	19	(45%)
Both activities:	13	(30%)

c. *Acinetobacter* (non-pigmented, non motile) Origin: sea water, mud, shellfish. Number of strains: 15.

Proteolytic:	3	(20%)
Nitrate reducing:	4	(25%)
Both activities:	3	(20%)

d *Erwinia* (non-pigmented, motile, gas producing) Origin: muds, fishes and shellfish (France, Algeria, Madagascar, New Caledonia). Number of strains: 29.

Proteolytic:	19	(65%)
Nitrate reducing:	27	(93%)
Both activities:	17	(58%)

e. *Chromobactereae* (Chromogen with non-diffusible pigment) (*Flavobacterium, Empedobacter*) Origin: sea water, shellfish. Number of strains: 15.

Proteolytic:	10	(66%)
Nitrate reducing:	8	(53%)
Both activities:	7	(46%)

Among the 131 studied strains, the highest proteolytic activity was observed for *Chromobactereae, Erwinia* and *Vibrio* organisms, and then, in a decreasing order, for *Achromobacter* and *Acinetobacter*. The denitrifying organisms belong mostly to the genera *Erwinia* (93%) and *Pseudomonas* (79%) and at a lesser extent to the genus *Vibrio*. Moreover, the organisms having both proteolytic and nitrate reducing activities belong mostly to

Erwinia and to *Chromobactereae*, and a lesser extent to *Vibrio* (30%), *Achromobacter, Pseudomonas* and *Acinetobacter.*

The role of these microorganisms in the processes of proteolysis and nitrate reduction is of topmost importance.

These observations confirm previous investigations on the microflora of fish and shellfish, on bacteria pathogenic for marine animals and on bacteria isolated from sea food by us and coworkers (2, 3, 4, 5), by Gianelli (7) in Italy, and by Fischer (6) from fishes of the Baltic Sea. These data show that the intestinal microflora of shellfish is predominantly represented by *Pseudomonadaceae* and more precisely by *Achromobacter, Erwinia* and *Flavobacterium*. These bacteria are the main agents responsible for proteolysis and decomposition of sea food.

The *Pseudomonadaceae* are also responsible for diseases of fishes and shellfish. All these data emphasize the importance of this microflora and its economical and practical incidences to protein degration and nitrate reduction. Many of these bacteria are very active on coagulated serum and some amino acids.

CONCLUSIONS

Present work demonstrates the very important role of *Pseudomonadaceae* and *Vibrio* (all of them halophilic) not only in the marine nitrogen cycle, but also in the aerobic processes of proteolysis and nitrate reduction in marine environment. Nitrite is metabolized by other bacterial species which are able to reduce it further to ammonia and/or free nitrogen. Many *Pseudomonadaceae,* concurrently with other bacterial groups, are also active in these further steps of denitrification.

Our observations have some pathologic and hygienic interest, due to the fact that a small number of investigations have already demonstrated the role of these bacteria in the diseases of marine animals and plants, and also due to the fact that these microorganisms are the agents of human intoxications and presumably of human diseases. Actually, some observations have shown the role of *Achromobactereae* and other *Pseudomonadaceae* in digestive and pulmonary diseases.

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Chapter 39

Researches on Nitrifying Bacteria in Ocean Depths on the Coast of Algeria

Huguette Vargues and Jean Brisou

Since the discoveries of Schloesing and Muntz and of Winogradsky, many investigations have been dedicated to the phenomena of nitrification; the mechanism of the oxidation of ammonium salts. From an ecological standpoint, nitrification in the marine environment has not failed to draw the attention of investigators, but the results obtained are quite divergent. The authors generally agree in recognizing that nitrifying bacteria do not exist in the water itself, but only in the sediment deposits. Brandt (1) notes their presence in the ooze of the Kiel sound, while Gran (3) finds none in the sediments of Norwegian fjords. Issatchenko (4) has obtained positive results with sediments from the Murmansk area. Thomsen (5) also found these bacteria in the ooze of the Kiel sound, the gulf of Naples and the Helgoland area.

From 1952 to 1958, we had the opportunity to study samples of marine muds and littoral sands from the depths of the Algerian area, and the results of these studies concerning nitrifying bacteria are reported here.

METHOD

Samples were inoculated into a medium containing a source of ammonia nitrogen. We have adopted Omeliansky's medium: $SO_4(NH_4)$:1.3%; KH_2PO_4:1.0%; MG SO_4:0.5%; ferrous sulfate: trace. In this selective medium, the appearance of nitrites and nitrates, and the release of nitrous or nitric nitrogen is investigated with the aid of chemical titrations at regular intervals of time.

This sensitive technique offers the advantage of a quantitative evaluation of the nitrifying activity in the marine muds. However, it does not permit the distinction of the bacteria re-

sponsible for these processes from the numerous other species which developed in Omeliansky's medium.

In practice we seeded 20 g of marine ooze or sand in 400 ml of Omeliansky medium. It is certain that this medium, containing no source of organic carbon, selects autotrophic bacteria, but even so, the possibility of development of other bacteria due to the presence of organic material carried with the inoculum cannot be overlooked.

Nitrite appearing in the medium was titrated with Griess reagent, and nitrate by the sulfophenic reaction. The results obtained at different time intervals are expressed in micrograms of nitrous or nitric nitrogen per 10 ml of culture medium.

DESCRIPTION OF SPECIMENS STUDIED

Geographic Distribution

The 44 samples studied were taken quite near the Algerian coast. Actually we had available marine muds taken by the laboratory of "Algerian Marine Station" from the bay of Algiers itself, or from the area around this bay. These samples were collected under the direction of Professor Bernard during a systematic exploration of the ocean depth in the bay of Algiers (geographic location is: 3° to 3°30′ east and the line 36°52′ north of the bay of Algiers). The depth explored extends from 50 to 1,000 m.

We also collected sands originating from the rather special sedimentary formation abundantly represented around the "Castiglione Station of Agriculture and Fishery." This formation, locally called "Mattes," constituted almost entirely of sands, is set in a substratum with a covering of vegetation consisting of *Zostera* and *Cymodocacae* (2). It consists of a sort of littoral marine basin covered by a thin sheet of water and is dry under certain atmospheric conditions (east wind, high barometric pressure).

In the deep areas a gravity corer was used. In the shallow basin we took advantage of dry periods and operated as for land, with a core sampler or a dredge. From the center of the plug thus obtained, or from the block taken up by the dredge, we took samples with a sterile scoop; the specimens then being stored

until use in sterilized flasks in the refrigerator. The cores have the obvious advantage of permitting us to compare several horizons of the sediments collected.

Physico-Chemical Study

The two types of sediments are quite different from each other, from a biological point of view. The temporary dry periods and the vegetative covering of "Mattes" set them apart from the muds of the bay, which belong to the true marine ooze type. One has to stress, however, that the last type of sediment is never very deep (with the exception of three samples) and the influence of land should not be overlooked. The physico-chemical analysis of these two formations also differentiates them quite clearly.

The grain size composition of the two formations is extremely different. The muds of the bay are classified as a clay type. The average content of bay clay is 60 per cent, whereas the basin is only 13 per cent clay and a sand content of about 79 per cent.

The pH is slightly different in the muds of the bay (7.3) and the sands (6.9).

The average carbonate content of muds is of 1.05 per 100 gm of dried mud, and 1.11 per cent of dry sand. Nitrogen content is quite similar to the two formations; 0.11 gm per cent in the muds, 0.07 per cent in the sands.

Comparative studies made at different depths of the same sediment samples have not shown a relationship between the nitrogen and carbon content and the vertical distribution.

The phosphorus and potassium content of the specimens are quite variable, but a relationship clearly exists between these values and the depth or geographical location of the station. Potassium varies between 70 and 110 mg per 100 g(dry weight). We have also observed abnormally low concentration to 20 mg per cent. The concentration of phosphorus fluctuates from a trace to 60 mg per 100 gm dry weight.

ANALYSIS OF THE RESULTS

We will not give a detailed analysis of our results, but will present the types of curves obtained when 20 gm of sediment

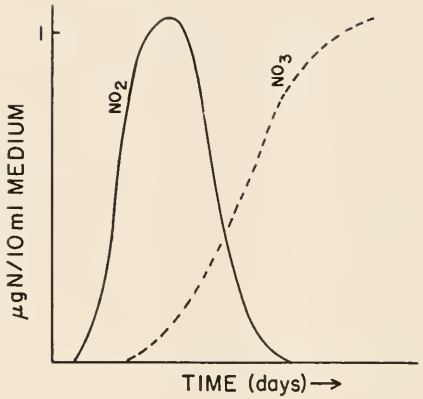

Fig. 1. Nitrification by organisms in sediment samples from the Bay of Algiers. Ammonia is provided by Omeliansky medium.

were inoculated in 100 ml Omeliansky's medium. In these curves arbitrary time is expressed on the abcissa, and the weight of nitrous or nitric N in micrograms per 10 ml of culture medium on the ordinate.

The average of these curves is reproduced in Figure 1, for samples taken at a depth of 63 m in the bay of Algiers. The nitrite curve is bell-shaped, diminishing rapidly when maximum production is reached, i.e., when ammonium nitrogen is exhausted. Simultaneously, the nitrate curve starts and has a sigmoidal aspect. But has to be stressed that if the curve was prolonged in time, a decrease in the percentage of nitrates would be observed, corresponding to the phenomenon of de-nitrification.

The nitrite and nitrate produced in the culture medium provide curves similar to bacterial growth curves, showing a lag phase, a period of rapid growth, a stationary phase, and a phase of regression; this last phase being faster in the present case, due to the fact that a second process utilizes the metabolic products of the first.

INTERPRETATION OF CURVES

Nitrite Production

The lag phase has a minimum duration of two days and a maximum duration of twenty days. According to the variation

duration of this period we can classify our curves into the following categories: lag phase less than ten days; lag phase between ten and fifteen days; and lag phase more than fifteen days. This lag phase corresponds to the adaptation of bacteria to the medium, and to the quantity of bacteria seeded. It thus expresses the potential activity of the sample studied, since all possible conditions of environment are similar, and only the nitrogenous substrate is added in excess.

The period of active nitrite production which begins at the end of the lag phase is very fast; the slope of the curve at the point of maximum derivation is steep. The slope of this curve is still related to the activity of the bacteria, but, even though the slope of the curve is often steeper when the latent period is short, we found some exceptions, and it is difficult to interpret these curves in great detail. The maximum points of the ordinates generally have similar values, and, with two exceptions, they correspond to the percentage of nitrogen obtained through total transformation of ammonium or nitrite nitrogen. These points seem, therefore, of no interest to us for judging the potential of our specimens.

The period of decrease is always rapid, often quite abrupt, the nitrite nitrogen generally disappearing after two or three days. This period has no relation to the nitrite-producing bacteria, but depends on the activity of bacteria which rapidly "consume" nitrite.

Nitrate Curve

The nitrate curve reproduces the shape of nitrite curve. The lag phase is necessarily a function of the production of nitrite nitrogen, since this is the source for nitrate production. Thus the nitrate activity cannot be foreseen from the lag phase. In our measurements, the lag phase extends from 8 to 27 days.

The slope of the curve of nitrate production is the only point susceptible of analysis for the purpose of determining the activity of this group of bacteria. However, the general shape of the curve is less regular than that of the nitrite curve, undoubtedly due to the interference of de-nitrification phenomenon.

Whatever the case, the nitrification should be considered as

a whole. For this reason, it is necessary to link the two categories of curves as often as possible.

ANALYSIS OF RESULTS OBTAINED ON THE MARINE MUDS

Variations in Nitrification Activity in Relation to Water Depth

We will consider only the activity of nitrite producing bacteria, as the nitrate production is subordinate to the former. The interesting data furnished by these curves are the lag phase and the ascending slope of the curves. In Figure 2 are grouped six nitrite curves corresponding to six different depths: 45, 63, 100, 185, 325, and 500 m. The rate at 63 and 45 m is rapid and nearly uniform; a medium rate is observed at the depths of 185 and 100 m, and finally a slow rate is produced by the sample from depths of 325 and 500 m. In this family of curves, the duration of the lag phase increases when the rate of nitrate formation decreases. By classifying the sample areas according to the duration of the lag phase, one finds:

1) A duration shorter than ten days; observed for depths of 45, 57, 63, 72 and 80 m.

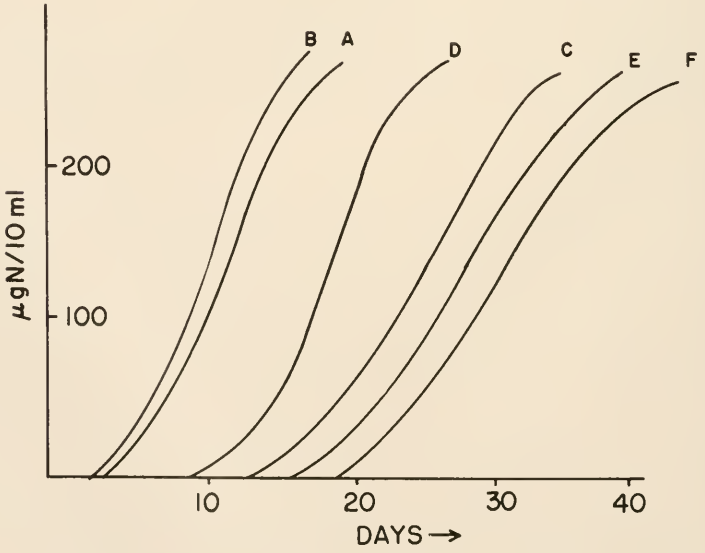

Fig. 2. Effect of water depth on nitrate production.

2) Between ten and fifteen days: for depths of 50, 80, 185, 325 and 500 m.

3) Fifteen days: for depths of 0, 120, 356, 526 and 650 m.

The first group includes only the depths of less than 100 m, the last includes a majority of depths over 300 m and the second group is intermediary with nearly equal representation of great and shallow depth. Certainly depth cannot be the sole determinant, which explains the exception in the second group and third; however, depth seems to play an important role in the distribution of the bacteria.

It is worth noting that all the sediments studied have proven capable, in this selective medium, of transforming ammonium salts into nitrates.

Variations in Nitrification Activity in Relation to the Depth of Sediment

Let us now consider the results of a single sediment in relation to the sediment depth. By using cores of 0.5 to 1.0 m in length, it has been possible to compare various horizons. Figure 3 represents a typical example obtained from sample area No. 235:

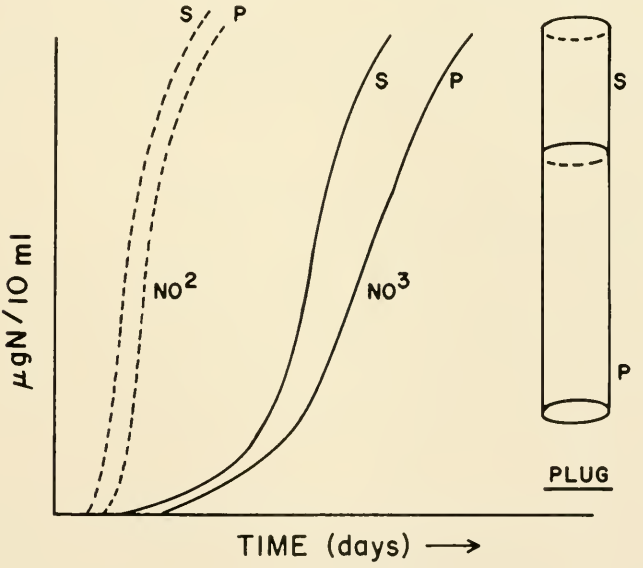

Fig. 3. Nitrification in the upper (S) and lower (P) portion of a sediment core.

in the upper part of the plug (S) the production of nitrites and nitrates is clearly more rapid than in the lower portion (P). Let us note at the same time, however, that a very strong nitrifying activity still exists in the deeper layer.

ANALYSIS OF THE RESULTS OBTAINED ON THE "MATTES"

These sands covered by vegetation have revealed themselves equally capable to transform ammonia into nitrate. If we examine the lag phase of the nitrite curve, we find that these sands have a potential even greater than that of the muds, since the time of latency is nearly always less than fifteen days.

Similarly, the nitrate curves generally start sooner in the sands than in the muds.

Now, it is indeed necessary to call attention to the fact that these sands were quite poor in numbers of other bacterial organisms. Let us note also that the most active sediment is sand from Castiglione beach. This sample is very poor in innocuous saprophytes, and contains no ureolytic flora. We believe it is of interest to compare the results with those we have obtained with some desert sands from Hoggar and Beni-Ounif. These sands, often extremely poor in organic matter and innocuous bacteria, have demonstrated an extremely intense nitrifying activity which is never observed in other soils.

Halophilic Character of Nitrifying Bacteria in the Studied Marine Sediments

The results reported above demonstrate the existence, in the marine muds and sands, of a nitrifying microflora whose potential activity is at least equal to that of an average terrestrial soil. But we do not know if this flora is active *in situ*. In this respect, it seems of interest to compare the behavior of marine strains with those of terrestrial strains in a medium enriched with sodium chloride.

Thomsen (5), in 1910, examined *Nitrosomonas* strains isolated from muds of Naples' gulf and Helgoland area. He noted a morphological and physiological identity between these species and the terrestrial strains, but calls attention to the fact that the

marine species develop equally well in media of weak and strong salinity. (Ubiquitous species). With terrestrial species, on the contrary Yankovitch and Yankovitch (6) have demonstrated that the beginning of nitrification is slower when the salt concentration is raised. Therefore, we have compared two marine strains, one isolated from ooze at a depth of 63 m (strain V) and the other from Castiglione Matte (Strain M), with two terrestrial strains taken from innocuous soils.

These strains, purified physiologically by successive transfer in the selective Omeliansky medium, were seeded in the same medium with various degrees of salinity.

The curves (Fig. 4) provide a comparison of the lag phase and lag phases of the marine nitrite-producing organisms with those of terrestrial strains, in media of increasing salinity. It is seen that lag phase of the terrestrial strains is much more affected by the increase of NaCl concentration than the strains of marine origin, but they do eventually grow in a concentration of 5%

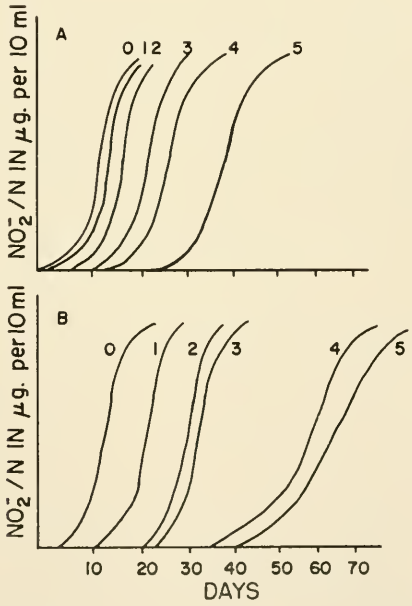

Fig. 4. Effect of NaCl concentration on the growth of Marine (A) and Terrestrial (B) nitrifying bacteria.

NaCl. We do not report here the results obtained with the nitrate curves which are similar to those obtained for nitrite.

In Tables 1 and 2 are reported the speeds of nitrite and nitrate production by marine and by terrestrial strains as a function of NaCl concentration in the culture media. It is clear that

TABLE 1

MICROGRAMS OF NITROUS NITROGEN PER 10 ML OF MEDIUM

MEDIUM 1: 0.1% NaCl MEDIUM 3: 1.2% NaCl
MEDIUM 2: 0.7% NaCl MEDIUM 4: 5.0% NaCl

		Incubation in Days									
		9	11	13	15	19	23	25	30	40	50
Marine strains medium:	1	20	55	81	250	475	470	435	200		
	2	18	45	78	135	400	455	490	125		
	3	4	20	40	100	245	305	395	280		
	4	0	4	4	6	10	10	15	0		
Terrestrial strains medium:	1	0	0	0	0	0	15	45	420	405	106
	2	0	0	0	0	0	0	10	175	450	250
	3	0	0	0	0	0	0	5	30	410	440
	4	0	0	0	0	0	0	0	0	5	5

TABLE 2

MICROGRAMS OF NITRIC NITROGEN PER 10 ML OF MEDIA OF THE SAME COMPOSITION AS IN TABLE 1

		Incubation in Days									
		9	11	13	15	19	23	25	30	40	50
Marine strains medium:	1	10	11	11	25	40	55	90	300	380	
	2	10	10	10	15	25	35	60	250	340	
	3	0	0	0	0	0	10	30	200	320	
	4	0	0	0	0	0	0	0	0	0	
Terrestrial strains medium:	1	0	0	0	0	10	25	35	40	385	440
	2	0	0	0	0	5	10	15	20	100	380
	3	0	0	0	0	0	0	5	5	45	260
	4	0	0	0	0	0	0	0	0	0	0

the marine strains grow consistently faster in NaCl containing media, but it emphasizes that terrestrial strains also produce 450 and 440 μg of nitrous nitrogen in media 2 and 3 after 40 and 50 days of incubation, and 5 μg in medium 4 with 5% NaCl concentration.

This seems to imply that the marine microorganisms are somewhat active in their original environment and that terrestrial bacteria carried in the sea water are able to become active after a lag for adaptation.

CONCLUSIONS

The studies on nitrifying bacteria in the marine depth of the Algerian coast have led to the following results:

(1) All the specimens studied have demonstrated active nitrification.

(2) The nitrification is more rapid in depths of less than 100 m, and slows down proportionally to the depth. For a given sample the upper horizons of a sediment are more active than the lower.

(3) In the sands covered by marine vegetation, nitrification is more active, even though other bacteria are represented.

(4) The nitrifying bacteria from a marine origin are more active at salt concentrations equivalent to those prevalent in their natural environment than the terrestrial strains. But the terrestrial bacteria, after a longer lag period, also show a great activity.

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Chapter 40

Some Biochemical Differences Between Fresh Water and Salt Water Strains of Sulphate-reducing Bacteria

F. W. Ochynski and J. R. Postgate

The dissimilatory, sulphate-reducing bacteria are among the commonest of microbes, being encountered in the majority of marine and fresh water environments (soil is included as a fresh water environment). Their economic activities and some aspects of their general physiology have been reviewed (15, 16) with more extensive documentation than will be provided here. Their ubiquity makes them a promising group for the study of the biological and physiological characters that distinguish marine from fresh water organisms because, at least in principle, one can expect to be able to study the same species as it occurs naturally in either environment, and one can further hope to observe the acclimatization of strains from either environment to the other. The present contribution records a few studies conceived from this point of view.

INTRODUCTION

The taxonomy of the sulphate--reducing bacteria has been the subject of some controversy in the literature; this has only recently begun to be resolved. Beijerinck's (3) original species *Spirillum* (now *Desulfovibrio*) *desulfuricans* was a fresh-water type, and its isolation was followed by van Delden's (6) *Microspira* (now *Desulfovibrio*) *aestuarii*. Baars (2) and Kluyver & Baars (9), referring to both types by the generic name *Vibrio*, showed that the specific types were inter-convertible by training. They did not favour the specific name *aestuarii*, Rittenberg (21) and Zobell and Rittenberg (24) could not train their marine strains to a fresh water habit. Littlewood & Postgate (11) ex-

amined populations of marine and fresh water strains from the point of view of the numbers of variants which were present enabling the strains to grow in media of unfamiliar salinities. Their findings are represented schematically in Figure 1. The marine types showed three classes of behaviour: (1) the whole population tolerated from zero to about 4% NaCl (these populations had small numbers of variants tolerating very high salt concentration); (2) an intermediate type in which only a small proportion of the population (*c* 0.1%) tolerated no NaCl; (3) an exigent type in which no variants present were able to grow without NaCl. The exigent type was not successfully "trained" to a fresh water habit; naturally, the others "adapted" at once. The fresh water type was readily trained to behave like the tolerant marine type; by repeated subculture of the tolerant marine type in 10% NaCl an exceptionally tolerant strain was obtained in which 100 per cent of the viable population grew within a salinity range of 0.25 to 11% NaCl. The exigent marine type differed from the others in that the chloride ion concentration, rather than the sodium ion concentration, conditioned the via-

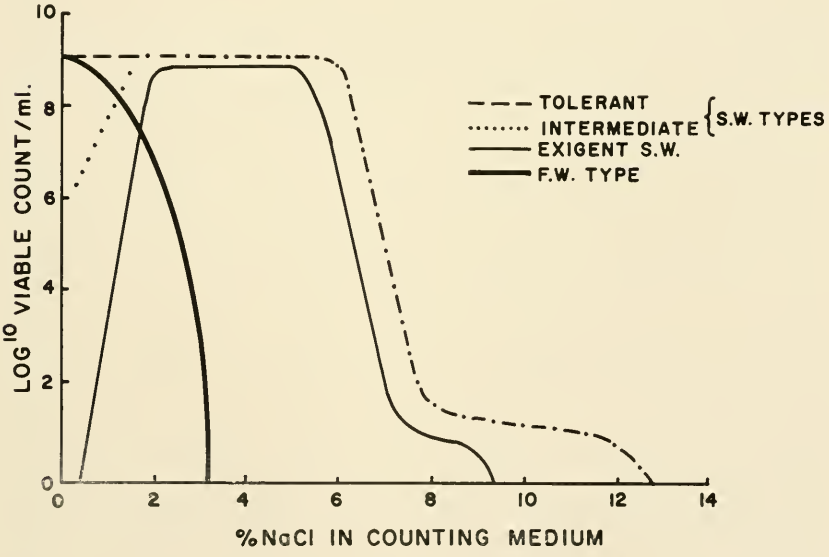

Fig. 1. Salt relations of *Desulfovibrio desulfuricans.* (After Littlewood & Postgate, (11)).

bility. These observations afford some reconciliation of the findings of Baars (2) with those of Rittenberg (21).

Whether or not the name *aestuarii* is accepted for the common marine type of *Desulfovibrio,* there is little doubt that the types are physiologically and morphologically similar. Both differ substantially from the two sporulating species of sulphate-reducing bacteria: the thermophile *Clostridium nigrificans* (earlier *Vibrio, Sporovibrio* or *Desulfovibrio thermodesulfuricans*) and the mesophilic *Desulfovibrio orientis.* It is a curious fact that, to the authors' knowledge, marine strains of the two sporulating species have not been reported formally in the scientific literature. One of us in collaboration with Professor C. B. van Niel and the late Miss M. E. Adams sought thermophiles from a marine environment, and indeed obtained enrichments of a conventional kind from the bay of Monterey (near Elkhorn Slough) showing blackening at 55° in saline media. Despite many efforts, including micro-manipulation and multiple colony isolations, a pure culture was not obtained; the crude culture contained organisms morphologically similar to *Cl. nigrificans* but since the maximum sulphide content of the cultures was low (*c* 30 mg. H_2S/L) (4) there remained doubt whether an enrichment of sulphate-reducing thermophiles had been obtained. There seems to be no reason in principle why marine thermophiles should not in fact exist; the authors have "trained" a type strain of *Cl. nigrificans* (Starkey's strain 74T; NCIB 8395) to grow in media containing 2.5% NaCl. The training process was relatively difficult, involving the sequential subcultures recorded in Table 1, but the adapted strain grew well in saline media and was morphologically normal. Filamentous growth restricted to the precipitate occurred during the training (in this respect the behaviour of the organism recalled that of fresh-water *D. desulfuricans* to be described later). *D. orientis* has only recently been defined as a species, and though it is more ubiquitous than was at first thought (1), the few marine samples that have been examined for this species (three to the authors' knowledge) have not shown it. In this case there is even less reason why marine types should not exist; the holotype strain of *D. orientis* (Singapore; NCIB 8382), though obtained from a fresh water environment, grows

in media containing 2.5% NaCl, though it has a lag of seven to ten days. It is morphologically normal when so grown.

TABLE 1

ADAPTATION OF CL. NIGRIFICANS TO A SALINE ENVIRONMENT

Stain 74T was Subcultured under N_2 at 50C. in Baars' (1930) Medium Containing Thioglycollate (c $10^{-3}M$) and Extra NaCl as Indicated Below. Growth was Visible in 24 hours.

Number of Subculture	*NaCl Concentrations (%) Highest Tolerated*	*Next Highest*	*Period Before Growth Visible (Days)*
1	1.25	2.5	2
2	2*	2.5	7
3	2	2.5	10
4	2.5		2

* This culture failed to grow again, and one with 1.5% NaCl was used as parent for the third subcultures.

PRESENT WORK

For the work reported in this paper two strains of *D. desulfuricans* were used, the fresh-water Hildenborough strain (NCIB 8303) and the salt water stain El Agheila z (NCIB 8380). These were the same strains as were studied by Littlewood & Postgate (11) who mentioned their origin; El Agheila Z was of the "intermediate halophilic type" illustrated in Figure 1. Two additional strains feature in the third section of the work reported here and are mentioned there (11). The strains were maintained as described by Littlewood & Postgate in lactate-yeast extract –sulphate media ("Tryptone," mentioned by those authors, was not included); media were supplemented with 2.5% NaCl for the salt water types.

For certain purposes a sub-strain of the fresh-water strain Hildenborough adapted to growth with 2.5% NaCl was obtained. Though the strain grew on first subculture in such a medium, growth was restricted to the walls of the tube and the organisms were morphologically most unusual, appearing as straight or broken-looking rods two to three times the length of the original vibrio; the mean length of 1.6μ (range 0.9 to 2.5) increased to 3.75μ (range 2.1 to 7μ). By the fourth passage, growth through-

out the bulk of the fluid was taking place (though wall growth was still predominant) and the organisms were now recognizably vibrios though still elongated and markedly pleomorphic. The strain retained these cultural and morphological characters for sixteen passages; the culture was pure by the criteria of Postgate (14) and regained its "ordinary" morphology on transfer back to a fresh water medium.

The natural salt and fresh water strains, as well as the trained salt water strain were studied from three points of view: (1) the fragility of their osmotically sensitive bodies resembling spheroplasts; (2) their secretion of a mucin; and (3) their pool of free amino-acid-like material.

RESULTS

(I) Preparation and Behaviour of Osmotically Fragile Forms

Salt water strains of *D. desulfuricans* do not show the spontaneous osmotic lysis typical of "strong" halophiles (11). Protoplasts, spheroplasts or similar osmotically sensitive bodies might be expected to show different osmotic fragilities according to the salinity to which the strain was accustomed. The following section reports the preparation of two morphologically distinct classes of osmotically fragile bodies from these bacteria and an experiment on the relationship of osmotic fragility to saline habit with one class.

Preparation of Spheroplasts

Lederberg's (10) penicillin procedure was adopted in the following form. The Hildenborough strain was grown in continuous culture at 30° under H_2 + 30% v/v CO_2 (19) in the following medium NH_4Cl, 1g; $(NH_4)_2SO_4$ 0.3g; KCl, 0.2g; $CaCl_2$ 0.1g; $MgSO_4$: $7H_2O$, 2g; Na_2SO_4, 12g; yeast extract (Difco dry 4g) distilled water 1 l., pH 7.4±0.2; cell yield *c* 0.5mg wt./ml. Effluent from the continuous culture was allowed to flow into 1 to 10 vol. of similar medium containing *M* to 0.05 *M* sucrose and 10^2 to 10^4 units penicillin G/ml., with precautions to minimize aeration and temperature shock. After incubation at 30 C under H_2+20% CO_2, a proportion of spheroplasts appeared. These had the typical "rabbit" or "tadpole" forms obtained with rod-shaped bacteria

(10), being essentially spheres with residual fragments of cell walls attached. In most instances, however, these forms constituted only 10 to 20% of the population; the best yields were obtained with 10^{-2} units penicillin/ml., 0.1 *M* sucrose and eighteen to twenty hours incubation. Then up to 40 percent of the population formed spheroplasts, the remainder being unchanged vibrios showing no osmotic sensitivity. Such a yield was inadequate for the purposes intended and this approach was abandoned.

Preparation of Osmotically Sensitive Vibrios

Repaske (17, 18) showed that lysozyme-insensitive bacteria could be rendered sensitive by adding ethylendiaminetetraacetate (EDTA) as well as lysozyme. Cells of the Hildenborough strain, harvested from continuous culture and suspended in dilute buffer lysed in the condition described by Repaske, though lysis was slower (c 40 min. at 37 C) than with his organisms. Papain was active in place of lysozyme; EDTA caused slow lysis on its own. The lysed suspensions were mucoid and the viscosity was reduced by adding ribonuclease. A typical lysis curve is given

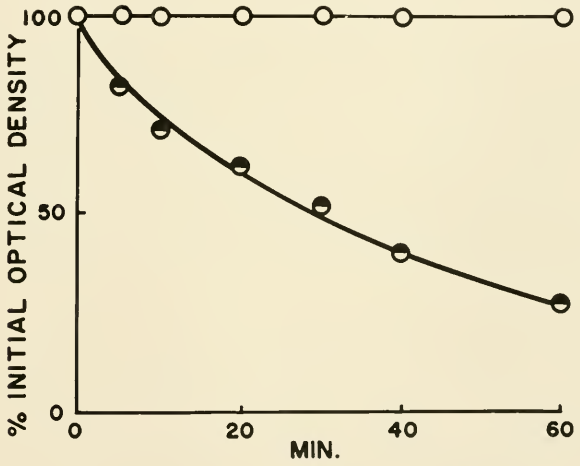

Fig. 2. Lysis of Desulfovibrio desulfuricans (Hildenborough). Organisms harvested from continuous culture were washed, suspended at 0.36 mg. dry wt./ml. M/100 KH_2PO_4 (pH 6.9) were incubated at 37 C with 50 μg/ml. lysozyme and 400/ml. EDTA and the changes in optical density in blue light consequent on lysis were measured. ○ control suspension, ● test.

in Figure 2. Repaske's procedure thus showed promise of yielding "protoplasts" and conditions for this were examined.

Most rapid lysis (15 to 20 min. at 37 C) took place with 50μg/ml. lysozyme + 400μg/ml. EDTA at 37 C in dilute (about 1 mM) potassium phosphate buffer. Alteration of the absolute amounts of lytic agents, or their ratio, slowed lysis. The process had a broad pH optimum about 7.6 but was little slower at values of 6.0 and 8.7; within the range 0.3 to 1.3 mg dry wt. cells/ml the rate of lysis was little influenced by the cell concentration.The EDTA concentration routinely used was 1.08 mM; antagonism of lysis by metal ions was tested using the following salts at 4 mM; $MgSO_4$, $CaCl_2$ $Fe(NH_4)_2(SO_4)_2$, NH_4 - molydate, $ZnSO_4$, $CuSO_4$, $MnSO_4$, NH_4-vanadate, $CoCl_2$. Ca, Mg, Mn or to a lesser extent Fe antagonized lysis; the other materials had no observable effect.

Concentrations of sucrose, phosphate buffer or NaCl in the region of 0.5 to 1M prevented lysis, and the organisms retained their vibrio form. Nevertheless, the cells had become osmotically fragile because they lysed rapidly when transferred to distilled water or dilute buffers. Lysis, observed under the phase contrast microscope, involved first a swelling of the vibrio until it became a symmetrical sphere, then an abrupt breakage of the sphere with loss of contrast and formation of a flat, circular "ghost." These bacteria, therefore, though rendered osmotically fragile by treatment with the lytic agents, formed in these conditions neither true protoplasts nor spheroplasts. Sufficient of the cell wall remained to retain morphological rigidity in isotonic media, but not to survive osmotic shock. These osmotically fragile vibrios will be referred to as "O.F.V.s."

O.F.V.s could thus be prepared in nearly 100 per cent yield. Batches of organisms from 100 ml cultures of strains Hildenborough, its salt-adapted variant and El Agheila Z were harvested from the stationary phase of growth by centrifugation and suspended at turbidities equivalent to 0.1 to 0.6 mg dry wt. cells/ml in NaCl solutions of various molarities. The optical densities were then measured; they were lowest with dilute salt solutions in accordance with Mager's *et al.* (12) optical effect. Lysozyme and EDTA were then added in a volume of water small enough not

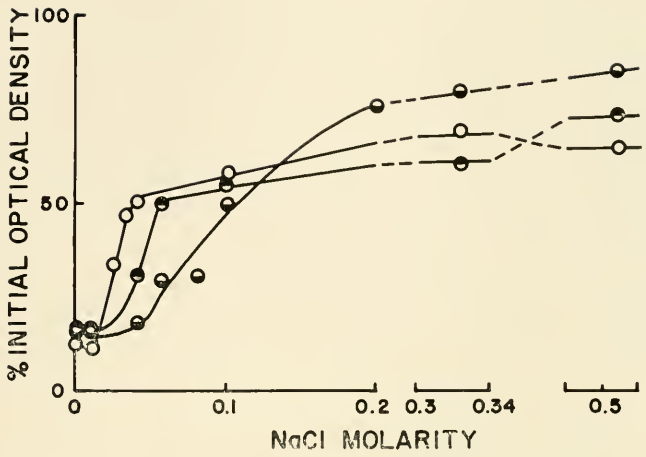

Fig. 3. Osmotic fragility of O.F.V.s from Desulfovibrio desulfuricans. Osmotically fragile vibrios were prepared (see text) in environments of diverse salinities. They were made from ○ the salt-water strain El Agheila Z, ◒ the fresh water Hildenborough strain and ◓ the Hildenborough strain adapted to a saline environment and the extents of lysis measured after 60 minutes at 37 C.

significantly to alter the optical density by dilution, and the tubes were incubated at 40 C for one hour. The optical densities of the suspension after incubation were used to obtain Figure 3. In spite of the cultural habit of the salt-water strain, its O.F.V.s were less fragile than those from the fresh water strain; training the fresh water strain to a salt water habit had caused its O.F.V.s to take on the characters of the natural salt water type.

(II) Secretion of Mucin

Slime formation by *D. desulfuricans* in various conditions has been mentioned by several workers (8, 20, 23). Casual observation at the National Chemical Laboratory had suggested that slime was to be found most often in old cultures of salt water varieties and a systematic experiment was designed to test this belief, making use of an increase in the viscosity of the culture that characterized the appearance of slime. Figure 4 shows the results of the experiment, in which the Hildenbrough strain, its salt-adapted variant and strain El Agheila Z were grown at

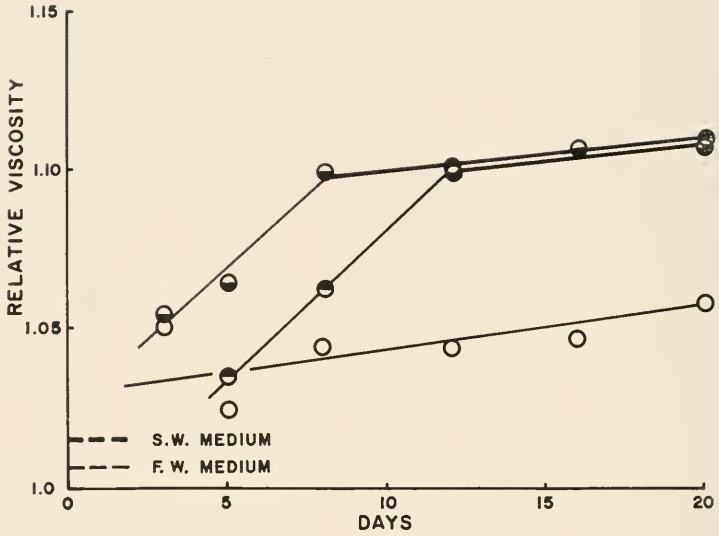

Fig. 4. Relative viscosities of cultures of Desulfovibrio desulfuricans. The viscosities at 18 C of cultures of ◒ the salt water strain El Agheila Z, ○ the fresh water Hildenborough strain and ◓ the Hildenborough strain adapted to saline media were measured at intervals after growth had finished.

30 C and the viscosities of whole cultures relative to distilled water measured in the Ostwald capillary viscometer at 18.2 C to 18.8 C. All the cultures were fully grown before the first reading was taken. The natural and adapted salt water organisms formed more mucin than did the fresh water strain.

Mucous material was formed in very large amounts when the fresh water strain was grown in continuous culture with nitrogenous supplements and a high salt concentration as sulphate (20). The effluent culture had the consistency of white of egg and separated spontaneously into a layer of zoogleal cell debris and an almost clear supernatant. This material was used for the chemical studies in the mucin reported below.

The mucin was precipitated from the supernatant fluid either by acidification with trichloracetic acid or by addition of ethanol to 50 per cent. Dialysis and desiccation of the ethanol-precipitated material gave a white powder with a slight anthrone reaction, positive Molisch and ninhydrin reactions; it charred on

heating. It was not reducing to cupric or argentammonium salts and gave no colour with iodine. These properties suggested that it was a mucoprotein. It was not hydrolysed by saliva, diastase, hyaluronidase, DNA-ase, RNA-ase, lysozyme + versene (as used in part I), suspensions of the Hildenborough strain whether live or acetone dried. Hydrolysis for 9 hr. at 100 C in 5 N HCL *in vacuo* yielded an amino-acid mixture and a sugar. These were analysed qualitatively by paper chromatography with the results shown in Table 2; the usual amino-acids were present and the only sugar was an aldo-hexose. This ran parallel with mannose in four solvent mixtures; we are indebted to Dr. D.A.L. Davies of Porton for confirming that it was truly mannose. Tryptophan was demonstrated in the unhydrolysed mucin.

It was possible that the mucin represented hyper-secreted

TABLE 2

Compounds Detected in Hydrolysed Preparations From *Desulfovibrio Desulfuricans*

	Mucin	*Cell Walls*
1. α -alanine	+	+
2. arginine and/or lysine	+	+
3. aspartase	+	+
4. cystine	+	+
5. diaminopimelate	—	+
6. glutamate	+	+
7. glutamine	—	—
8. glycine	+	+
9. histidine	+	+
10. hydroxyproline	+	tr.
11. the leucines	+	+
12. methionine	+	+
13. phenylalanine	+	+
14. proline	+	+
15. serine	+	+
16. threonine	+	+
17. tyrosine	+	+
18. valine	+	+
19. tryptophan*	+	+
20. mannose	+	—
21. glucosamine	—	+
22. "ribose"	—	+

* Demonstrated in un-hydrolysed material.

cell wall material. Therefore cell walls of the Hildenborough strain, harvested from continuous culture when no detectable mucin was being formed, were prepared by disruption with glass ballotini and repeated washing (22). The product was hydrolysed and analysed qualitatively for amino-acids and sugars. Those observed are recorded in Table 3; the cell wall preparation differed from the mucin in containing diaminopimelic acid. Hydroxyproline was scarcely detectable and the relative concentration of other amino acids was different from those in mucin hydrolysates. Mannose was not present, carbohydrate being represented by glucosamine and a material having the Rf of ribose.

TABLE 3

AMINO-ACID MATERIAL CONTAINED IN STATIONARY POPULATIONS OF SALT AND FRESH WATER STRAINS OF *D. Desulfuricans*

(For Procedeure see Text. One Unit Material is that Giving a Color Reaction Equicalent to 1 μg Casein Hydrolysate)

Strain	*Habit*	*Content (units/mg. dry wt.)*	*Age of Culture (Days)*
El Agheila Z	Salt water	72	3
		65	3
		70	5
Hildenborough	Fresh water	52	3
		36.5	5
		53.5	5
Hildenborough	trained to salt water	82	5
		80.5	8

If follows from these observations that the mucin produced by these bacteria is not over-produced cell wall or capsular material. It is a mucopolymannoside produced in response to media of appropriate salinity.

(III) "Free Amino-Acid Material"

Mattick *et al.* (13) have used chromatography of the ninhydrin-positive material released from bacteria by acidification for taxonomic studies. During an investigation of the applicability of this procedure to the sulphate-reducing bacteria we observed

marked differences among the chromatographic patterns of extracts of marine and fresh water strains. All studies of this kind were done with stationary phase populations grown in 100 ml batches of medium; organisms from continuous culture were not used since the phase of growth is known to influence the character of the extracts (e.g., 5). Such cultures yielded live cells equivalent to 30 to 50 mg dry wt. organisms. Lots of fresh cells equivalent to 10 mg dry wt. were extracted by suspending in 10 per cent acetic acid, the cells centrifuged off, and the whole extracts dried onto paper. They were then chromatographed in a conventional manner in two dimensions using phenol and butanol/ammonia. The patterns obtained are illustrated in Figure 5, which consists of sketches of tracings of the actual chromatograms. Individual strains showed reasonable consistency when extracted and chromatographed a second time, but the differences between strains were wide and suggested that a diagnostic test on these lines might be difficult to develop. Most marked, however, was the qualitative observation that the salt water strains released relatively large amounts of material containing at least 5 components, and the fresh water strains released less material with fewer components; sketches of chromatograms from another salt water strain (Canet 41, NCIB 8393) another fresh water strain (Essex 6, NCIB 8307) are included in Figure 5. This figure also includes patterns obtained with salt and fresh water strains trained to the alternative environment; the fresh water strain took on some of the characters of the salt water strain on acclimatization to the appropriate medium, but the reverse change did not occur.

Table 3 shows that the qualitative impression that salt water strains released none of such material was correct. In these experiments the material was released with 10 per cent acetic acid and analysed for free primary amino-groups by the method of Frame, Russell & Wilhelmi (7). The standard for this assay was Difco "Casamino-acids"; 1 unit is an amount of material giving a colour equivalent to 1 μg "Casamino-acids" (*c* 10% N).

The nature of the material released from these strains was not studied further. None of the spots corresponded to known amino-acids and it is likely that they were peptide in nature.

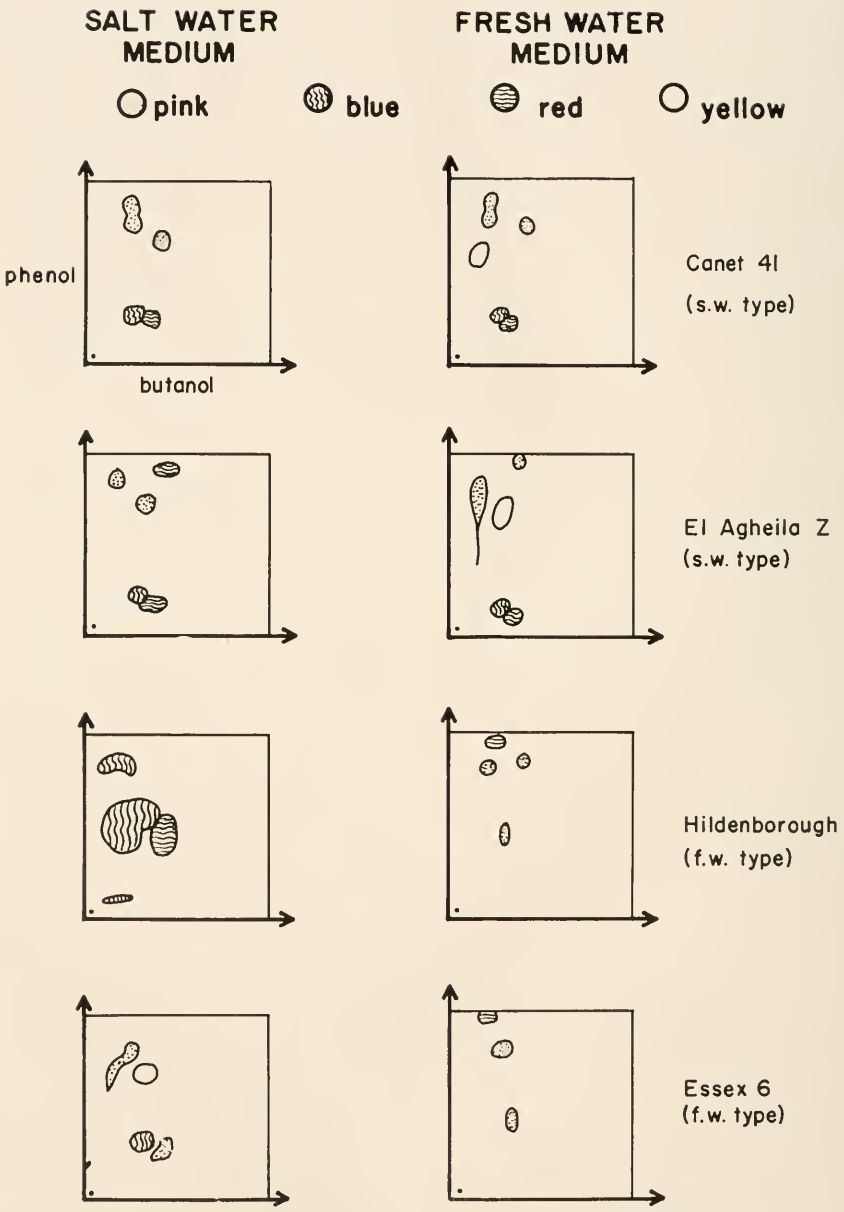

Fig. 5. Sketches of chromatograms of extracts of Desulfovibrio desulfuricans. (For description see text.)

DISCUSSION

Knowledge of the general biochemistry of *Desulfovibrio desulfuricans* is still rather fragmentary, but the published data reviewed recently (15) show no indication of essential differences in the contents of pigments and metabolic enzymes between salt water and fresh water strains. Hence such differences as must exist are likely to be of more subtle kinds. The present study has demonstrated three physiological differences that seem to be consistently maintained and we can put forward four tentative conclusions concerning growth of the strains studied in a saline environment:

(i) Growth in a saline environment increased the osmotic stability of O.F.V.s prepared from the organisms.

(ii) Growth in a saline environment was associated with production of a mucopolymannoside which was not chemically related to the cell wall.

(iii) Growth in a saline environment led to an increased content "free amino-acid material" within the cell and to a change in its quality.

(iv) Adaptation of a fresh water strain to a saline environment included the acquirement of the three characters listed above, together with a morphological change. For an adaptive change in the reverse direction only the last character was studied and it was not lost.

It is reasonable to suppose that the possession of the appropriate characters has survival value for the organisms in a given environment, but the connection between some of the characters reported here and survival in the marine environment is not immediately clear. The data presented here may be a useful pointer to further work, but studies of more strains as well as investigation of other characters are necessary before a useful comprehensive rationalization of these findings could be attempted.

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Chapter 41

Organic Factors Responsible for the Stimulation of Growth of *Desulfovibrio desulfuricans*

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The present paper describes in outline some recent experiments on the nutritional requirement of *Desulfovibrio desulfuricans* already reported by us (11), and presents some fresh insight into the roles of growth stimulating factors in the metabolism of this microorganism.

It has already been found by many workers that the growth of sulfate-reducing bacteria was more or less stimulated by certain factors contained in natural organic substances of indefinite composition. Since the work of Bunker (4), who showed that yeast, yeast water, and filtered yeast culture stimulated the growth of *Desulfovibrio desulfuricans*, more than ten papers (1, 6, 7, 10, 12, 13, 14, 16, 17, 18, 19, 23) have been published on the presence of such stimulating factors in peptone, yeast extract, liver extract, beef extract, potato juice, protein hydrolysates, milk and other natural substances. Although several workers (2, 5, 6, 20, 21, 22, 24) reported that *Desulfovibrio* grew autotrophically in simple mineral media without organic nutrients, the apparent growth observed by these authors was attributed recently by Mechalas and Rittenberg (14), and by Postgate (19) to the utilization of organic substances present as impurities in the autotrophic media. Such organic impurities might serve not only as hydrogen donors in energy-yielding reaction but also as growth stimulants for these bacteria.

Some of the previous works (12, 13, 26) also indicated that these bacteria could easily be cultivated without added natural

substance when they were contaminated with certain other microorganisms. These facts suggest that the contaminants supplied some factors possessing the growth-stimulating activity for *Desulfovibrio.*

It was found by Grossman and Postgate (7), and also by Kimata *et al.* (12, 13) that pure strains of *Desulfovibrio desulfuricans* could be cultivated without any supplement bearing growth factors when very large inocula were employed. This suggests that the cells added as inocula possibly served as a source of growth-stimulating factors in those cultures.

In view of these previous works, it may be concluded that factors stimulating the growth of *Desulfovibrio* are commonly present in various biological matter, including the cells of the organism itself.

Although attempts to isolate such factors from yeast extract and peptone were made by Postgate(16), a substance possessing the growth-stimulating activity could not be obtained. The factors in yeast extract, however, were found by him to be relatively stable to heat, non-volatile, water-soluble, and not removed by various extraction procedures. Postgate (16), and Kimata *et al.* (12, 13) also tested a number of pure, biologically-active substances including vitamins, amino acids, purines, etc., for ability to replace peptone and yeast extract, in this respect, but they could not find any substance showing such an activity markedly. Although Postgate (16) found, with purified cultures, that cysteine stimulated the growth, and serine, ornithine and isoleucine acted synergistically with it, these amino acids did not account for the whole activity of peptone and yeast extract.

All these previous studies suggest that the factors in question are substances which have not been regarded as growth factors for microorganisms, and are expected to be distributed widely in various biological matter.

ADENOSINE-5'-TRIPHOSPHATE AS GROWTH-STIMULATING FACTOR IN DESULFOVIBRIO DESULFURICANS STRAIN MAIZURU 1

Based on the above presumption, the present authors (11) tested adenosine-5'-triphosphate (ATP), which is a biologically

active substance commonly present in animal, plant and microbial tissues and has not yet been examined with this respect in the previous works, for its ability to stimulate growth of a salt water strain of *D. desulfuricans* (strain Maizuru 1).

TABLE 1

EFFECT OF ATP ON THE GROWTH *of Desulfovibrio desulfuricans* (MAIZURU 1) IN A DEFINED MEDIUM SUPPLEMENTED WITH PEPTONE OR CASAMINO ACIDS OR EIGHTEEN AMINO ACIDS AS NITROGEN SOURCES

*Supplement**	*Growth, O. D. at 610 mμ*
Peptone	0.39
Peptone + ATP	0.40
Casamino acids	0.06
Casamino acids + ATP	0.34
18 amino acids	0.05
18 amino acids + ATP	0.42
None (basal medium)*	0.00

* Basal medium was composed of minerals, lactate and asorbic acid only.

The data illustrated in Table 1 are the results of experiments carried out to demonstrate the effect of ATP on the growth of this microorganism in media based on metallic salts, sulfate, lactate and L-ascorbic acid, and supplemented as nitrogen sources with peptone or Casamino acids or a mixture of eighteen amino acids which was composed of L-arginine, L-histidine, L-lysine, L-tryptophane, DL-tyrosine, L-phenylalanine, L-cystine, DL-methionine, DL-serine, DL-threonine, L-leucine, L-isoleucine, L-valine, L-glutamic acid, L-aspartic acid, glycine, DL-alanine, and L-proline.

When supplemented with ATP, the partially defined medium containing Casamino acids as nitrogen sources enabled good multiplication of the microorganism tested, whereas very little growth occurred in such a medium without added ATP. The growth obtained in the Casamino acids medium with ATP was similar to that in the peptone medium. The replacement of Casamino acids by a mixture of eighteen amino acids in the medium supplemented with ATP also supported the normal growth which was approximately equal to that in the peptone

medium. The amino acids medium without added ATP, however, did not by itself support the growth of this microorganism.

These data revealed that ATP and the eighteen amino acids together could completely be substituted for peptone, in respect to the growth-stimulating activity for this microorganism.

We attempted next to replace ATP by its possible precursors and some related compounds (adenosine-5'-monophosphate (AMP), adenosine-3'-monophosphate, adenosine, inosine, adenine, hypoxanthine and guanine) by using a chemically defined medium composed of minerals, lactate, ascorbic acid and eighteen amino acids as the basal. For reference acid-soluble nucleotides preparation from the cells of *D. desulfuricans* (Maizuru 1) itself (prepared after Tsuboi and Price (25)), which was found by paper chromatography (3, 27) to contain ATP, AMP, adenosine-5'-phosphosulfate (APS) and three other unidentified nucleotides, was also tested for ability to stimulate the growth in place of ATP.

By this experiment, the results of which were given in Table 2, we found that AMP, adenosine-3'-monophosphate, and the nucleotides preparation from the cells were equally active as ATP in stimulating the growth of *D. desulfuricans* (Maizuru 1); ATP in this respect was perfectly replaced by the above nucleotides.

TABLE 2

RESPONSE OF *D. Desulfuricans* (MARIZURU *1)* TO ATP AND ITS RELATED COMPOUNDS IN CHEMICALLY DEFINED MEDIUM

Supplement	*Growth, O. D. at 610 mμ*
ATP	0.47
AMP	0.47
Adenosine-3'-monophosphate	0.47
Adenosine	0.06
Inosine	0.03
Adenine	0.05
Hypoxanthine	0.06
Guanine	0.05
Nucleotides preparation from the cells of Desulfovibrio desulfuricans (Maizuru 1)	0.47
None**	0.05

** Basal medium contained minerals, lactate, ascorbic acid and eighteen amino acids.

Adenosine, inosine, adenine, hypoxanthine and guanine, however, failed to support the normal growth.

These facts suggest that the ability of this microorganism to synthesize some nucleotides was limiting for its own growth, probably at the initial stage.

TABLE 3

EFFECT OF AMINO ACIDS ON THE GROWTH OF *D. Desulfuricans* (MAIZURU 1) IN CHEMICALLY DEFINED MEDIA

*Amino Acid Omitted****	*Growth, O. D. at 610 mμ*
DL-Alanine	0.01
L-Arginine	0.02
L-Aspartic acid	0.01
L-Cystine	0.15
L-Glutamic acid	0.03
Glycine	0.28
L-Histidine	0.31
L-Isoleucine	0.01
L-Leucine	0.01
L-Lysine	0.01
DL-Methionine	0.33
L-Phenylalanine	0.01
L-Proline	0.13
DL-Serine	0.01
DL-Threonine	0.01
L-Tryptophane	0.29
DL-Tyrosine	0.31
L-Valine	0.01
None	0.34

*** Each amino acid was omitted from the complete medium based on minerals, lactate, ascorbic acid, ATP and eighteen amino acids.

RESPONSE OF DESULFOVIBRIO DESULFURICANS (MAIZURU 1) TO AMINO ACIDS

To elucidate the response of *D. desulfuricans* (Maizuru 1) to amino acids in detail, we examined subsequently the respective effects of the above described eighteen amino acids on the growth of this microorganism, by observing its growth in chemically defined media based on minerals, lactate, ascorbic acid, ATP and seventeen amino acids (out of the complete eighteen amino acids each one to be tested was omitted respectively).

The results of this experiment, which were illustrated in

Table 3, indicate that the growth of this microorganism did not take place by deleting arginine, lysine, phenylalanine, serine, threonine, leucine, isoleucine, valine, glutamic acid, aspartic acid, or alanine from the complete medium. By omitting cystine or proline, the growth was drastically reduced. Omission of each of the remaining five amino acids, however, had little effect on the growth.

From these results it is considered that the first mentioned eleven amino acids were indispensable to this microorganism, and that cystine and proline were significantly stimulating. To confirm this, we observed next the growth of the organism in a defined medium containing ATP and the above thirteen amino acids, in comparison with that in the media having somewhat reduced compositions in amino acids from the above (Table 4).

As shown in this table, the medium containing the thirteen amino acids supported the normal growth which was similar to that in the complete medium containing all eighteen amino acids. When cystine and proline or cystine, proline, arginine and glutamic acid, which were found in the preceding experiment to exhibit relatively low activity as compared with the others, were omitted from these thirteen amino acids, the growth obtained was about three quarters and one-fifth of the normal respectively.

From these data it was confirmed that the thirteen amino

TABLE 4

GROWTH OF *D. Desulfuricans* (MAIZURU 1) IN CHEMICALLY DEFINED MEDIA CONTAINING AMINO ACIDS IN DIFFERENT COMPOSITIONS

*Amino Acid Added*****	*Growth, O.D. at 610 mμ*
All 18 amino acids	0.40
13 amino acids (Arg, Lys, Phe, Ser, Thr, Leu, Isoleu, Val, Glu, Asp, Ala, CyS, and Pro)	0.37
11 amino acids (Arg, Lys, Phe, Ser, Thr, Leu, Isoleu, Val, Glu, Asp, and Ala)	0.30
9 amino acids (Lys, Phe, Ser, Thr, Leu, Isoleu, Val, Asp, and Ala)	0.07

****Basal medium was composed of minerals, lactate, ascorbic acid and ATP.

acids described above were more or less seriously required by this microorganism for its normal growth.

These experiments have also led to the development of the minimum synthetic medium, for supporting the normal growth of the organism, which had the composition as illustrated in Table 5.

TABLE 5

COMPOSITION OF CHEMICALLY DEFINED MEDIUM FOR *D. Desulfuricans* (MAIZURU 1)

$MgSO_4 \cdot 7H_2O$	2.0 g	L-Isoleucine	0.144 g
$FeSO_4 \cdot 7H_2O$	0.05	L-Leucine	0.216
K_2HPO_4	0.1	L-Lysine	0.175
NaCl	25.0	L-Phenylalanine	0.136
Ca-lactate	3.0	L-Proline	0.176
L-Ascorbic acid	0.1	DL-Serine	0.165
DL-Alanine	0.067	DL-Threonine	0.090
L-Arginine	0.092	L-Valine	0.147
L-Aspartic acid	0.075	ATP (or AMP)	0.1
L-Cystine	0.007	H_2O	1000.0 ml
L-Glutamic acid	0.532	pH	7.2

RESPONSE OF OTHER STRAINS OF DESULFOVIBRIO DESULFURICANS TO ATP AND AMINO ACIDS

To ascertain whether the nutritional pattern thus obtained with the organism is applicable to other strains, we observed subsequently this growth of several representative strains of this species including both salt-water types and fresh-water types in the above described defined medium (in the cases of fresh-water strains NaCl as an ingredient was reduced to 5.0 g/L). For reference, the growth of these microorganisms in the media which contained, as nitrogen sources, peptone or Casamino acids or eighteen amino acids in place of the thirteen amino acids was also observed. The results of this experiment, which were given in Table 6, indicate that all the strains tested grew well in the medium containing the eighteen amino acids, although the growth maxima obtained in this case were somewhat lower, in general, than those in the medium based on peptone. In the medium containing the thirteen amino acids, however, the growth of strains Wandle, Hildenborough, Miyazaki, and El Agheila Z were dras-

tically suppressed as compared with those in the remaining media. These facts suggest that the pattern of the amino acid requirement in these four strains differed to a certain extent from that in strain Maizuru 1.

TABLE 6

GROWTH OF VARIOUS STRAINS OF *D. Desulfuricans* IN THE DEFINED MEDIUM BASED ON MINERALS, LACTATE, ASCORBIC ACID AND ATP, AND SUPPLEMENTED WITH AMINO ACIDS OR CASAMINO ACIDS OR PEPTONE, AS NITROGEN SOURCES

	Growth (O.D. at 610 mμ) in the Medium Supplemented With:			
Strain	*Peptone*	*Casamino Acids*	*18 Amino Acids*	*13 Amino Acids*
Wandle* (NCIB 8305)	0.32	0.15	0.14	0.03
Hildenborough* (NCIB 8303)	0.37	0.16	0.15	0.05
Miyazaki*	0.24	0.14	0.12	0.07
El Agheila Z** (NCIB 8380)	0.42	0.28	0.26	0.08
Maizuru 1**	0.43	0.40	0.41	0.37

*Fresh-water strain.
**Salt-water strain.

The population densities obtained with the cultures of the salt-water strains (El Agheila Z and Maizuru 1) at the maximum growth phase were generally higher than those of the fresh-water strains (Wandle, Hildenborough and Miyazaki), especially in the cases that chemically defined media were employed. The times required for attaining the maximum growth of the salt-water strains were also more or less shorter than those of the fresh-water strains. These may be due to the lower concentration of NaCl in the fresh-water medium as compared with that in the salt-water medium; certain heavy metals which probably contaminated to reagent NaCl might be responsible for the stimulation of growth of these microorganisms to some extent.

CONCLUSION

The general conclusion which can be drawn from all these experiments is that most of sulfate-reducing bacteria belonging to genus *Desulfovibrio,* irrespective of their response to the NaCl

concentration, require some nucleotides (AMP, ATP, APS, etc) and amino acids (all or some of the following: arginine, lysine, phenylalanine, serine, threonine, leucine, isoleucine, valine, glutamic acid, aspartic acid, alanine, cystine, glycine, histidine, methionine, proline, tryptophane, and tyrosine) for the normal or nearly normal growth. These substances seem to be required by these organisms as growth-stimulating factors rather than essential growth factors in the strict sense of the word.

The high activity of nucleotides as growth-stimulating factors in the present case may principally be due to the extraordinarily great need for ATP by these microorganisms in the energy-yielding reactions indispensable for the multiplication of the cells; it was recently found by Peck (15) and Ishimoto (8, 9) that ATP participated in the energy-yielding reaction which was linked to the reduction of sulfate to sulfide ion through the following pathways when sulfate was present:

$$SO^{2-}_4 + ATP = APS + PP$$
$$APS + H_2 = SO^{2-}_3 + AMP$$
$$SO^{2-}_3 + 3H_2 = S^{2-} + 3H_2O$$

Accordingly, the amount of ATP required for the growth (especially, for the initiation of growth) by these microorganisms is expected to be considerably larger than that in the cases of usual microorganisms; the amounts of AMP, ATP and APS initially present in the inoculum of ordinary size are thought to be too small for supporting the normal multiplication of the cells of these microorganisms. Although purines and nucleosides appear to be hardly available for the synthesis of ATP or APS in the cells, AMP and adenosine-3'-monophosphate seem to be relatively easily transformed by the cells to APS via ATP in the presence of sulfate, and subsequently be used for the sulfate reduction.

The previously reported facts that these bacteria were able to be grown in the media without any natural substance of indefinite composition only (1) when an inoculum of abnormally large size was employed; (2) or when certain other microorganisms coexisted in the culture, may reasonably be explained by the above described findings.

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Chapter 42

Mechanisms in the Microbial Oxidation of Alkanes

R. E. Kallio, W. R. Finnerty, S. Wawzonek and P. D. Klimstra

It seems well established that the primary enzymatic attack on alkanes by microbial action occurs at the terminal carbon atom and results in the early formation of a fatty acid corresponding in chain length to the alkane (1, 9, 10, 14)). Rupture of the hydrocarbon structure and introduction of oxygen into the molecule could conceivably occur in at least two ways; dehydrogenation of the alkane to an olefin followed by water addition to the double bond or by hydroperoxidation of the alkane leading to the formation of an alkyl hydroperoxide. Since there appears to be evidence for both mechanisms these are examined in the discussion which follows. Space stricture dictates the brief and incomplete form of the treatment.

MICROBIAL HYDROPEROXIDATION OF ALKANES

Formation of alkyl hydroperoxides involves incorporation of atmospheric oxygen into the alkane molecule either directly or through the participation of another peroxide the formation of which, in turn, is dependent upon the presence of free oxygen. That atmospheric oxygen is necessary for bacterial alkane oxidation has been shown indirectly by Hansen and Kallio (5) who showed that while strains of nitrate-reducing pseudomonads readily oxidized alkanes aerobically the organisms were unable to do so anaerobically in the presence of nitrate. Updegraff and Wren (15) in a careful re-assessment of earlier work demon-

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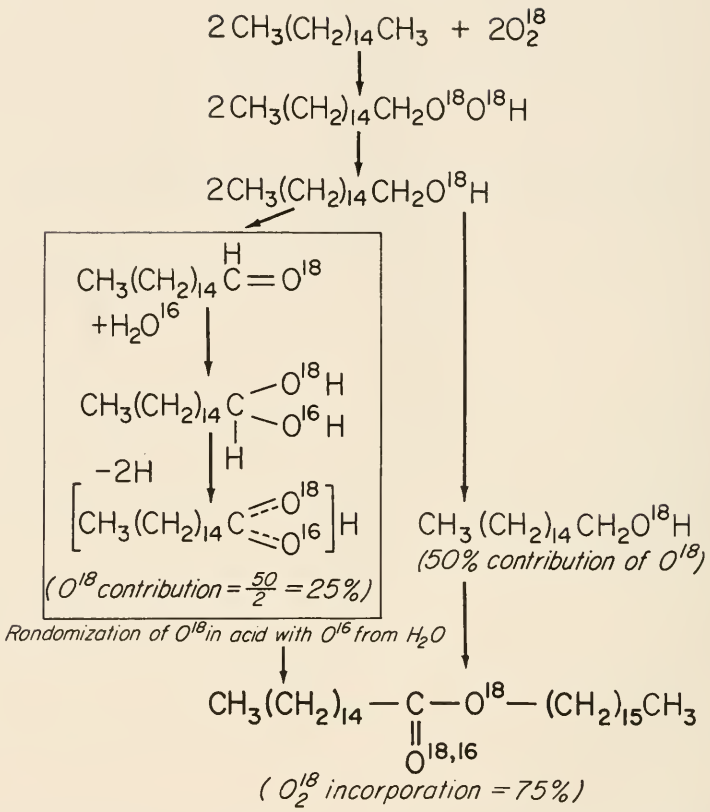

Fig. 1. Hypothetical mechanism for bacterial oxidation of *n*-hexadecane and subsequent formation of cetyl palmitate [from Stewart *et al.* (14)].

strated that there appears to be no significant anaerobic oxidation of pure alkanes by *Desulfovibrio* as evidenced by failure to observe H_2S formation in sulfate containing minerals-alkane media.

Direct evidence for the participation of gaseous oxygen in alkane oxidations was found by Stewart *et al.* (14), in a study of the oxidation of *n*-hexadecane by a gram negative micrococcus characterized by ester excretion when growth at the expense of alkanes. Cetyl palmitate, the ester produced from hexadecane by the organism was isolated after incubation under O^{18} enriched atmosphere and was shown to have 75 percent incorporation of atmospheric oxygen. Based upon these findings the mechanism for alkane oxidation and ester formation shown in Figure 1 was

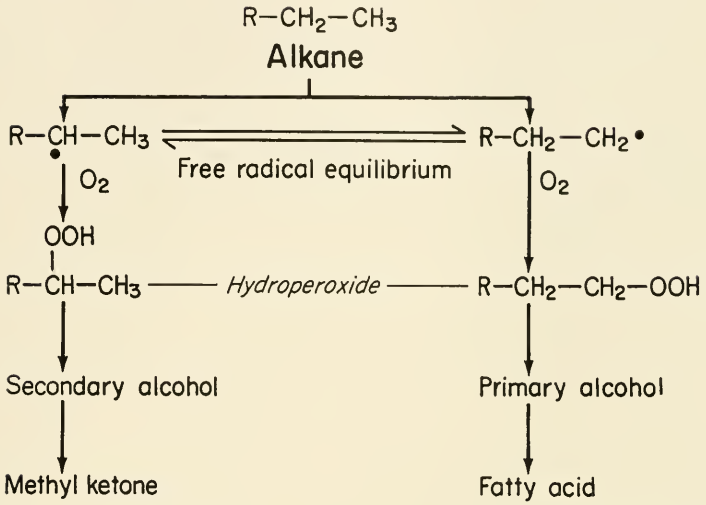

Fig. 2. Formation of *n*-alcohols and methyl ketones from alkyl free radicals [Leadbetter and Foster (9)].

proposed. Independently, Leadbetter and Foster (8, 9) arrived at the same conclusion after investigating the oxidation of alkanes by *Pseudomonas methanica* and other alkane utilizing bacteria. Incorporation of O^{18} into cell material of organisms grown in various *n*-paraffins was found to decrease markedly as the length of the paraffin increased. The results were taken as evidence that the first reaction involved in alkane oxidation was one requiring participation of molecular oxygen and subsequent reactions involved the oxygen of water. Thus, oxidation and assimilation of intermediates with long alkyl chains would necessarily involve a larger number of reactions, thereby diluting the original O^{18} incorporation levels of cells utilizing long chain paraffins.

The formation of methyl ketones observed by Leadbetter and Foster (9), acetone from propane, for example, does not necessarily mitigate against either the terminal carbon attack or the hydroperoxidation hypothesis. Hydroperoxidation very likely involves a free radical mechanism and one factor involving the nature of the products formed may lie in the relative stabilities associated with free radical intermediates. Stabilities of alkyl radicals increase in the order I° < II° < III° and also increase

with the size of the groups bonded to the carbon atom carrying the unpaired electron. Analysis of microbial alkane oxidation literature indicates fatty acids, *n*-alcohols, and methyl ketones predominate as identifiable products from *n*-alkanes of low molecular weight (C_{10} or less). Acids, alcohols and esters are the major products of microbial oxidation of *n*-alkanes longer than ten carbon atoms. The division of end products seems, in any case, to be quite clear: methyl ketones but no esters are produced by bacterial action on low molecular weight alkanes whereas the reverse is true of high molecular weight alkanes. Possible relationships between free radical equilibria, terminal alkane oxidation and end products are shown in Figure 2, from the original suggestion of Leadbetter and Foster (9). Harris (6) on the basis of sequential induction experiments with an alkane oxidizing micrococcus concluded that methyl ketones did not lie on the direct pathway of alkane oxidations and thus it is tempting to speculate that the methyl ketones observed by Leadbetter and Foster may have been the result of free radical equilibria rather than "direct" products of alkane oxidation. It is equally conceivable that methyl ketone formation is the major pathway followed in microbial oxidation of certain short chain alkanes.

Although 'peroxides" (4) have been detected in culture fluids of bacteria utilizing alkanes, the nature of such peroxides has not been established. Reduction of hydroperoxides by bacteria has been reported by Updegraff, and Bovey (15) and Stewart *et al.* (14) showed that 1-decyl hydroperoxide was oxidized by a species of *Micrococcus*. These fragmentary indications stimulated attempts to establish more firmly the role of hydroperoxides in biological paraffin oxidation processes and led to the synthesis of a series of 1-alkyl hydroperoxides (C_{12} to C_{18}) by treating the appropriate alkyl methane sulfonate with H_2O_2(17). Alkane oxidizing micrococci grown in minerals-alkane media readily oxidized all 1-alkyl hydroperoxides as measured by conventional manometry. Results of a typical experiment in which intact cells were tested for their ability to oxidize an alkane, the corresponding 1-alkyl hydroperoxide, and other mono-terminally oxygenated derivatives of the alkane are shown in Figure 3. No attempt was made to assess the significance of rate differences since no satis-

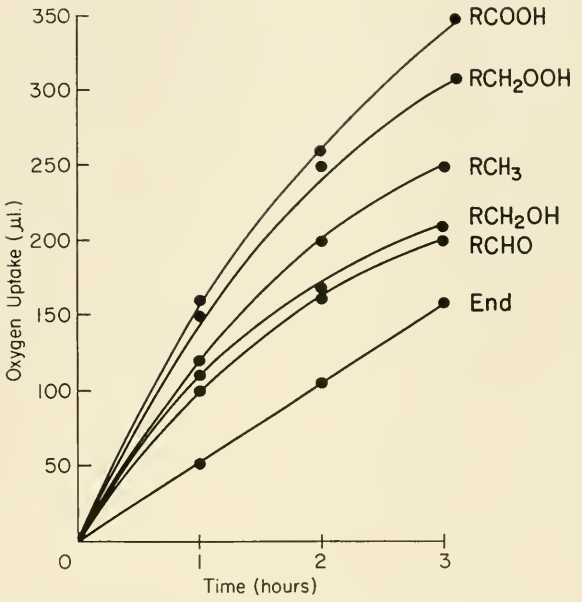

Fig. 3. Oxidation of *n*-dodecane, 1-dodecyl hydroperoxide, and other oxygenated analogues of dodecane by *Micrococcus* sp. grown in minerals-dodecane medium. Each vessel contained 5 μmoles substrate, 0.5 ml. of 0.1 *M* phosphate buffer (pH 8.0) containing 200 mg. $MgSO_4.7H_2O$, 1.0 mg. $CaCl_2.2H_2O$ and 1.0 mg. $FeSO_4.7H_2O$ per 100 ml. buffer, 10 mg. (dry wt.) of cells. $V_t = 3.0$. Incubation at 30 C.

factory way has been developed to compare the substrate-water-bacterium interface which may be a real factor in any rate study particularly when the substrates vary in solubility and physical form (solid and liquid).

Stewart *et al.* (14) have pointed out that 1-decyl hydroperoxide is relatively toxic, but our tests with the series of these materials indicates the toxicity decreases with an increase in the length of the alkyl chain. At low concentrations (5 μmoles per experimental vessel) non-proliferating cells rapidly oxidized all hydroperoxides tested. The toxicity of the hydroperoxides was high enough to preclude meaningful growth experiments. However, heavy, non-proliferating cell suspensions harvested from alkane-minerals medium and incubated with 400 μmoles of 1-alkyl hydroperoxide (the least toxic of the hydroperoxides) produced

60 μmoles of ester/100 ml in a twenty-four hour test period as compared with a yield of 50 μmoles of ester/100 ml from *n*-octadecane under similar conditions. The hydroperoxide assay of Eise and Giesecke (3) was adapted to measurement of alkyl hydroperoxides and yielded evidence of hydroperoxides in cells grown at the expense of *n*-hexadecane. Cells from six liters of medium were extracted with hot reagent grade toluene and yielded as much as 1 μmole of hydroperoxide, and while these are relatively low levels of hydroperoxide it seems significant that cells grown on non-hydrocarbon substrates never contained such peroxides. The low intracellular levels of hydroperoxide, moreover, are consonant with the relative toxicity of 1-alkyl hydroperoxides.

These data, to be presented in detail elsewhere, support the hydroperoxide hypothesis in bacterial oxidation of normal long chain alkanes.

DEHYDROGENATION OF ALKANES

The long held notion that dehydrogenation is a step in biological alkanes oxidation seems based upon incomplete identifications of "olefins," the ability of paraffin oxidizing microorganisms to utilize olefins as well as paraffins for growth and energy, and the obvious relationship of chemical structures between the alkane and alkene [see (9) for complete citations]. Support for the hypothesis has come from recent experiments demonstrating that *n*-alkanes function as hydrogen donors for reduction of indicators by cell extracts under anaerobiosis (11, 18). Senez and Azoulay (10) have demonstrated a diphosphopyridine nucleotide (DPN) dependent heptane dehydrogenase (which requires the presence of pyocyanine) in extracts of *P. aeruginosa* cells grown at the expense of *n*-heptane. We have extensively tested alkane utilizing species of *Candida*, *Nocardia*, *Pseudomonas*, *Mycobacterium*, and *Micrococcus* both in the intact state and as extracts for ability to reduce various indicators anaerobically in the presence of *n*-alkanes from decane through octadecane. Our results have been uniformly negative.

It is difficult to reconcile the findings of Senez and Azoulay (11) with other findings which seem to favor the hydroperoxida-

tion theory. First, so far as the writer is aware, the crucial isolation and identification of an appropriate olefin has never been accomplished in what appears to be anaerobic alkane oxidation. Another difficulty lies in attempting to reconcile dehydrogenation of paraffins with the elegant study of Leadbetter and Foster (9) on the oxidation of ethane by *P. methanica*. In the presence of methane *P. methanica* oxidizes ethane to acetic acid. Cultures provided with hexadeuteroethane (and methane) yielded acetic acid which was shown by mass analysis to have the structure CD_3COOH, thus eliminating ethylene ($CD_2 = CD_2$) as an intermediate in the oxidation of ethane. Nevertheless, it does seem significant that in Senez' study glucose-grown *P. aeruginosa* cell extracts have no heptane dehydrogenase activity. Hexadecane is utilized for growth by the pseudomonad used by Senez, but such cells are devoid of hexadecane dehydrogenase (Senez, personal communication).

Since a fatty acid with the alkane carbon skeleton unchanged

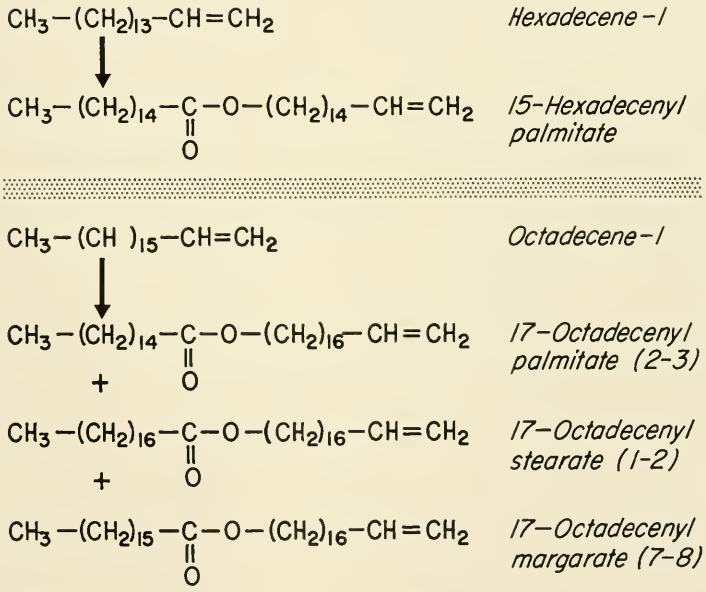

Fig. 4. Esters isolated from culture fluids of *Micrococcus* sp. grown at the expense of hexadecene-1 and octadecene-1 (12). Numbers in parentheses refer to proportional amounts of esters produced.

results from bacterial alkane oxidation, the olefin resulting from dehydrogenation must be a terminal, or 1-olefin. At this writing no such olefins have been isolated from bacterial oxidations of saturated hydrocarbons. Furthermore, if the 1-olefin is an intermediate in alkane oxidation it should be handled by alkane oxidizing microorganisms in the same manner, i.e., oxidation to fatty acids (or methyl ketones or esters). Bruyn (2) isolated hexadecane-diol-1,2 from culture fluids of *Candida lipolytica* growing at the expense of 1-hexadecene, but apart from this report little is known concerning microbial utilization of 1-olefins. Stewart *et al.* (11) isolated esters from *Micrococcus* strains growing in minerals-olefin media and the esters were shown to have alcohol moieties with a carbon skeleton identical to that of the olefin substrate including an intact double bond (Fig. 4). The findings suggest that olefins are utilized differently by *C. lipolytica* and *Micrococcus* but even more significant is the finding that the oxidative attack by bacteria does not apparently occur at the olefinic bond, thus suggesting that the terminal olefin is not an intermediate in the oxidation of long chain alkanes.

A reasonable reconciliation of these diverse findings based on the activities of different organisms toward various alkanes would seem to be assigning of at least three major pathways to the microbial utilization of alkanes. Long chain alkanes (C_{12} - C_{18}) are oxidized by the way of 1-alkyl hydroperoxides and the formation of *n*-alcohols which in turn are oxidized to *n*-fatty acids. Certain short chain length alkanes (C_3 - C_5) produce methyl ketones probably *via* rearrangement of 1-alkyl free radicals and, finally, intermediate length alkanes (C_6 - C_{10}) are dehydrogenated to the corresponding 1-alkene. Much work remains to be done to establish the pathways more firmly and, considering the metabolic flexibility of microorganisms, it is not unlikely that other metabolic sequences for microbial alkane oxidations will be uncovered. Kester and Foster (7), for example, have already demonstrated an intriguing case of bacterial diterminal oxidation of alkanes. Interestingly, later evidence suggests mono-terminal oxidation (hydroperoxidation?) followed by ω-oxidation (Foster, personal communication).

SUBSTRATE	PRODUCT
OCTADECANE·····	1:1 Mixture of : $CH_3(CH_2)_{16}COOCH_2(CH_2)_{16}CH_3$ and $CH_3(CH_2)_{14}COOCH_2(CH_2)_{16}CH_3$
HEXADECANE·····	$CH_3(CH_2)_{14}COOCH_2(CH_2)_{14}CH_3$
TETRADECANE·····	$CH_3(CH_2)_{14}COOCH_2(CH_2)_{12}CH_3$
DODECANE ·····	$CH_3(CH_2)_{14}COO - X$

Fig. 5 Esters obtained from culture fluids of *Micrococcus* growing on alkane substrates. From Stewart *et al.* (13).

ORIGIN OF CARBON IN ACID MOIETIES OF ESTERS FROM BACTERIAL ALKANE OXIDATIONS

Esters produced by alkane utilizing micrococci are predominantly palmitates regardless of the length of *n*-alkane used as substrate as shown in Figure 5 [from Stewart and Kallio, (13)]. To establish the origin of the carbon skeletons of palmitic acid in cetyl palmitate (from hexadecane) and myristyl palmitate (from tetradecane) hexadecane-1-C^{14} and tetradecane-1-C^{14} were supplied to cultures of the organisms in otherwise mineral media. Esters were isolated, saponified and the palmitic acid and alcohol moieties were recovered. In each case carbon atoms 1, 2, 3, and 4 of palmitic acid were isolated (as benzoate) and C^{14} activities of each carbon atom were determined.

Data for the palmitic acid from cetyl palmitate are shown in Figure 6 and leave little doubt that the ester is formed by condensation of two 16 carbon units derived from the hexade-

Activity of Fragment (cpm)	1486	0	0	0	1161
	C–(C)(=O)–	C	C	C	COOH
% activity	65	0	0	0	52

Fig. 6. Distribution of C^{14} in palmitic acid moiety of cetyl palmitate isolated from cultures of *Micrococcus* sp. utilizing hexadecane-1-C^{14} for growth.

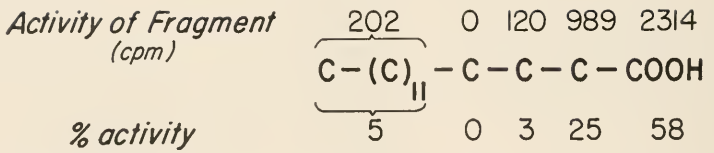

Fig. 7. Distribution of C^{14} in palmitic acid moiety of myristyl palmitate isolated from cultures of *Micrococcus* sp. utilizing tetradecane-1-C^{14} for growth.

cane without change in the carbon atom structure. Total C^{14} activity of cetyl alcohol equalled that of the palmitic acid.

Palmitic acid from myristyl palmitate (derived from tetradecane) represents a somewhat more complex problem. Radioactivity of the myristyl alcohol moiety, presumably labeled in positions 1 and 14 equalled the C^{14} activity of carbons 3 and 16 of palmitate, thus suggesting these fragments arose more or less directly from tetradecane-1-C^{14}. Carbon atoms 1 and 2 of palmitic acid showed considerable labeling - far greater activity than in the remainder of the palmitic acid (Fig. 7). No explanation is available for the high activity in C_1 and C_2 of this palmitic acid but it does seem quite clear that palmitate is formed from a 14-carbon atom unit derived from tetradecane without change in carbon skeleton to which have been added 2 carbon atoms either by condensation of a 2-carbon unit in which considerable C^{14} randomization has occurred, or by successive 1 carbon additions, or by a combination of these two processes. The data give the appearance of some selective process whereby the first acetyl group removed from myristic acid (which would be labeled in the C_1 position) is almost exclusively used to construct the palmitic acid of the ester formed.

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Chapter 43

Pathway of Lower Alkane Oxidation by Pseudomonads

Jacques C. Senez and Edgard Azoulay

The existence of bacteria, yeasts and molds able to metabolize the paraffins was discovered by Söhngen in 1913, and since this pioneer work the repartition of these organisms in natural environments, their systematics and their nutritional requirements have been the subject of an extensive literature which has been reviewed by Zobell (14) and Beerstecher (3). Particularly, it has been shown that the bacteria responsible for the breakdown of hydrocarbons are widespread in the sea where they play an important role in limiting, at least to some extent, the industrial nuisance of water pollution by oil and petroleum wastes.

After the end of World War II, the pathways of the bacteria oxidation of hydrocarbons received an increasing interest and several important contributions to this field have been published, namely by Imelick (4), Treccani *et al.* (13), Kallio and coworkers (11), Thijsse and van der Linden (12). For our own part we have studied the oxidation of lower (C_6 to C_{10}) alkanes by Pseudomonads of marine and terrestrial origin and a brief summary of this work, some preliminary accounts of which have already appeared (1, 7, 8, 9), will be reported in the present contribution.

EXPERIMENTAL OBSERVATIONS

Most of our work has been made with a typical strain of *Pseudomonas aeruginosa* (S 20) which was isolated from soil and produces both pyocyanin and pyoverdin (8). This organism grows profusely on a mineral medium containing a C_6 to C_{16}

alkane as the only carbon and energy source, but does not utilize the branched paraffins nor the aromatic hydrocarbons.

Intermediary Production of Fatty Acids

The resting cells of the studied strain actively consume oxygen in the presence of *n*-heptane. Under these conditions the recorded respiratory quotient is not significantly different from the theoretical value for the complete oxidation of the hydrocarbon into CO_2 and H_2O. However, the production from *n*-heptane of small quantities of acidic products was strongly suggested by the fact that bacterial cultures from this substrate have a distinct red tinge due to the natural pigment pyocyanin in its acidic form.

The chemical identification of the acids formed in the cultures has been achieved as follows (9). Five liters of a five to eight days old culture on *n*-heptane (10 g/L) were centrifuged and the supernatant concentrated to 150-200 ml in the presence

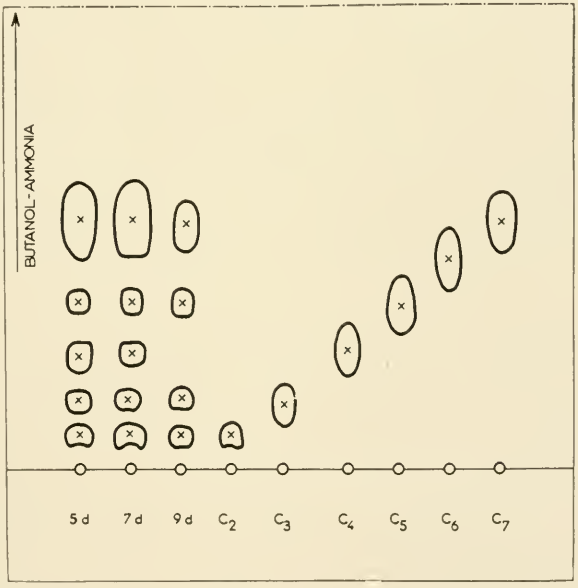

Fig. 1. Chromatogram of fatty acids accumulated in aerobic cultures on *n*-heptane. 5d, 7d, 9d,: steam distillates of cultures after respectively 5, 7, and 9 days of incubation at 32° C; C_2 to C_7: fatty acids standards.

of a large excess of alkali. After reacidification with tartaric acid, the steam distillation of the concentrate yielded a total volatile acid fraction corresponding to 0.55 meq per liter of original culture, i.e., to about 0.1 per cent of the initial hydrocarbon. The steam distillate, concentrated to 10 ml in excess alkali, was chromatographed on Whatman No. 1 paper with butanol-ammonia. On these chromatograms (Fig. 1), the presence of enanthic acid, i.e., of the fatty acid corresponding to the initial alkane, is unequivocal. No caproic acid (C_6) is visible, but the presence of other acids belonging to the even series and namely of butyric acid (C_4), could suggest a breakdown through an α-oxidation pathway.

Results Obtained By the Simultaneous Adaptation Method

Conclusive evidence that the degradation of the fatty acids actually proceeds through a β-oxidation process has been obtained by the method of simultaneous adaptation (10). It was previously observed that the enzymes responsible for the metabolism of both the alkane and the corresponding fatty acid are adaptative. This was demonstrated by the fact that resting cells grown on glucose only consume O_2 in the presence of *n*-heptane or enanthic acid after a lag phase and that no oxygen consumption is observed when chloramphenicol is added.

The simultaneous adaptations of the resting cells have been determined in the presence of chloramphenicol (50 μg/ml), in order to supress any secondary adaption on the course of the manometric experiments. The results obtained under these experimental conditions are very significant. From the data recorded in Table 1, it is clear that the cells grown on *n*-heptane are simultaneously adapted to the corresponding fatty acid and to the lower acid belonging to the odd series, but not to be corresponding dicarboxylic acid (pimelic) nor to octanoic and hexanoic (caproic) acids. Conversely, the cells grown on an even alkane are simultaneously adapted to the corresponding and lower acids of the same series, and are not adapted to the acids of the odd series.

The experiments reported in Table 2 show that the cells grown on *n*-heptane are adapted to the corresponding mono-

TABLE 1

OXIDATION OF FATTY ACIDS BY CELLS GROWN ON VARIOUS ALKANES

Substrate	— QO_2* of Cells Grown on: *n-hexane* (C_6)	*n-heptane* (C_7)	*n-octane* (C_8)	*n-decane* (C_{10})
Corresponding alkane	163	175	116	88
Caprylic acid (C_8)	—	0	153	12
Enanthic acid (C_7)	—	175	15	0
Caproic acid (C_6)	109	12	110	20
Valerianic acid (C_5)	0	84	15	0
Butyric acid (C_4)	207	132	165	80
Propionic acid (C_3)	0	42	97	8
Acetic acid (C_2)	207	138	150	28
Formic acid (C_1)	—	0	—	—
Pimelic acid (C_7)	—	0	—	—

*Activity measured manometrically at 37 C, pH 7.1, in the presence of chloramphenicol (50 μg/ml) and expressed in μL O_2 consumed per hour, per mg of cells (dry weight). Endogenous activities ($-Q_{O_2}$ less than or equal to 10) subtracted.

alcohol and aldehyde. Moreover, these experiments clearly demonstrate the sequence of the intermediary steps between the hydrocarbon and the fatty acid. It is seen that the cells grown on the primary alcohol are only adapted to this substrate and to the aldehyde and acid, and that the cells grown on the aldehyde are only adapted to the aldehyde and to the acid.

These observations are consistent with a metabolic pathway in which the oxidation of the hydrocarbon molecule takes

TABLE 2

SIMULTANEOUS ADAPTATIONS OF THE CELLS GROWN ON *n*-HEPTANE AND ON THE CORRESPONDING ALCOHOL AND ALDEHYDE

Substrate	*-Q_{O_2}* of the Cells Grown on: *n-heptane*	*1 n-heptanol*	*n-heptanal*
n-heptane	175	9	0
1 *n*-heptanol	175	184	0
n-heptanal	150	169	142
enanthic acid (C_7)	120	136	135

*Same conditions as in Table 1.

place on a single terminal carbon with successive formation of the mono-alcohol, aldehyde and fatty acid, then proceeding further through a β-oxidation mechanism.

The demonstration of a β-oxidation for the higher (C_5 to C_{10}) fatty acids is quite conclusive. However, it must be noticed that the cells grown on hexane and on heptane are respectively adapted to propionate (C_3) and to butyrate (C_4). This is correlated with the observed accumulation of butyrate with bacteria cultivated on heptane (Fig.1). As suggested by Thijsse and van der Linden (12) propionic and butyric acids most likely do not enter into the specific pathway of alkane oxidation and are formed through some independent "side" metabolism.

Initial Step of Alkane Oxidation

Cell free extracts have been prepared from heptane grown cells by grinding in the cold with alumina as already described (7). The dehydrogenase activities of these preparations were measured in Thunberg tubes under vacuum with purified pyocyanin as the final electron acceptor, the quantities of pyocyanin reduced to its leuco-form being determined colorimetrically from the absorbance of this pigment at 380 mμ (A_5 = 17.2).

The results recorded in Table 3 show that the cell free extracts actively reduce pyocyanin in the presence of *n*-heptane or 1 *n*-heptanol. The reduced form of the pigment is strongly fluorescent in U.V. and recolorizes immediately to blue upon the introduction of air into the systems. A sharp optimum of activity has been observed at pH 7.3.

The crude extracts have a high DPNH oxidase activity, which is 75 per cent inhibited by mercapto-ethanol, 0.03 M. In the presence of this compound, the extracts reduce DPN, but not TPN, at the expense of *n*-heptane, 1 *n*-heptanol and heptanal (Table 3). No activity is observed when the extract has been previously boiled or when heptane is omitted. Furthermore it is interesting that in the absence of mercapto-ethanol (Fig. 2, curve 2), DPN is not appreciably reduced by heptane. This seems to indicate that mercapto-ethanol not only inhibits the interfering activity of DPNH-oxidase, but even more activates

TABLE 3

DEHYDROGENASE ACTIVITIES OF EXTRACTS FROM HEPTANE GROWN CELLS

Substrate	*Hydrogen Acceptor*	*Activity* *
n-heptane	Pyocyanin	0.58-0.70
n-heptane	DPN	0.40-0.55
n-heptane	TPN	0.075
1 *n*-heptanol	Pyocyanin	0.95
1 *n*-heptanol	DPN	0.60
n-heptanal	DPN	0.80

* Measured at 23°C and expressed in μmoles of the acceptor reduced per hour, per mg. of nitrogen. For DPN and TPN reduction, systems containing 0.03 M mercaptoethanol.

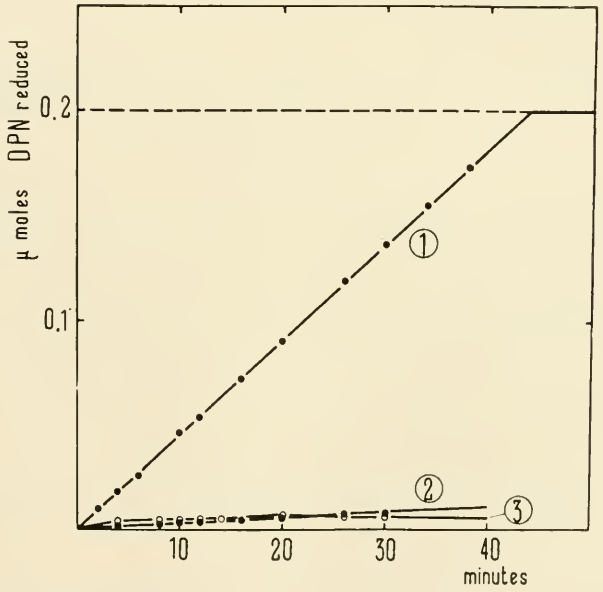

Fig. 2. Reduction of DPN by cell free extracts. Quantities of reduced DPN determined at 340 mμ, Curve (1): complete system containing bacterial extract (0.8 mg. protein N), DPN 0.2 μmole, *n*-heptane 0.1 ml., mercaptoethanol 0.03 M. Curve (2): mercapto-ethanol omitted; curve (3): *n*-heptane omitted.

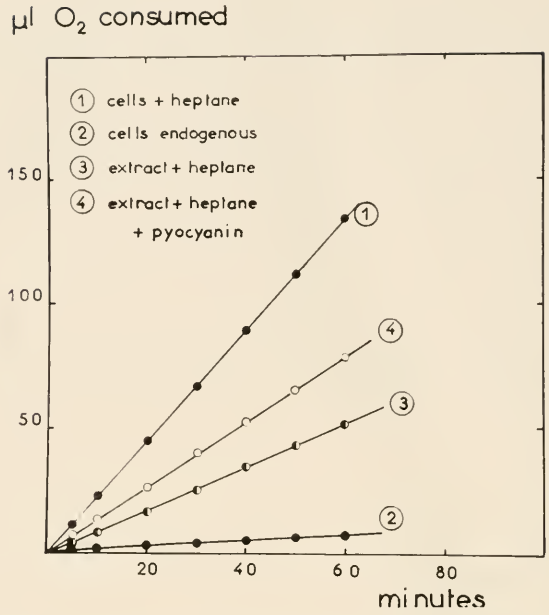

Fig. 3. Respiratory activities of intact bacteria and cell free extracts. Experimental conditions: t°: 37° C; pH 7.1 (phosphate buffer, 0.05 M); ± *n*-heptane 0.1 ml, ± pyocyanin 0.7 mg.

the hydrocarbon dehydrogenase, as previously observed by Jakoby (5) for the aldehyde dehydrogenase of *P. fluorescens*.

The crude extracts contain a complete respiratory system and in aerobiosis actively consume O_2 in the presence of *n*-heptane (Fig. 3). The respiratory activity of the extracts is low in comparison with the intact cells (Table 4) and consistently increased by the addition of either DPN or pyocyanin. This fact confirms the role of DPN and pyocyanin in the transfer of hydrogen from heptane and suggests that the activity of cell free extracts is partly limited by the concentration of the electron carriers.

From these data, it is clear that the primary attack of the alkane molecule consists in an anaerobic dehydrogenation coupled with either DPN or pyocyanin. Moreover, the dehydrogenation of 1-heptanol and heptanal by the extracts provides additional evidence that these compounds are intermediates in alkane

TABLE 4

RESPIRATION OF RESTING CELLS AND EXTRACTS IN THE PRESENCE OF *n*-HEPTANE (t°:37°C)

Addition	$-Qo_2$ *Cells*	*Extracts*
none	56	15
+Pyocyanin (700 μg)	56	27
+DPN (700 μg)	56	24

oxidation. Concerning this last point, similar observations have been made by Baptist and Coon (2) who have prepared from a soil bacterium grown on *n*-octane cell free extracts oxidizing this hydrocarbon to octanoic acid and containing a DPN dependent *n*-octanol dehydrogenase.

The product of heptane dehydrogenation has not yet been identified by chemical analysis. However, one may assume that it is most likely the corresponding olefin and consequently the whole pathway of alkane oxidation by *P. aeruginosa* can be tentatively represented as shown in Figure 4.

DISCUSSION

The existence of a β-oxidation pathway had been previously suggested by Treccani *et al.*(13) and by Thijsse and van der

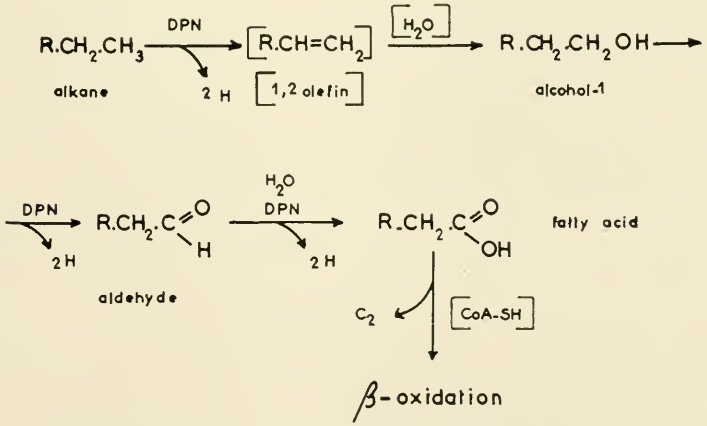

Fig. 4. Proposed scheme for *n*-alkane oxidation by *Pseudomonas aeruginosa.*

Linden(12) from experimental evidence obtained by the simultaneous adaptation method. These investigators noticed, however, that the cells grown on a given alkane had a small but not negligible activity towards the fatty acids with one more or one less carbon atom, so that the parallel occurrence of an α-oxidation process could not be conclusively eliminated. In the case of our strain of *P. aeruginosa* tested in conditions where any secondary adaptation was supressed by chloramphenicol, the existence of an exclusive β-oxidation is clearly demonstrated.

The initial dehydrogenation of *n*-heptane is of special interest and deserves some comments. Studying the oxidation of cyclo-hexane by several strains of *Pseudomonas*, Imelick (4) has obtained some experimental evidence that the first oxidation step in the case of this substrate consists in a direct oxygenation with the production of a hydro-peroxide. More recently, Stewart *et al.* (11) have made similar observations with an unidentified Gram-negative coccus which grows on *n*-hexadecane with the accumulation of cetyl-palmitate. From the incorporation and repartition of 0^{18} into the products, these investigators have concluded that the first intermediate is 1-hexadecyl-hydroperoxide.

Thus, it appears that there are two different mechanisms for the initial attack of the paraffin molecule, one of them involving a direct participation of atmospheric oxygen, and the other starting with an anaerobic dehydrogenation. It may be that the second mechanism is restricted to the lower alkanes of less than 10 carbon atoms. This interpretation is supported by the fact that the cells of our *P. aeruginosa* strain grown on hexadecane and the cell free extracts prepared from these bacteria do not reduce anaerobically pyocyanin in the presence of hexadecane and are apparently devoid of the corresponding dehydrogenase.

It must be stressed that the alkanes lower than C_{10} are slightly soluble in water. At 16 C, the water solubility per liter is still 130.8mg for *n*-hexane and 15 mg for *n*-octane. Moreover, previous experiments (1) have demonstrated that *P. aeruginosa* is able to grow from *n*-heptane dissolved into the water phase of the media. In the case of cyclo-hexane and *n*-hexadecane, which are quite insoluble, the bacterial attack must obviously take place at the surface of the hydrocarbon particles dispersed into

the medium and this observation is possibly correlated with the fact that these substrates are oxidized through a different pathway.

Concerning the role of pyocyanin as electron acceptor and carrier for the hydrocarbon dehydrogenases in *P. aeruginosa*, it was observed previously by Ramakrishnan and Cambell (6) that this natural pigment is a very active electron acceptor for the gluconic dehydrogenase of the same organism. Unpublished experiments recently made in our laboratory have given preliminary evidence that pyocyanin takes place in the physiological electron transfer between DPNH and cytochrome *c*, as demonstrated by the fact that pyocyanin increases the activity of DPNH-cytochrome *c* reductase in sub-cellular particles.

ADDENDUM

Since this contribution was presented, the anaerobic formation of *n*-1 heptene from *n*-heptane has been demonstrated by infra red spectrophotometry, in collaboration with Dr. J. Chouteau. Details of this work have been published elsewhere. (*Nature*, London, *194*:576, 1962).

SUMMARY

Experimental evidence obtained by the simultaneous adaptations method show that *Pseudomonas aeruginosa* oxidizes the lower (C_6 to C_{10}) paraffins through an adaptive pathway involving successive formation of the corresponding mono-alcohol, aldehyde and fatty acid, and then proceeding further by a β-oxidation mechanism.

Cell-free preparations obtained from cells grown on *n*-heptane contain DPN dependent dehydrogenases for the corresponding alkane, mono-alcohol and aldehyde. These dehydrogenases are equally active with the natural pigment pyocyanin as the electron acceptor.

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Chapter 44

Degradation of 2-Methylhexane by a *Pseudomonas*

A. C. Van Der Linden and G. J. E. Thijsse

In recent years our knowledge of bacterial metabolism of alkanes has been greatly enlarged. As late as 1956, at the Symposium on Petroleum Microbiology, Kallio had to inform the audience of the "murky state of the problem" and further stated that - in the absence of positive identification of intermediates - "any discussion of mechanisms was premature (1)."

At present most investigators will agree on the following major principles of alkane oxidation:

1) *n*-Alkanes are oxidized far more easily than branched ones.

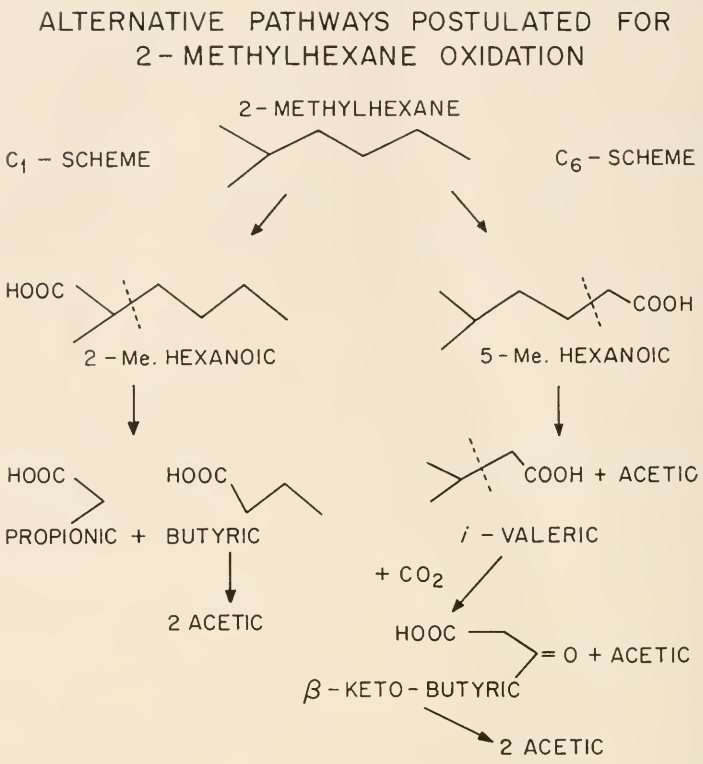
2) The predominant alkane oxidation system attacks the molecule at a terminal methyl group.

3) The corresponding fatty acids are formed via the primary alcohol and the aldehyde.

4) The fatty acids are broken down by β-oxidation, excluding participation of a decarboxylation reaction.

This distinct progress in our knowledge prompted us to extend our work to the branched paraffins. These hydrocarbons generally contain non-equivalent methyl groups, and it would be interesting to see what is the preferred oxidation route. Consequently, an investigation into 2-methylhexane oxidation has been started, the central question being whether the oxidation of 2-methylhexane starts at C_1 or - as we must expect from the high oxidation rates of *n*-alkanes - at C_6.

Applying existing knowledge in the field of methyl-substituted fatty acid metabolism (3) we may postulate two degradation schemes, *viz.* a "C_1-scheme" and a "C_6-scheme."



Inspection of these two schemes shows that the C_1-scheme is characterized by the occurrence of 2-methyl-hexanoic and propionic acid, whereas 5-methylhexanoic and *iso*-valeric acids are typical for the C_6-scheme.

MULTIPLE ADAPTATION EXPERIMENTS

Using the multiple adaptation principle we have investigated whether the 2-methylhexane-grown cells were adapted simultaneously to 2-methylhexanoic and propionic acids, or to 5-methylhexanoic and *iso*-valeric acids. For comparison, the oxidation rates of the same acids by *n*-heptane-grown cells were also determined.

n-Heptane metabolism by our *Pseudomonas* strain had been investigated earlier and the pathway was shown to include heptanoic, *n*-valeric and propionic acids by multiple adaptation tech-

niques (4) as well as by isolation of the fatty acids and their mass-spectrometric identification (2).

The micro-organism used throughout all our studies was isolated from brackish harbour water and is believed to be closely related to, or identical with *Ps. aeruginosa* (4).

Some of the results of the multiple adaptation experiments are presented in Table 1.

TABLE 1

Q_{O_2} WITH VARIOUS FATTY ACID SUBSTRATES OF PS. CELLS GROWN ON 2-METHYLHEXANE AND *n*-HEPTANE

Substrate	*Ps Adapted to* 2-*Me. hex.*	*n-Hept.*	*Pref. for Scheme*
Propionic	42	118	C_6
Iso-valeric	100	58	C_6
2-Me. hexanoic	80	47	C_6
5-Me. hexanoic	132	47	C_6

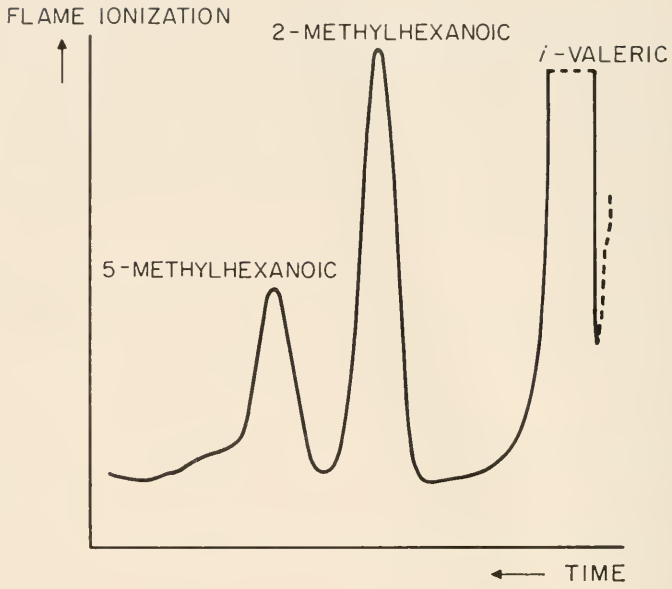
As expected the figures show a high Q_{O_2} for propionic acid oxidation by heptane-grown cells. Slow propionic acid oxidation by 2-methylhexane-grown cells can be explained by the assumption that the C_6-pathway is followed in 2-methylhexane oxidation, which pathway does not incorporate propionic acid. This conclusion is substantiated by a high Q_{O_2} for *iso*-valeric acid, which indeed is an intermediate in the C_6-scheme.

The two methyl-substituted hexanoic acids are only slowly oxidized by the heptane-grown cells, which, of course is not astonishing. The 2-methylhexane-grown cells clearly show preference for the 5-methyl isomer and thus, again the C_6-pathway seems favoured by the micro-organism.

The oxidation of 2-methylhexanoic acid is, however, not negligible (Q_{O_2} = 80 versus Q_{O_2} = 47 for the heptane-grown cells). A conclusion that the 2-methylhexane molecule is also oxidized at C_1 is, however, not warranted on the basis of multiple adaptation only.

FATTY-ACID ISOLATION AND IDENTIFICATION

The conclusions from the multiple adaptation experiments are well supported by the results of fatty acid identification.



CHROMATOGRAM OF FATTY ACIDS FROM 2-METHYLHEXANE INCUBATION WITH HEPTANE-GROWN *PSEUDOMONAS* CELLS

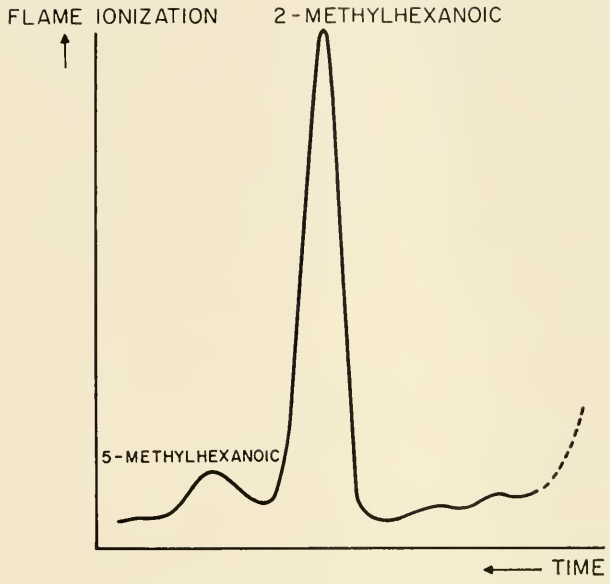
61.03.1090

FIGURE 1

n-Heptane-, as well as 2-methylhexane adapted cells were incubated with 2-methylhexane in one-liter flasks containing 200 ml of mineral medium. The flasks were stoppered and the oxygen supply, therefore, was restricted. After sixteen hours of incubation the cells were harvested and the liquid analysed for traces of fatty acids by gas-liquid chromatography of their methyl esters.

Figure 1 shows the recorder tracing in an experiment where heptane adapted cells were used.

Three peaks were identified by addition of model compounds, *viz.* 2-, and 5-methylhexanoic acids as well as *iso*-valeric acid. Consequently, we must assume that heptane-adapted cells oxidize the C_1 as well as the C_6-terminal methyl group. The *iso*-valeric acid must have arisen from β-oxidation of 5-methylhexanoic acid and - in addition - we know from the multiple adaptation experi-



CHROMATOGRAM OF FATTY ACIDS FROM
2-METHYLHEXANE INCUBATION WITH
2-METHYLHEXANE-GROWN *PSEUDOMONAS* CELLS

61.03.1091

FIGURE 2

ments quoted above - that *iso*-valeric acid is hardly oxidized by heptane-adapted cells.

Figure 2 shows a chromatogram of fatty-acid methyl esters obtained from 2-methylhexane incubation with 2-methylhexane-adapted cells.

It is seen that here no *iso*-valeric acid has accumulated, presumably because of its high oxidation rate.

However, both methyl-substituted hexanoic acids were found to be present. The presence of the 2-methyl derivative proves that both the 2-methylhexane-adapted cells and the heptane-adapted ones can oxidize C_1. Decrease of the ratio of the 5-methyl- to the 2-methyl isomer as compared with Figure 1 further demonstrates the increased oxidation of 5-methylhexanoic acid by cells adapted to 2-methylhexane. This also is quite in line with the multiple adaptation experiments.

Summarizing, we may conclude from the analytical experi-

ments that in 2-methylhexane oxidation by 2-methylhexane-grown *Pseudomonas* cells both pathways are actually followed. From the ratio of the isolated methyl-substituted hexanoic acids it further follows that the C_6-pathway is the predominant one. This conclusion closely agrees with the results of the multiple adaptation experiments, which, e.g., by the slow observed rate of propionic acid oxidation - suggest that the C_1-pathway is of minor importance only.

A full account of this study, including experimental details, appeared elsewhere (5).

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Chapter 45

Studies on the Stability of the Na^+ Requirement of Marine Bacteria

Robert A. MacLeod and E. Onofrey

Much information has accumulated indicating a preference of marine bacteria for sea water over fresh water in the medium for growth. Korinek (2) believed that marine bacteria could be distinguished from non-marine forms on the basis of their salt tolerance. Dianova and Voroshilova (1), using a fish broth medium, observed that Na^+ salts were required for the growth of a number of marine isolates and could not be replaced by equimolar concentrations of K^+ salts. Zobell and Upham (8) defined marine bacteria as bacteria from the sea which on initial isolation require for growth a medium containing sea water as the diluent. In a study of the relation of sea water to the growth of a number of marine bacteria, MacLeod and Onofrey (4) using chemically defined media found that the need for sea water was due to its ability to supply the inorganic ions required for growth by the organisms. A study of the qualitative and the quantitative requirements of the marine bacteria for inorganic ions revealed among other things an almost unique and highly specific requirement for Na^+. This requirement for Na^+ could not be replaced in whole or to any significant extent in part, either by any one of a number of related inorganic ions or by organic compounds added to increase the osmotic pressure of the medium. It seemed probable that true marine bacteria could best be distinguished from land contaminants present in sea water by the possession of this need for Na^+.

Evidence has been presented which suggests that the requirement of marine bacteria for sea water or for Na^+ may not be a stable one. Zobell and Michener (7) found that nine of twelve cultures requiring sea water in the medium on initial isolation

grew in the same medium prepared with fresh water, after the cultures had been held for five months without transfer. It was subsequently reported by Zobell (9) that 56 of 60 species of marine bacteria had developed a capacity to grow in fresh water media. Paradoxically, attempts to train the original cultures of these organisms to grow at lower sea-water concentrations met with only limited success. Since then, Stanier (6), MacLeod and Onofrey (4), and Littlewood and Postgate (3) have all reported difficulty or failure to train marine bacteria to grow at lowered sea-water or salt concentrations. In 1959, Pratt and Waddell (5) reported the selection of what appeared to be mutants of a marine bacterium on a trypticase medium prepared without added NaCl but containing the other ions of sea water.

All of the reports of the growth of marine bacteria in media prepared without sea water or NaCl have been based on observations made using non-chemically defined media which would be expected to be contaminated with, in some cases, relatively high concentrations of Na^+ as well as other inorganic ions. Since the requirement of marine bacteria for Na^+ is the one feature of their cultural requirements which distinguishes them most readily from land forms, and since the evolution of these bacteria to land forms would represent the development of a capacity to grow without Na^+, it was of interest to know more about the stability of the Na^+ requirement. Attempts have therefore been made to train and to produce mutants of marine bacteria that will grow in media prepared with chemically pure components containing at the most only traces of Na^+ as a contaminant.

Three organisms of marine origin were studied initially - B_9, a *Flavobacterium*, B_{10} a *Pseudomonad* and B_{16}, a *Pseudomonad* or *Spirillum*. The qualitative and quantitative inorganic requirements of these three organisms were studied previously and have been reported. A salt solution containing Na^+, K^+, Mg^{++}, Ca^{++} and Fe^{++} salts and providing amounts of the ions known to be required for growth by the organisms has been used as the diluent for the various media. Trypticase medium was prepared by making this salt solution 1 per cent with respect to B.B.L. typticase. Synthetic medium was made by adding glucose and the three amino acids, alanine, aspartic and glutamic acids to the salt solution. When

solid media were required, 1.5 per cent agar was added. Special precautions were taken to purify the chemicals used in the preparation of the chemically defined medium. All glassware was specially washed in a mixture of concentrated HNO_3 and H_2SO_4 and then rinsed with glass distilled water. In running responses of the organisms to Na^+ the usual procedures of microbiological assay were used.

Efforts were first made to train cultures to grow either at lower Na^+ concentrations or in the absence of added Na^+ in the chemically defined medium.

We have shown previously that Na^+ affects both the rate and extent of growth of marine bacteria, Table 1. Organism B-16 which grew rapidly and to the maximum extent in the presence of 0.20M Na^+, also grew at 0.03M Na^+, though not maximally, after a sufficiently long incubation period.

TABLE 1

THE RESPONSE OF MARINE BACTERIUM B-16 TO Na^+ IN A Na^+ DEFICIENT MEDIUM (FROM MACLEOD AND ONOFREY, 1957)

Na^+[1] M	*Incubation Time (Hrs.)* 48	72	120	312
	Percent Incident Light Transmitted[2]			
0	100	100	100	100
0.01	100	100	100	100
0.03	100	100	100	44
0.05	100	100	89	—
0.07	98	39	35	—
0.10	86	39	46	—
0.13	34	30	60	—
0.20	26	30	52	—
0.30	25	34	51	—

[1] Added as NaCl.

[2] Evelyn colorimeter readings, 660 mμ filter. Uninoculated medium = 100.

In the present study, efforts were made to acclimatize cultures to grow at still lower Na^+ concentrations by serially subculturing into media containing Na^+ lowered progressively in steps of 0.005M. Organism B-16 which grew initially, after long incubation at 0.03M Na^+ could not be induced to grow at less than 0.02M Na^+. At this Na^+ concentration, the organism became pro-

gressively more feeble on being successively transferred into media containing the same low Na^+ concentration. On the fourth or fifth transfer at the 0.02M Na^+ level growth failed to occur. The other organisms behaved similarly. This observation is in agreement with that of other investigators who have also failed to train marine bacteria to grow at appreciably lowered sea water or Na^+ concentrations.

By plating heavy suspensions of marine bacteria on trypticase medium prepared without added Na^+, Pratt and Waddell (5) obtained a few colonies which they assumed were mutants of marine bacteria which no longer required Na^+ for growth.

We were unable to obtain colonies of our organisms under similar conditions but found that one of the organisms, B-16, could be trained to grow well on the trypticase agar medium containing no added NaCl if it was streaked serially onto the surface of plates of the medium containing progressively lower concentrations of Na^+.

A flame photometric analysis of the trypticase medium prepared without added NaCl revealed a concentration of 0.028M Na^+ present as a contaminant. By comparison, the chemically defined medium used in these studies contained only 6.5×10^{-5}M Na^+.

Since the culture of organism B-16 adapted to grow in the trypticase medium without added NaCl was actually growing at

TABLE 2

Comparison of Na^+ Response of B-16 and of B-16 Adapted to Growth on Trypticase Agar Without Added Na^+

Na^+ M	*B-16*			*Adapted B-16*		
	Incubation Time (Hrs.)					
	65	120	168	65	120	168
	*Per Cent Transmission**					
0	100	100	100	100	100	100
0.01	100	100	100	100	100	100
0.03	100	100	100	100	100	31
0.05	100	85	46	99	55	70
0.10	85	28	—	38	58	—
0.20	26	48	—	35	—	—

* Evelyn colorimeter readings, 660mμ filter.

about the same Na^+ level as the unadapted culture would grow after long incubation in the chemically defined medium, it was of interest to know how the Na^+ requirement of the adapted and unadapted cultures compared. The results, Table 2, show that the adapted culture, like the unadapted organism, still required Na^+ when tested in the synthetic medium, though the adapted one did grow a little more quickly and at a slightly lower Na^+ concentration than the unadapted parent.

We also found that the organisms which eventually grew after long incubation in the chemically defined medium at a level of 0.03M Na^+ also grew without training when transferred to the trypticase medium prepared without added Na^+.

When the synthetic medium used in this study was solidified with 1.5 per cent agar, the chief source of Na^+ contamination was the agar added. Such a medium contained about 0.007M Na^+. The plating of heavy suspensions of the marine bacteria (2 to 4 x 10^9 organisms) on this medium failed to produce colonies unless Na^+ was added. Efforts were therefore made to induce mutation to growth on this medium by ultraviolet irradiation. The results of one such experiment are recorded in Table 3. Irradiated suspensions of organism B-16 plated both immediately and after a period of incubation under conditions designed to produce a limited number of cell divisions, when plated on the chemically-defined medium without added Na^+ gave rise to a very limited number of colonies after three weeks of incubation.

Because we were endeavouring to produce organisms which

TABLE 3

Induction of Mutants of Marine Bacterium B-16 by Ultra-violet Irradiation

Irradiation time	*Survival Per Cent*	*No. of Viable Organisms Plated*	*No. of Mutants*
5 min.	22	1.62 x 10^9	2
5 min. followed by incubation		1.1 x 10^8	4
7 min.	3	2.7 x 10^9	1
7 min. followed by incubation		4.0 x 10^7	1

did not require Na^+ from organisms which did and since non-sodium requiring organisms are prevalent in the laboratory environment, special precautions had to be taken to avoid contamination. Cell suspensions were irradiated in a sterile quartz flask - which accounts for the length of the irradiation time required to achieve an appreciable kill. I may also say that many attempts were made to increase the yield of mutants by increasing the irradiation dosage, but none of these attempts succeeded. We either got no mutants or a few which failed to survive on subsequent transfer.

The response of three of these mutants to Na^+ in a chemically defined medium prepared with purified components is compared with that of the parent culture, Table 4. It is evident that whereas the original culture failed to grow in the absence of Na^+, the three isolates grew appreciably under these circumstances. All three however, grew more extensively when Na^+ was added to the medium.

TABLE 4

Comparison of the Na^+ Response of Organism B-16 and Three Mutants Derived From it by Ultra-violet Irradiation

Na^+ M	*B-16*			*Mutant A_2*			*Mutant B_3*			*Mutant C_1*		
	Incubation Time (Hrs.)											
	22	72	192	22	72	192	22	72	192	22	72	192
	*Per Cent Transmission**											
0	100	100	100	45	39	27	53	45	34	53	33	41
0.01	100	100	100	39	34	28	32	20	21	31	25	24
0.02	100	100	100	32	20	20	38	27	27	32	25	25
0.03	100	100	98	31	19	19	40	27	24	38	33	26
0.05	100	100	57	32	25	24	47	35	29	47	44	39
0.10	100	28	66	53	42	30	79	46	36	48	46	40
0.20	95	30	76	96	93	59	93	88	79	97	94	64

* Evelyn colorimeter readings, 660 mμ filter.

A total of 14 isolates have been obtained by ultraviolet irradiation (capable of growing) in the chemically defined medium without Na^+ added. These were compared morphologically and in biochemical tests employing standard methods with the original culture of organism B-16 and with B-16 adapted to grow

in trypticase agar prepared without Na^+. Morphologically the isolates were all found to be gram negative pleomorphic motile rods indistinguishable from the parent culture. A summary of the results of a few biochemical tests is presented in Table 5. All of the cultures behaved in the same way except in the gelatin liquefaction and nitrate reduction tests. None of the derived cultures was capable of liquefying gelatin either in the presence or absence of Na^+, though the original culture was an active gelatin liquefier. Eight of the fourteen isolates behaved like the parent culture in being unable to reduce nitrate, while three reduced it slightly and three gave a strong positive test. Another difference between the parent and the cultures derived by U. V. irradiation lay in their capacity to autolyze. It is typical of organism B-16 and other marine bacteria examined here that once growth has ceased in a culture the cells autolyze rapidly on further incubation. The mutant cultures, however, did not autolyze detectably under the same conditions even when Na^+ was added. The culture adapted to grow in the trypticase medium without added NaCl was indistinguishable from the parent in its reactions in all of the tests.

Since ultraviolet-induced mutants of organism B-16 still

TABLE 5

Comparison of Results of Biochemical Tests on Marine Bacterium B-16, Adapted B-16 and 14 Isolates Derived From B-16 by Ultra-Violet Irradiation

Organism	*No. of Isolates*	*Gelatin Liquefaction*	*Nitrate Reduction*	*Indole*	*H_2S*	*Starch Hydrolysis*	*Citrate Utilization*	*Carbohydrate Fermentations*[3]
B-16	1	+++[1]	−	−	+	+	+	−
Adapted B-16	1	+++[1]	−	−	+	+	+	−
Mutant A	8	−[2]	−	−	+	+	+	−
Mutant B	3	−	+	−	+	+	+	−
Mutant C	3	−	++	−	+	+	+	−

[1] Stratiform.

[2] One of the eight isolates demonstrated slight stratiform liquefaction. The cells in all sixteen cultures were morphologically indistinguishable and were gram negative, motile rods.

[3] Acid and gas formation negative from glucose, arabinose, maltose, sucrose, dulcitol, lactose.

responded to added Na^+ in a Na^+ deficient medium, it was of interest to know if this residual requirement for Na^+ for maximum rate and extent of growth could be eliminated by training. Three cultures were selected for study. They were representative of the three types of mutants obtained - those which did not reduce nitrate, those which did slightly and those which did strongly. These were each transferred serially into flasks of chemically defined medium containing no added Na^+. The inoculum at each transfer was also used to inoculate a series of flasks of the same medium containing increasing concentrations of Na^+ to keep a check on the response of the organism to Na^+ after each serial transfer. The results, Table 6, show that by the eighth transfer all three cultures grew to the same ultimate extent in the presence and in the absence of added Na^+. Two of the isolates still responded slightly to the addition of 0.01M Na^+ in the early stages of growth but the third, C, did not.

TABLE 6

Results of Experiments Designed to Train Three Mutants to Grow as Well in the Absence of Na^+ as in its Presence

Na^+	*Mutant A-2*				*Mutant A-8*				*Mutant C-1*			
M	*No. of Transfers*											
	1		8		1		8		1		8	
	Incubation Time (Hrs.)											
	24	90	23	88	24	90	25	74	26	72	23	88
	*Per cent Transmission**											
0	97	47	70	26	93	60	95	20	71	34	56	28
0.01	83	30	57	24	58	26	78	21	51	30	63	30
0.03	93	31	68	25	69	26	94	19	52	30	66	29
0.05	94	32	79	25	81	30	95	20	60	39	74	29
0.10	95	42	93	27	97	46	100	30	78	48	84	30

* Evelyn colorimeter readings, 660 mμ filter.

The responses of two organisms of terrestrial origin to Na^+, *Pseudomonas aeruginosa* 9027 and a strain of *E. coli*, were also determined in a chemically defined medium. Maximum growth of these organisms occurred in the absence of Na^+ and no stimulation of the rate of growth of the organisms was observed on addition of Na^+.

The findings reported here indicate that the Na^+ requirement of the marine bacteria examined is a very stable one. Mutants, however, have been derived from one culture, though with difficulty, which have a capacity to grow either in the absence of Na^+ or at least at very low levels of Na^+. This finding provides some support for the hypothesis that land and fresh-water bacteria could have evolved from marine bacteria through mutations giving rise to organisms with a capacity to grow either in the absence of Na^+ or at the low levels of Na^+ which would be expected to be present as a contaminant in most terrestrial environments.

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Part 5
Distribution and Function of Marine Bacteria

Chapter 46

The Immediate Tasks of Marine Microbiology

A. E. Kriss

The extension of microbiological investigations in various seas and oceans from the ecological and geographical points of view is one of the primary tasks of marine microbiology. For its speedy fulfilment, it is necessary to ensure a more systematic participation of microbiologists in oceanographical expeditions.

The present scale of microbiological studies in seas and oceans cannot be regarded as sufficient. They are still sporadic in character, being carried out by a small number of scientists and lagging behind the work expended on other oceanographical disciplines. Oceanographical sections and surveys, basic methods for the comparative study of the horizontal and vertical distribution of the physical, chemical and biological elements of the environment in various areas of seas and oceans has, so far, not been fully exploited by microbiologists, who usually work only on isolated stations.

In addition to the organization of systematic microbiological studies on stationary bases, which can include only the sea not more than some dozens of miles from the shore, expeditions should be made during various times of the year, traversing the main areas of the sea, in order to elucidate the seasonal changes in the biomass of microorganisms in the coastal as well as the central areas of the sea. Daily stations, constituting a part of the practice of oceanographical expeditions, should also serve the task of studying the dynamics of the microbial populations.

Estimates of the biomass of microbial populations of seas and oceans or their separate parts, however, even when sampling is sufficiently frequent, do not produce a true picture of the dynamics of the living cells, if the reproduction rates of microorganisms under the conditions of their natural habitat are un-

known. All we know so far about the rate of reproduction of bacteria is based on experiments with laboratory cultures. Only recently we have worked out a method for estimating the time required for the division of a bacterial cell under natural conditions. It is true that it is not completely flawless, but at present, it is better than the methods used in hydrobiology for the quantitative estimates of the production of animal and plant organisms.

It is now possible to determine the value for the coefficient, expressing the ratio of the production of microorganisms to their biomass (P:B) in the sea. Due to the differences in the conditions existing in various parts of seas and oceans and also to fluctuations in these conditions with the season of the year in the same localities, it is necessary to carry out systematic studies of the rate of reproduction of microorganisms in the coastal and open areas of the seas and oceans. Such studies will clarify the process of "turnover" of living matter in the form of microbial cells throughout the water and on the bottom of seas and oceans within an annual cycle or several years, and find out the amount of mineralization of dead organic matter and of utilization of microorganisms by aquatic animals.

In this connection it is extremely important to ensure the development of one of the important branches in the hydrobiological sectors of marine microbiology, namely, that of determining the part played by microorganisms as a direct link in the food chains in the sea. Studies in this field have been conducted in the field only recently. After it was demonstrated that growth and reproduction of a number of aquatic animals can take place exclusively on a microbial diet, a logical question was raised, whether microorganisms are used as food and, if so, to what extent in natural habitats in which various food material exists.

For this purpose investigations were carried out under natural conditions with some representatives of the bottom fauna in the Caspian Sea. With the aid of specially developed methods it has been shown, for example, that about half the microbial cells found in mud are digested by *Nereis succinea* during the passage of the mud through the alimentary canal of that worm. This case is offered as an example of a food chain,

microorganisms - nereids - fish. At present, the possibility of the existence of shorter food chains is not excluded, characterizing the nutritional importance of microorganisms at early stages in the development of fishes.

Much remains to be done to clarify the complicated and varied inter-relations between microorganisms and phytoplankton, phytobenthos, zooplankton and zoobenthos in marine habitats. Only very broad lines have so far been drawn, but even those lines need confirmation and correction. In the past, only the relations between marine animals and plants and comparatively small groups of microorganisms belonging to heterotrophs, which are able to develop only on the easily assimilable organic matter have been studied. The use of direct methods opens new possibilities in the study of these phenomena. The immediate task, of interest also to planktonology, is a study of the dynamics of microbial populations in connection with biological processes of such importance in the seas and oceans as the diurnal migrations of zooplankton and the "blooming" of phytoplankton.

The progress of marine microbiology in explaining the regularities underlying distribution of many biogenic substances in seas and oceans is also still very insufficient. The obvious part played by microorganisms in the biocatalytic changes of organic and inorganic matter on land and in the ocean prompts hydrochemists and geochemists to expect from microbiology an explanation of the causal relationships which determine the existing regularities in distribution of a number of chemical compounds. However, the data of marine microbiology until recently were often of a purely qualitative character. It has been established that in the water, and on the bottom of the seas, and in the coastal areas of the oceans are found representatives of various physiological groups of microorganisms, taking direct part in the carbon, nitrogen, phosphorus, sulphur, iron, manganese, calcium cycles, but there are only few data as to what numbers of these microbial forms are present and how active they are.

Wide scale investigations of the distribution of physiological groups of microorganisms and the degree of their activity were carried out in the Black Sea. They were to elucidate the reasons

for the peculiar vertical distribution of ammonia, nitrates, nitrites, hydrogen sulphide, thiocompounds and phosphates in the sea. Due to the use of the comparative quantitative method it became clear that the bottom of the Black Sea is a giant biochemical laboratory, determining the present day hydrochemical regime of that sea. It must be mentioned that these investigations proved the erroneous nature of some of the conclusions of hydrochemists, as, for instance, the origin of hydrogen sulphide, the fate of the dead organic matter in the Black Sea and the reasons for the presence of thiocompounds in its hydrogen sulphide zone.

We must not, however, over-estimate the present quantitative methods for estimating the physiological groups of microorganisms. Like other quantitative methods of culturing, they, in relation to the microorganisms living in natural environments, are inaccurate, strongly underestimating the real values. In their present state these methods are useful not for an absolute estimate but for a relative estimate of the intensity of microbiological transformations of matter, and only when the gradients are sufficiently steep. We can state that on the bottom of the Black Sea at its greatest depths there is considerably more organic matter easily assimilable by microorganisms than on the bottom of the deep region of the Sea of Okhotsk, since the differences in the numbers of the corresponding physiological groups are of the order of 1000. Smaller differences in the results of examining water and bottom samples can lead to incorrect conclusions due to the considerable experimental error of the methods used for the quantitative study of the physiological groups of microorganisms.

The improvement of the old and the working out of new methods for the quantitative study of the biochemical activity of microorganisms under the natural conditions of their environments is a most important task of microbiology. Progress in this field will allow hydrochemistry to explain many phenomena in the distribution of biogenic substances in the waters of seas and oceans. This also applies equally to the chemistry of the bottom deposits of marine habitats, which so far have gained very little from the data of marine microbiology to clarify the processes of diagenesis of various compounds on the bottoms of the seas

and oceans. Only very generalized data have so far accumulated concerning the socalled geological activity of microorganisms, which cannot satisfy marine geology and geochemistry, that try to reveal particular features in the transformation of organic and mineral components into various types and layers of bottom deposits.

Hydrology has also areas of contact with microbiology in which, as in the border areas of other sciences, new possibilities arise for the solution of problems of interest to both branches of science.

More and more examples accumulate, indicating the possibility of utilizing microbiological data for a study of the dynamics and origin of water masses, and for indicating deep currents which are poorly defined.

Technology which finds it important to take into account the role of microorganisms in the corrosion of metal and concrete structures in the sea, in fouling of the submerged parts of ships' hulls and industrial equipment utilizing sea water, makes demands on microbiology. Microbiological studies indicate that building materials might possibly be destroyed as a result of the activity of microorganisms. It has also been established that bacteria, rapidly settling on the surfaces of objects in the sea, favor the formation of biocoenoses of animal and plant-fouling communities, interfering with the efficiency of exploitation of ships and industrial equipment. But the data concerning the extent to which microorganisms participate in corrosion and in the processes of fouling are far from sufficient, and a quantitative knowledge of this kind is absolutely essential if efficient measures of prevention of these phenomena are to be evolved.

The qualitative knowledge of microbial biocoenoses of seas and oceans is insufficient. The lists published of the species of microorganisms, isolated from the sea water, give knowledge of only a very small part of the microscopic inhabitants of the sea, which are able to develop on artificial nutritive media under laboratory conditions. These, predominantly heterotrophic microorganisms which metabolize only easily assimilable organic substances even from the point of morphology, do not reflect the

variety of the microbial forms living in the depths of seas and oceans.

The microbiologist has now at his disposal: the "bathysphere" which allows him to look into the deepest localities of the oceans; the method of ultrafiltration, on membrane filters of considerable amounts of water, compared with the size of a bacterial cell (in the ultrafiltration of 15-20 mls the microbial population is studied in a volume of water some 10^{14} times greater than the mean volume of a bacterial cell); as well as the application of the submerged slides method. These allow one, as it were, to descend with a microscope to the required depth and to become acquainted with its microbial population. Such "descents" first carried out in the Black Sea, Caspian Sea and Sea of Okhotsk, in the Pacific and Arctic Oceans, revealed an entirely new world of microscopical organisms which, so far, have been unknown. Tentative studies with the use of the electron microscope suggest that, together with the smallest microorganisms, there exist ultramicroscopic noncellular forms of life in the seas and oceans.

Naturally, the study of the biology of the common forms of microorganisms in seas and oceans is one of the direct tasks of marine microbiology. The difficulties here are great when we take into account the poor progress made in the development of methods for culturing microorganisms living in nature. The development of the ecological and physiological aspects of a study of microbial species in their natural environment would overcome these difficulties.

It is necessary to intensify, in every possible way, marine microbiological investigations in order to satisfy the demands placed upon them by the field of oceanography and general microbiology.

Without a wide participation of microbiologists it is impossible to study the basic oceanographical problems, and consequently to realize the regularities underlying the productivity of the waters of seas and oceans in the interests of practical human activities.

Chapter 47

The Differentiation of Certain Genera of Gram Negative Bacteria Frequently Encountered in Marine Environments*

J. M. Shewan

To most of us with any experience in marine microbiology, the classification of our isolates has always been a problem. Some of you no doubt may consider classification or taxonomy a somewhat sterile field of enquiry. It is more important, you may say, to know what the micro-organisms do in the marine environment rather than to know what they are or to give them a name. A number would do just as well. Precise identification may, however, be of more than academic interest. For many practical problems it is becoming increasingly evident that we must be able to identify our organisms if any headway is to be made, and in particular, if we are to be able to compare our data with those of other workers. For example, in our work on fish spoilage, we know that fish from the same area but caught at different seasons, spoil at different rates, as do fish caught at the same time of year but from different fishing grounds. It seems almost certain that these differences are due to differences in the microbial flora, but unless we have the means of analysing the flora present on the fish, quickly and reasonably accurately, at least to the generic level, - indeed we think it must often go to the species level - we cannot disentangle the importance of the bacterial flora from other factors, such as the physical condition or chemical composition of the fish. Or to take another example. One of the most important features of Professor Kriss's recent book on Marine Microbiology (38) has been the demonstration of the usefulness of marine bacteria as indicators of ocean currents. It is clear,

* The work described in this paper was carried out as part of the programme of the Department of Scientific and Industrial Research.

however, that if this method is to be successful one must be able to identify accurately and quickly the microorganisms concerned.

Our first problem, then, was differentiation at the generic level, and our early results (45) suggested some broad categories corresponding roughly to the *Pseudomonas, Achromobacter, Vibrio* and *Bacteraceae* of Bergey (6) and from the experience gained since then, we have developed the scheme now to be described (46).

THE DETERMINATIVE SCHEME

The medium used for the isolation and cultivation of our bacteria was "Lab-Lemco" nutrient agar ("Oxoid") made up either in tap water or with 75 per cent aged sea water (61) as required. We are, therefore, purposely excluding from our discussion any of the Gram negative bacteria, requiring specialised media, such as the *Azotobacter, Acetobacter, Cellulomonas* sp. etc.

The original isolate is plated out to obtain a pure culture and then put through the series of operations outlined in Table 1. Colony appearance, Gram's stain, morphology and the Kovacs' (37) oxidase test are carried out on the agar plate culture; motility and morphology (under phase contrast) are done on the "Lab-Lemco" broth culture. This latter culture is also used to surface seed on agar plate for the sensitivity tests of Shewan *et al.* (45); and to inoculate the two tubes required for the Hugh and Leifson (30) test. The production of diffusible fluorescent pigment is determined by using the media of King *et al.* (35) or that of Paton (43). When growth occurs in the latter medium the production of 2 keto-gluconic acid from the glucose present, an important diagnostic feature for many *Pseudomonas* sp. (6), is recorded, using an aniline oxalate or aniline phthalate reagent. A sheet of chromatographic paper (Whatman No. 1) is impregnated with a saturated solution of recrystallised aniline oxalate or phthalate (29) and the culture is heavily spotted on the paper which is then heated in an oven at 105C for two to three minutes. A red spot indicates the presence of the 2 keto-gluconic acid. Occasionally, the gluconate (or the glucose) appears to be dissimilated beyond the 2 keto-gluconic acid stage and gives rise to yellow colors with the aniline reagents (Floodgate, private com-

TABLE 1

SUMMARY OF TESTS USED IN DETERMINATIVE SCHEME

Medium	*Observation*
Nutrient agar	Colony appearance Gram stain Morphology Oxidase test (37) Antibiotic and 0/129 sensitivity tests (45)
Nutrient broth	Motility Morphology
King's media	Diffusible fluorescent pigments detected under U.V. light
Paton's medium	Ditto + 2 keto-gluconic acid
Hugh and Leifson's medium	Dissimilation of carbohydrates
Nutrient agar + 30% skim milk	Pigment production in *Flavobacterium* sp.
Nutrient agar slope	Flagella stain (Casare-Gils)

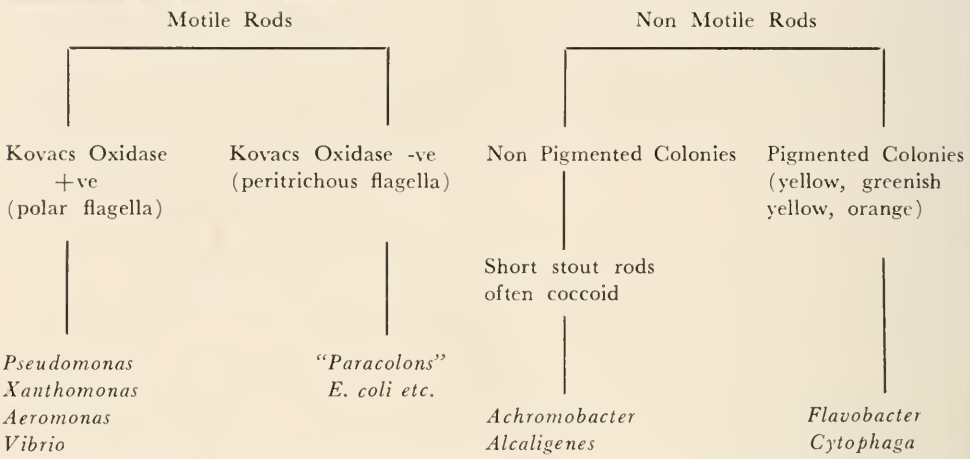
munication). For the detection of intra-cellular pigments, as in the flavobacteria, a nutrient agar, incorporating 30 per cent skim milk, has been found to be most useful. In the motile cultures the type of flagellation is determined by a modified Cesare-Gils (42) flagella stain or by electron microscopy. Most of the above tests are carried out at 20C and only occasionally at 37C.

On the basis of the above tests, the broad groupings given in Table 2 are obtained. These groupings are, of course, strictly applicable only to microorganisms from marine environments. We have, however, tested this differential scheme against many strains isolated by other workers from widely differing environments and it is clear that while the great majority of these can be classified by our scheme, some important exceptions occur.

As will be seen from Table 2, an important differentiating test for the motile rods is that of Kovacs' - the oxidase test. In our experience it is invaluable in differentiating the Enterobacteraceae (oxidase -ve) from the Pseudomonadaceae (oxidase + ve). This contention has recently been supported by Ewing and Johnson (15) who examined a total of 1301 cultures, 1222 of which were Enterobacteraceae with representatives of all the recognised genera and the remainder consisting of members of the Pseudomonadeceae (and including *Flavobacter*, *Xantho-*

TABLE 2

DETERMINATIVE GROUPINGS



monas, Aeromonas, Vibrio, etc.) In our hands these are oxidase -ve. Some *Xanthomonas* and *Pseudomonas* sp., associated with plants which are also Gram -ve and polar flagellated, have also been found to be oxidase -ve.

DISCUSSION

The polar-flagellate, oxidase positive groups:- These comprise members of the genera *Pseudomonas, Aeromonas* and *Vibrio.* From their reaction in Hugh and Leifson's media they can be differentiated into four groups giving 1) an oxidative reaction (*Pseudomonas* sp.); 2) no reaction (*Pseudomonas* sp.); 3) an alkaline reaction (*Pseudomonas* sp.) and 4) a fermentative reaction (*Aeromonas* and *Vibrio* sp.). Group I can be further subdivided on the basis of the presence or absence of fluorescence under U.V. light in the media of King *et al.* (35) or Paton (43).

It is suggested, therefore, that the *Pseudomonas* sp. can be divided into four groups (Table 3).

Group I, includes those which are oxidative in Hugh and Leifson's medium, and produce a diffusible fluorescent pigment. Typical species are *Pseudomonas fluorescens* and *Ps. aeruginosa.*

Group II strains attack glucose oxidatively but never produce a diffusible pigment.

Group III strains, like Group IV, also produce no diffusible

TABLE 3

GROUPINGS OF THE GRAM NEGATIVE ASPOROGENOUS RODS (POLAR FLAGELLATE, OXIDASE POSITIVE AND NOT SENSITIVE TO 2.5 I.U. PENICILLIN)

Hugh and Leifson's Test

Oxidative		Alkaline	No action	Fermentative	
Produce Green fluorescent diffusible pigment	Produce No diffusible pigment	Produce No diffusible pigment	Produce No diffusible pigment	Produce (1) No diffusible pigment (2) Acid and no gas in glucose (some strains produce small traces of gas) (3) Sensitive to the pteridine compound (0/129)	Produce (1) No diffusible pigment (2) Acid and large amounts of gas in glucose at 20°C (3) Insensitive to the pteridine compound (0/129)
Pseudomonas Group I	Pseudomonas Group II	Pseudomonas Group III	Pseudomonas Group IV	Vibrio	Aeromonas

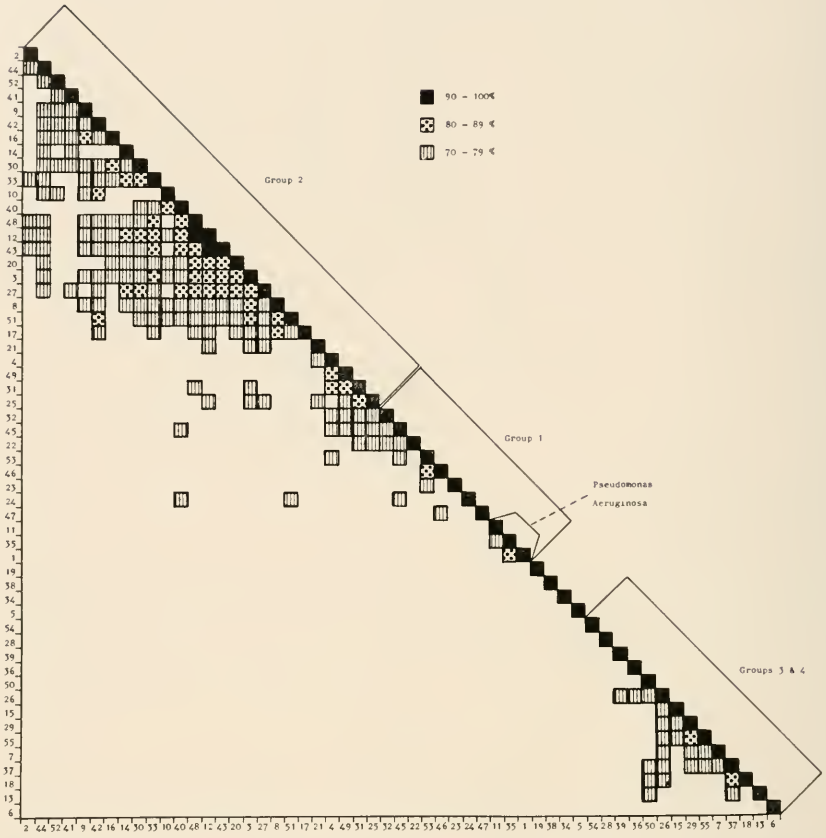


Fig. 1 Diagram showing the similarity values (S) of a series of *Pseudomonas* spp. isolated from marine sources,

fluorescent pigment and have no action on glucose but produce an alkaline reaction in Hugh and Leifson's medium, whereas Group IV produces no change at all.

Groups II, III and IV correspond to one of the *Achromobacter* groups recently postulated by Brisou (7) in his suggested classification of the Pseudomonadeceae.

As many of you are aware, Sneath (50, 51) has recently suggested that the most logical way of classifying bacteria is to compare their overall similarities, applying the Adansonian principle of giving equal weight to each character. It is of interest, therefore, to examine the application of this principle to

Pseudomonas species isolated from marine environment and see how far these fit into the four groups just postulated above. Approximately 60 of these organisms, isolated by various workers at Torry, were classified by them as Pseudomonas sp. (55). Their overall similarities in about 80 tests, for each organism, were calculated, using the formula of Sneath (51). The results (Fig. 1) show that at least three well defined groups can be differentiated, although there is some overlapping, and that these correspond generally to Groups I, II, III and IV, the latter two being almost indistinguishable. It might just be mentioned here that of the *Pseudomonas* sp. recently deposited in one or other of the National Type Culture Collections now housed at Torry, *Pesudomonas halocrenaea* fits into Group I; *Pseudomonas fragi* into Group II; *Pseudomonas aurefaciens* (36) into Group III and *Pseudomonas rubescens* (44) into Group IV.

Aeromonas and *Vibrio* groups:- According to our scheme the *Aeromonas* and *Vibrio* sp. are differentiated from the *Pseudomonas* sp. by acting upon glucose fermentatively. *Aeromonas* sp. are characterised by the production of acid and gas from glucose (at 20 C), whereas the *Vibrio* sp. ferment glucose anaerogenically. Moreover, only the latter are sensitive to 2:4 diamino - 6:7 di-isopropyl pteridine (0/129) (45).

The validity of the genus *Aeromonas* has recently been questioned by Stevenson (54) who suggested that all its members could be regarded as non-chromogenic species of the genus *Serratia*. Several authors, Stevenson points out, have stated that flagellation in the genera *Aeromonas* and *Serratia* may vary according to conditions of culture, and he believed that his *Aeromonas margarita* was in reality a non-chromogenic strain of *Serratia marcescens*. It is not surprising that there should be some confusion between the *Aeromonas* sp. and some members of the Enterobacteraceae, owing to the similarities of their biochemical reactions. Differentiation depends then on the accurate determination of their type of flagellation. Careful studies by electron microscope at Torry Research Station has shown that *Aeromonas margarita* is in fact peritrichous (Figs. 2 and 3) and this, along with its biochemical propensities, including its being oxidase -ve, indicates that it is a typical member of the Enterobacteriaceae.

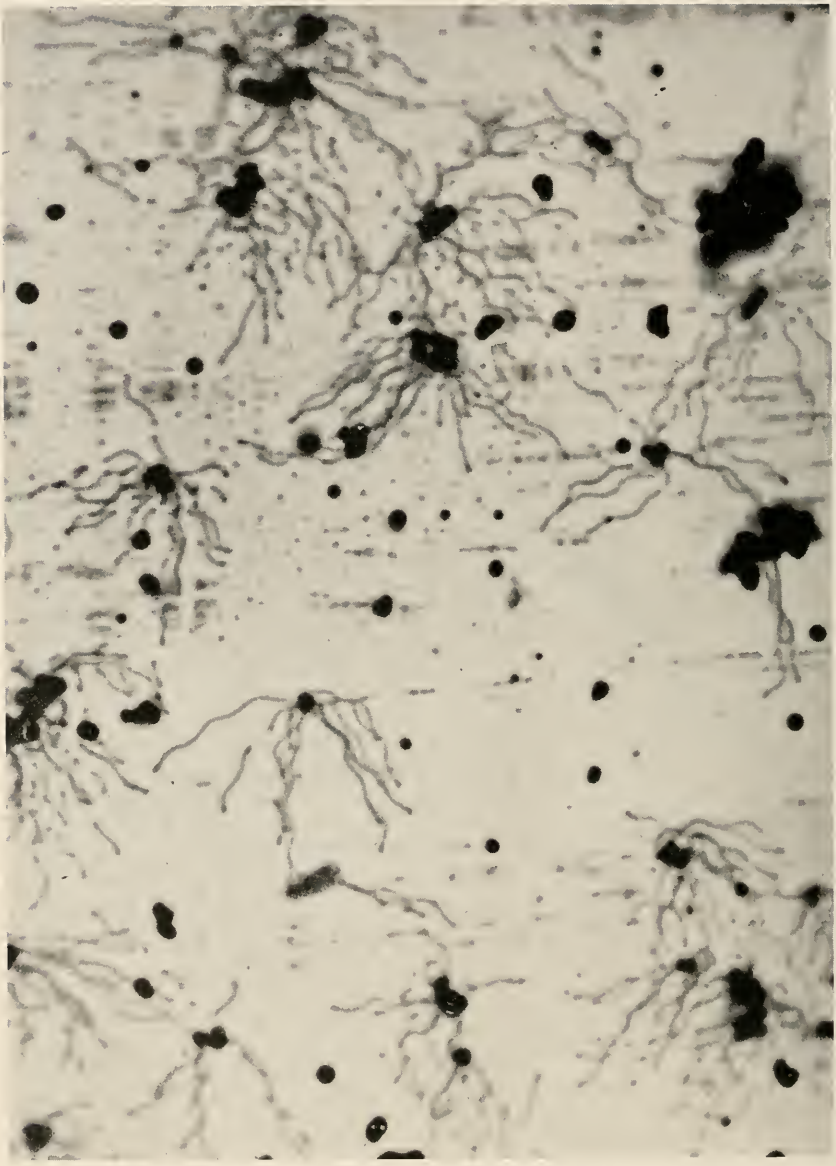


Fig. 2. Stevenson's (54) *Aer. margarita*? *Serratia margarita*?

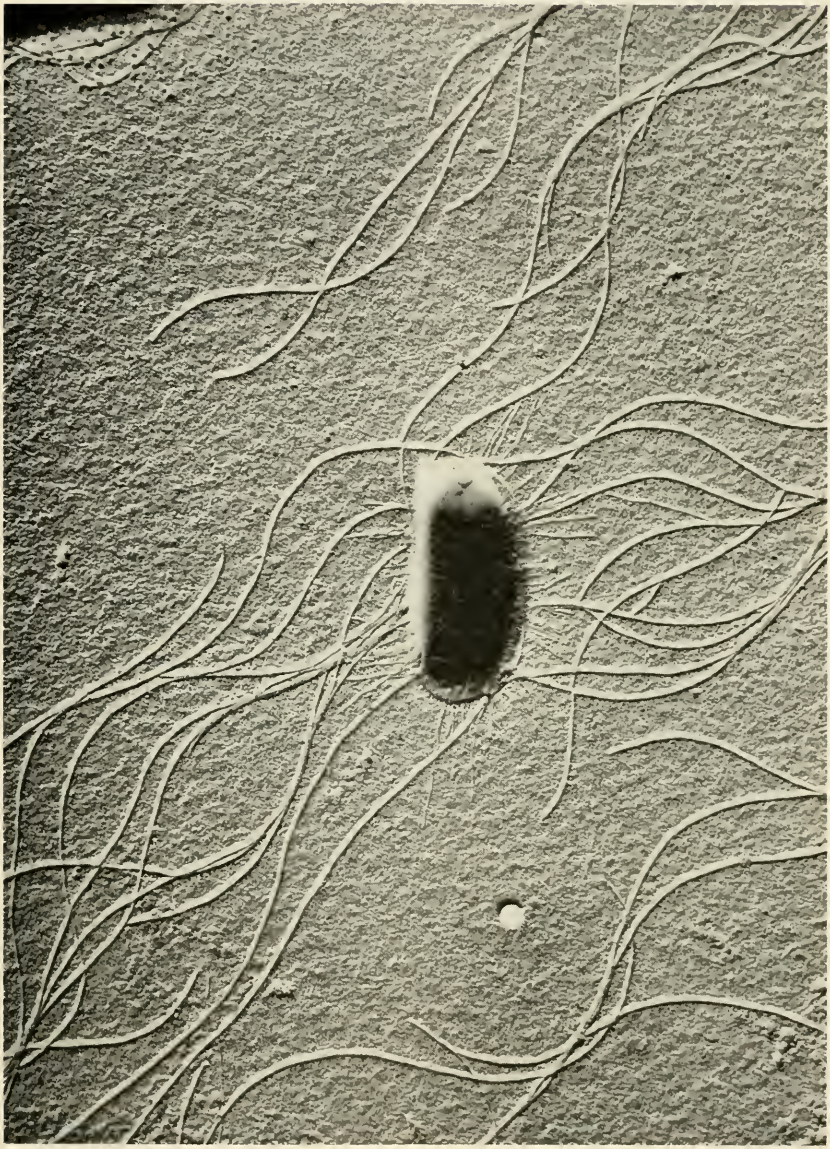


Fig. 3. Stevenson's (54) organisms *S. margarita.*

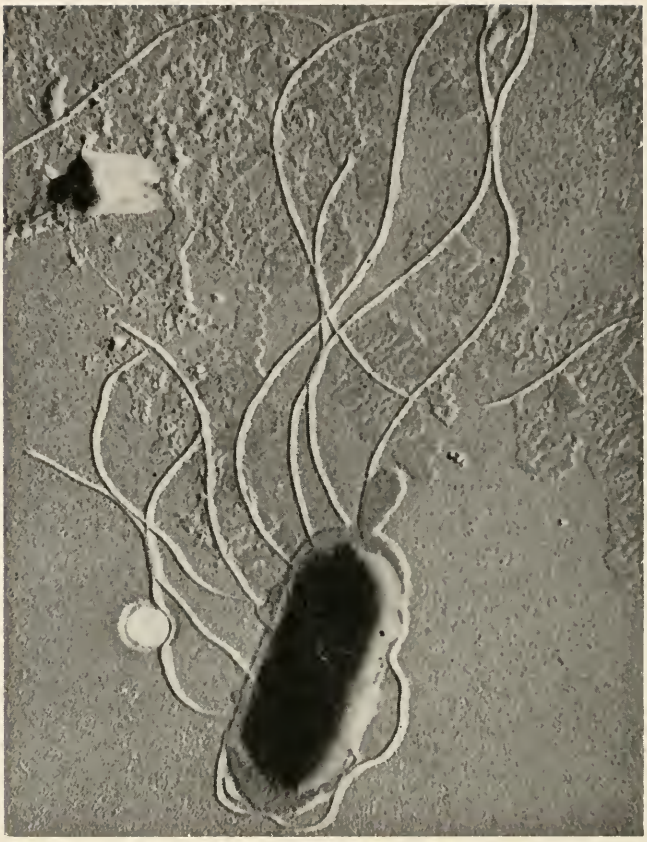


Fig. 4. Kluyvera citrophila NCIB 9138 Twenty-four hours 20° agar. Pl S-0.188 μ.

Asai and his colleagues, have recently described a group of polar flagellate Gram negative rods belonging to so-called genus *Kluyvera* which attack glucose fermentatively; and Cook (13) has also published a description of a so-called polar flagellate member of the Enterobacteriacea. All these organisms were found, however, to be peritrichous (Fig. 4).

Two well known organisms, *viz. Pseudomonas formicans* (14) and *Bacterium salmonicida*, both regarded by many workers (21 and 52) as *Aeromonas* sp., appear to occupy anomolous positions in our suggested scheme. *Pseudomonas formicans* is a Gram negative, oxidase positive, polar flagellate, asporogenous

rod, which is fermentative in Hugh and Leifson's medium, forms no gas from glucose and yet is insensitive to the pteridine compound (0/129). *Pseudomonas formicans* appear to lack the enzyme hydrogenlyase, otherwise morphologically and biochemically it is more closely related to the *Aeromonas* group than to the *Vibrios* and recent work by Liu (41), using the extracellular antigens as a means of classification, suggests that in fact the species *Aeromonas liquefaciens* and *Pseudomonas formicans* are closely related.

Bacterium salmonicida is also a Gram negative rod, oxidase positive, forming acid and gas from glucose, is fermentative in Hugh and Leifson's medium but is non-motile. Liu believed *B. salmonicida* to be a different organism from the *Aeromonas* strains, with its lower optimum temperature, pigment formation and lack of motility, yet he found some cross reactions with the *Aeromonas* sp. and *B. salmonicida* and concluded that the latter in fact belonged to this group.

Recently we have been examining a large number of named strains of *Vibrios*, particularly in relation to the definition of *Vibrio* given in our determinative scheme. The results are given in Table 4. It will be noted that *Vibrio comma*, fresh water vibrios of the *Parachlora* group, *Vibrio foetus* and *Vibrio anguillarium* all fit in well to this definition. In agreement with Hugh and Leifson (30), we consider that *V. percolans* and *V. cuneatus* are not related either morphologically or biochemically to *Vibrio comma* (39), and we would also exclude *V. cyclosites*, *V. neocistes* (31), *Vibrio jamaicaensis* (11) and a few others. In our hands, *V. cuneatus* appears to be a green fluorescent Pseudomonad (Group I), and *V. percolans*, *V. cycloistes* and *V. neocistes*, Group III *Pseudomonas* spp. and *V. jamaicaenis*, as Caselitz later pointed out (12) is an *Aeromonas sp. Vibrio alcaligenes*, it was proposed by Galarneavelt and Leifson (19), should be given the new generic name of *Lophomonas*, but from data available it fits well into our Group IV *Pseudomonas* sp.

It will also be noted from Table 4 that some organisms formerly described as *Pseudomonas* sp. should, on physiological and morphological grounds, now be placed in the *Vibrio* genus.

TABLE 4

NAMED VIBRIO SP. AND OTHER BACTERIA AND THEIR TAXONOMIC POSITION IN RELATION TO SUGGESTED CRITERIA FOR VIBRIO SP.

Culture	*Source*	*Suggested Taxonomic Position*
Vibrio cyclosites	NCIB 2581	Group III Pseudomonas
Vibrio neocistes	NCIB 2582	Group III Pseudomonas
Vibrio percolans	NCIB 8193	Group III Pseudomonas
Vibrio cuneatus	NCIB 8194	Group I Pseudomonas
Vibrio 01	NCIB 8252	?
Fresh water Vibrios (5 strains)	NCIB 9047-9052	Vibrio sp.
SH 1 Vibrio (from saithe)	Torry Research Station	Vibrio sp.
Vibrio anguillarum (cod)	Bagge and Bagge (3)	Vibrio sp.
Vibrio piscium (2 strains) (trout)	Hoshina (28)	Vibrio sp.
Vibrio paracholera	NCTC 30	Vibrio sp.
Vibrio elter	NCTC 4715	Vibrio sp.
Vibrio Group VI	NCTC 4716	Vibrio sp.
Vibrio Group II	NCTC 8042	Vibrio sp.
Vibrio sp. No. 5)		Vibrio sp.
Vibrio sp. No. 6)	Szturm-Rubinsten et al. (56)	Vibrio sp.
Vibrio sp. C.I. Pasteur 3638 (Piéchard))		?
Vibrio costicolus (Gibbons)	Baxter and Gibbons (4)	Vibrio sp.
Vibrio foetus	Sandwick	Vibrio sp.
Vibrio spp. (diseased finnock and trout - 2 strains)	Smith (49)	Vibrio sp.
Vibrio metchnikovii	NCTC 8443	Vibrio sp.
Vibrio proteus	NCTC 8503	Vibrio sp.
Vibrio alcaligenes	NCTC 2902	Group III Pseudomonas
Photobacterium splendidum)		Vibrio sp.
Photobacterium phosphoreum)	Spencer (53)	Vibrio sp.
Photobacterium pierantonii)		Vibrio sp.
PL1 Pseudomonas ichthyodermis	Hodgkiss and Shewan (27)	Vibrio sp.

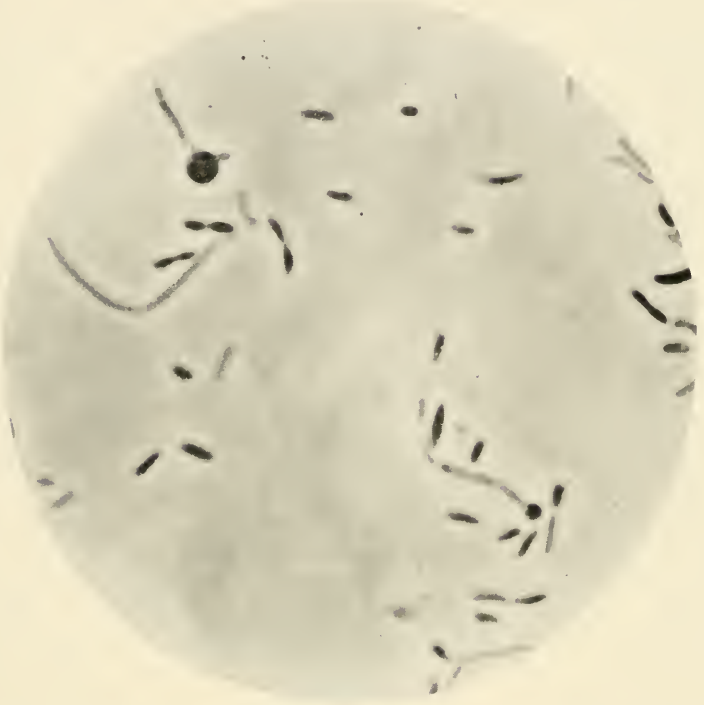


Fig. 5. Gut group *Vibrio* (39) showing curved rods and the characteristic round bodies. Phase Contrast X1200.

These include *Pseudomonas ichthyodermis* (27) and *Achromobacter ichthyodermis* (58, 60).

Previously it has always been difficult to differentiate the saprophytic vibrios causing disease in poikilothermic animals from other Gram negative polar flagellate rods. This was usually done on the basis of the curvature of the cell, quite an unsatisfactory criterion. The fermentative character of their attack on carbohydrates, together with their sensitivity to the pteridine compound (0/129) are thus valuable additional criteria. In addition to the curvature of the cell, *Vibrio comma* was characterised by early workers by the pleomorphism of the cell and in particular by the presence of round or coccoid bodies (24, 26). Hallock (22, 23) has recently claimed that this is a distinguishing feature of the *Vibrio* group as a whole and more attention might be given to

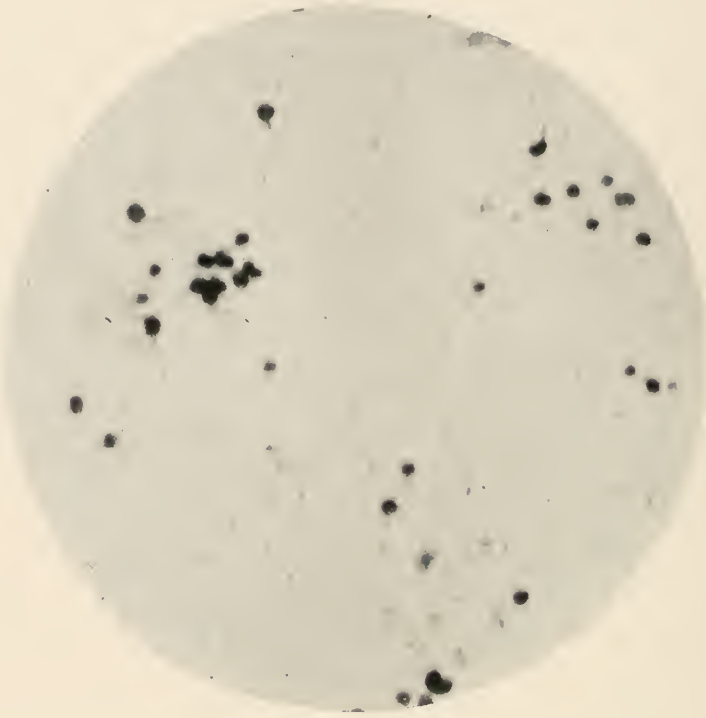


Fig. 6. Gut group *Vibrio* (39) showing rounded bodies on sea water agar. Phase contrast X1200.

this characteristic than hitherto. Coccoid bodies have been described in certain luminous bacteria (32), which, on our criteria are believed to be *Vibrio* sp. (53), and they are a regular feature of many of the vibrios we have recently been examining at Torry Research Station (Figs. 5 and 6), e.g., *Vibrio anguillarum* and *V. sp.* (from fish intestines).

The *Achromobacter* Group:- While the differentiation of the *Pseudomonas, Aeromonas, Vibrio* groups is fairly clear-cut and straight forward in our suggested scheme, that of the *Achromobacter* group does pose certain difficulties. Part of these reside in the fact that the original type species, *Achromobacter liquefaciens,* defined in Bergey as being peritrichous, is absent from any known Collection and partly because the data concerning this organism in Bergey are insufficient to identify it with

any degree of certainty. The organism named *Achromobacter liquefaciens* in the Collection of the Microbiology Department, Brno, Czechoslovakia, in our hands appears to be a typical "paracolon" rather than *Achromobacter* sp. and the *Achromobacter liquefaciens of* Youatt (59) (48) has only very recently been made available to us. A motile peritrichous flagellated Gram negative rod, originally named *Achromobacter histaminium* (33) was found to be fermentative in its action on glucose, producing acid and gas, and has in fact recently been assigned to the genus *Proteus* (34). In Table 2 it will be noted that the *Achromobacter* sp. are all recorded as being non-motile and non-pigmented. This is because in our experience over a number of years we have not so far encountered an organism from the marine environment which is peritrichous and yet possesses the typical morphology given in the original description by Frankland and Frankland (17). Buttiaux and Gagnon (10) have also recently concluded that members of the genus *Achromobacter* are non motile, since they never once encountered a peritrichous organism corresponding to *Achromobacter liquefaciens*, despite the fact that they were working with organisms isolated from very widely differing environments. Moreover, Thornley (57) working with 390 bacteria isolated from chicken carcasses, found only two peritrichous flagellated Gram negative rods among the 190 cultures which she designated as *Achromobacter* sp. - and even these she hesitated to designate to this group. All our species which have been assigned to the genus *Achromobacter* are non motile, non pigmented, short, stout or coccoid rods occurring singly, in pairs, like diplococci, or in short chains, forming a grey, off white slightly opaque colony on agar, sensitive to penicillin and usually biochemically inactive, i.e., having the so called "negative characteristics" of the literature. In Hugh and Leifson's medium most strains produce an alkaline reaction, some no reaction, and a few strains are oxidative. In general, these organisms correspond to the *Acinetobacter* genus proposed by Brisou and Prévot (8). It is becoming increasingly clear to many workers that the species described under the general *Moraxella*, *Diplococci* and even *Neisseria* could all be grouped under the genus *Achromobacter*. The *Achromobacter* sp. are distinguished from the *Pseudomonas*

sp. primarily on motility and morphology; are usually less active biochemically. In addition, most strains are sensitive to penicillin, whereas the majority of *Pseudomonas* strains are not. It might just be mentioned in concluding this section on the *Achromobacter* that the only organism which the author has encountered so far which accords with the description of *Achromobacter liquefaciens* is the *Agarbacterium alginicum* of Payne and his co-workers (1). This organism has the morphological and biochemical properties of our *Achromobacter* group, yet is motile by peritrichous flagella.

Very occasionally, organisms are encountered which are Gram negative, peritrichous and not obviously Enterobacteria. Morphologically they are quite different from the *Achromobacter*, being rather long rods with square ends, e.g., the *Achromobacter* sp. of Beppu and Arima (5). The taxonomic position of these organisms is at present not known.

The Flavobacteria and Cytophagas:— It is only quite recently that we have begun to pay some detailed attention to the Flavobacteria and Cytophaga groups and our work on these is still in progress. So far as the Flavobacteria are concerned the position has been made somewhat more difficult by the recent proposal of Ferrari and Zannini (16) that there is no justification for the exclusion of Gram positive strains from the genus *Flavobacterium* as has been done in the recent Bergey (6). These authors postulate three groups — each including Gram negative and Gram positive microorganisms — on the basis of their morphologies and life cycles. Whatever the merits of these proposals we shall deal here only with the Gram negative strains. Even so, the differentiation of the yellow or orange pigmented *Cytophaga* sp. from the *Flavobacterium* sp. has proven to be one of some difficulty. The Flavobacteria are described in Bergey (6) as non-motile or motile (peritrichous) short rods while the Cytophagas are described as consisting of long thin rods, often with pointed ends, which show flexing and hence gliding motility on solid surfaces. Consequently, on agar plates, the colonies have a characteristic diffuse margin spreading outwards into the agar.

Hayes, working recently at Torry Research Station, chose about 60 strains of yellowish pigmented asporogenous rods from

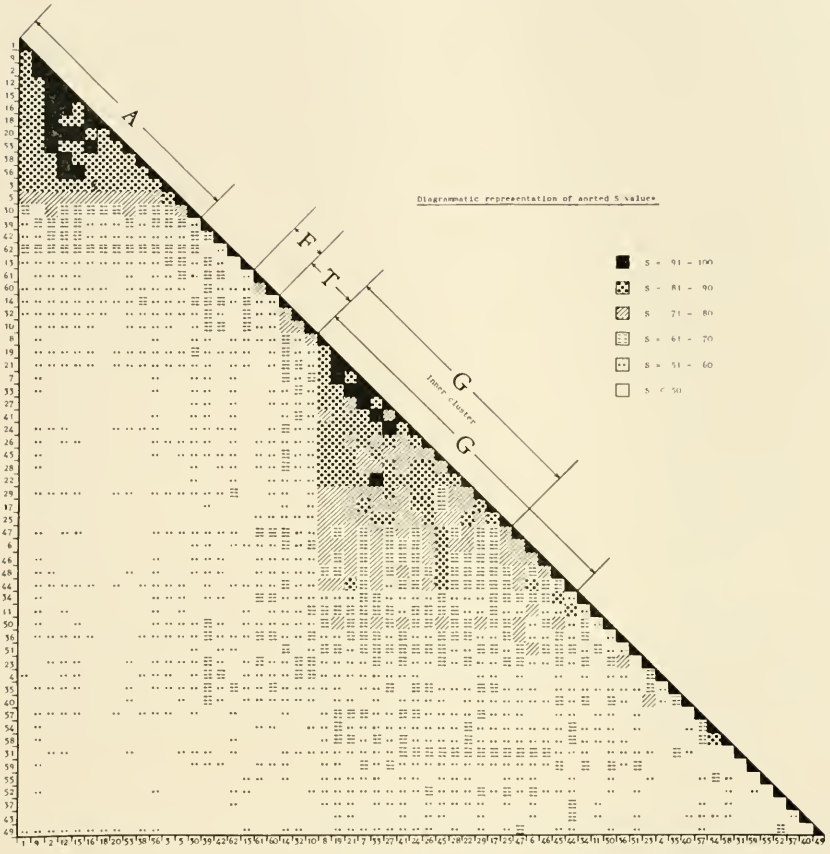


Fig. 7. Diagram showing the similarity values (S) of the yellow to brown pigmented rods from marine sources.

marine sources and put them through a variety of tests (25). Of the total of 54 organisms finally available only six were motile, three being polar and three peritrichous. His results, analysed according to the formula of Sneath, showed that amongst the non-motile strains there were at least two well-defined major groups, A and G. (Fig.). Their main differentiating features are shown in Table 5.

If swarming on agar and morphology count for anything, then it might be concluded that Group I are rather similar to the typical *Cytophaga* sp. of Bergey. Yet Hayes, despite persistent

THE DIFFERENTIAL FEATURES OF TWO MAJOR GROUPS OF YELLOW TO ORANGE PIGMENTED, GRAM -VE ASPOROGENOUS RODS FROM MARINE SOURCES

Characteristic Feature	*Group I*	*Group II*
Heat Resistance		
(1) 45 mins. at 45°C	All viable	All dead
(2) 48 hrs. at 37°C	All viable	All dead
Reaction to the pteridine compound 0/129	All sensitive	All resistant
Formation of NH_3 from peptone	None reactive	All reactive
Acid from xylose, fructose, mannose and sucrose	All +	All -ve
Pigment	Yellow to yellow green or lime	Yellow to orange-yellow
Upper limit of growth	30°C	Generally below 25°C
Nutritional requirements	Fairly complex	Fairly simple
"Swarming" of colonies on agar surface	Most "swarm"	None "swarm"
Morphology	Long slender Gram -ve rods, non motile.	Gram -ve, non motile pleomorphic rods - ranging from stout, oval cells to long slender rods

attempts, was never able to demonstrate convincingly that Group I possessed, at any time, flexing cells, and further work will be required to settle this problem. Hayes's three motile (peritrichous) rods show very little similarity to either of the other two groups and probably belong to another genus.

The above determinative scheme has now been in use at Torry for a number of years and has been used during this time by several workers, e.g. (40, 20, 47), in their examination of thousands of isolates from marine sources. By its use they have usually been able to classify their cultures, at least to the generic level, quite quickly. In addition, we have examined over 200 named strains from Type Culture Collections and individual workers, which have included *Pseudomonas*, *Achromobacter*, *Vibrio*, *Aeromonas*, *Xanthomonas* and *Flavobacterium* sp. In most instances we have been able to identify them unequivocally by our scheme although in some cases this classification has differed from that originally given. Our scheme has also been in use for some time in other laboratories and other workers, to whom the scheme has been demonstrated at Torry, have published data showing its general usefulness (9, 57). It is of course a determinative scheme and does not claim to express any phylogenetic relationships between the groups.

ACKNOWLEDGMENT

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Chapter 48

Regional Variability of Bacteria in North Atlantic Sediments

E. H. Anthony

INTRODUCTION

The accuracy with which bacteria in lake sediments may be counted was discussed in a previous paper (4). The errors exhibited by agar plate and membrane filter counts of bacteria in 309 sediment samples from the depths of sixteen lakes were analysed and the following conclusion drawn. If four trips are made to a lake, preferably over several seasons, and if upon each trip four cores of sediment are taken, and if from each of these cores quadruplicate membrane filter counts are made, then the mean number of bacteria in the sediment may be estimated with a standard percentage error of about $\pm$ 36 per cent. The objectives of the present study were to assess the precision with which bacteria in marine sediments can be counted by the filter method, and to determine whether sediments from different regions can be distinguished from one another on the basis of such counts. In addition, filter counts have been compared with direct counts by fluorescence microscopy, and the possibility of freezing marine sediments without changing their bacterial numbers has been examined.

PROCEDURE

Coastal sediments were obtained with a Naumann sampler; Labrador and Scotian shelf sediments with a Phleger corer. These devices were lined with plastic tubing which also served to transport the coastal sediments to the laboratory. Supernatant water was removed by aspiration as the core was pushed to the top of the tube by a plunger. A bacterial sample was obtained by thrust-

ing a sterile glass tube vertically into the surface of the core. The bore of the tube was known, hence the volume of the sample was determined from its length. The samples were either immediately prepared for counting or frozen in the glass tubes for later study. Dilutions of 250 or 2500 fold were made in 80 per cent sea water; the lower dilution tends to set a limit for the method because of the amount of sediment deposited upon the filter. The medium recommended by Carlucci & Pramer (1) was used in liquid form, at double concentration, and supplemented by vitamin B12. Further details of the counting technique are given in Hayes & Anthony (4). Direct counts were made by fluorescence microscopy (6). An ordinary household "deepfreeze" was used to freeze and store sediment samples.

RESULTS

Precision of Counting Bacteria in Marine Sediments by Membrane Filters

Under ideal conditions, plate counts of bacteria constitute a Poisson series (3). It is characteristic of the Poisson distribution that the variance (standard deviation squared) is equal to the mean. Advantage may be taken of this characteristic to check the goodness of fit between observed and theoretical numbers. For lake sediments, Hayes & Anthony (4) plotted the standard percentage error of the mean against the number of colonies per filter and compared the observed values with the Poisson expectation. This analysis has been repeated for 117 quadruplicate filter counts of marine bacteria. The results are shown in Figure 1A.

The Poisson series describes a situation in which it is presumed that all error is inherent, i.e., due to the random distribution of objects in time or space. Counts of biological material may be expected to incur additional error due to handling. In Figure 1B, the line describing the inherent error of the Poisson series is dependent upon the mean, i.e., it forms a rectangular hyperbola falling from high values associated with low mean counts toward infinitely small values as the mean increases (within the practical range shown here, the error tends to level off at about $\pm$ 3%). Handling error, on the other hand, should be more or less constant and independent of the mean count. Figure 1C is a plot of

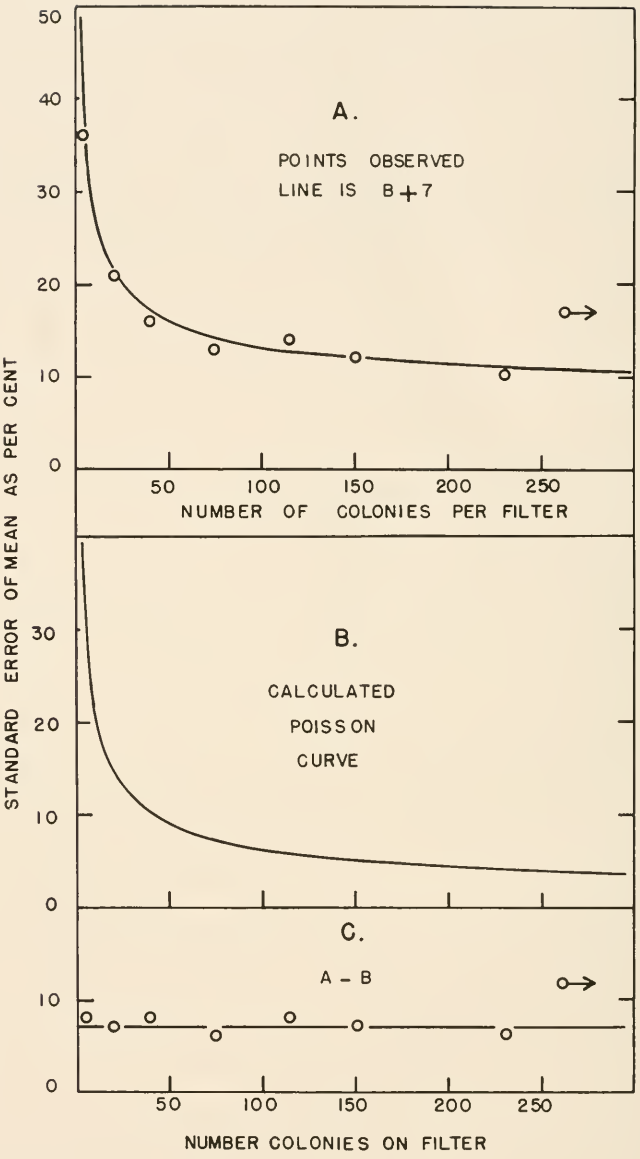

Fig. 1. Standard error of the mean in bacterial colony counts where quadruplicate membrane filters are prepared from the same dilution jar. Each point in A is a mean of twelve to eighteen tests. The line drawn through the points is curve B plus 7 percent. B is the calculated Poisson curve for quadruplicate samples, constructed on the presumption that the standard

differences between observed and calculated values in the other two parts of the same figure. The points exhibit those characteristics expected of handling error and are thought to represent it. The line drawn through the points is an abscissa at ± 7 per cent and this may be compared with a handling error of ± 9 per cent found by Hayes & Anthony (4) for lake sediment counts.

The point bearing an arrow in Figure 1A & C calls attention to the fact that above a certain count (say 500 colonies per filter) agreement with the theoretical distribution begins to break down, even when allowance is made for handling error. The D^2 (Fisher's X^2) values for these high counts also indicate that they cannot be accepted as part of a Poisson series (2 and 3), hence they were excluded from present treatment.

Estimation of bacterial populations involves further errors that have also been examined by Hayes & Anthony (4), whose paper gives methods of calculation. Their results are shown in brackets for comparison with similar analysis of marine sediment counts. The numbers are standard percentage errors of the mean. The average error of colony counts plotted in Figure 1A is ± 17 per cent (± 23%). Measurement of mud volume is assumed to be subject to the same error, namely ± 10 per cent, and the error involved in converting the volume to weight was found to be the same, namely ± 9.6 per cent, hence the error combined in counting four sediment samples is ± 11 per cent (± 13%). The variability between four samples from one station at one time averaged ± 33 per cent (± 31%). If four samples collected at the same time were each taken from a different station, the error was found to be ± 21 per cent (± 35%). If, on the other hand, the four samples were collected one at a time on each of four trips, the variability was found to be ± 27 per cent (± 71%). Finally, sixteen samples, one from each of four stations upon each

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error is:

$$\sqrt{\frac{M}{4}} \times 1.25$$

C shows the result of subtracting the theoretical values indicated by B from the observed points in A. The line is an abscissa passing through ± 7 percent.

of four trips, reduced the error to ± 13 per cent (± 36%), of which almost half is due to handling.

Apparently the membrane filter technique achieves greater precision with marine sediments than it displayed with lake sediments. This may reflect a greater inherent stability in the marine population, but it must be borne in mind that the present observations cover only one season, whereas those of Hayes & Anthony extended over several years.

In view of the practice of averaging trip means via their logarithms (see Table 1), it would be more appropriate to express the standard error as $\times\!\div$ 1.13 instead of ± 13 per cent, but for sake of direct comparison with Hayes & Anthony, the latter method of expression has been retained.

TABLE 1

BACTERIA IN TOP 5 CM OF MARINE SEDIMENT

"Station" does not mean a precisely located spot, but one of the sites chosen at random upon each visit to a region. Each station provided one sediment sample from which four membrane filters were prepared. A mean colony count per filter was determined for each station, the station means were averaged arithmetically to provide a trip mean colony count, and the logarithms of the trip means were averaged to obtain the final mean counts shown in this table and in Figure 2. Column 6 records, as percent of the arithmetic mean, the reduction in recorded numbers resulting from averaging trip means logarithmically. For example, the Fundy count, had it been averaged arithmetically, would have been $\frac{125 \times 100}{100 - 4} = 130$. A small difference between the two means reflects a high degree of agreement between replicates. The rationale of averaging by logs is discussed by Robertson (5) and Hayes and Anthony (4).

1	*2*	*3*	*4*	*5*	*6*
				Bacterial Colonies	
Number	*Region*	*Number of Trips*	*Number of Stations*	*Thousands Per Gram Dry Sediment*	*Per Cent Reduction Via Logs*
1	Fundy	4	13	125	4
2	Halifax	6	23	114	3
3	Northumberland	11	41	275	16
4	Labrador and Scotian Shelf	8*	22	13	19

* Strictly speaking, only one cruise was made to this region. Explanation in text.

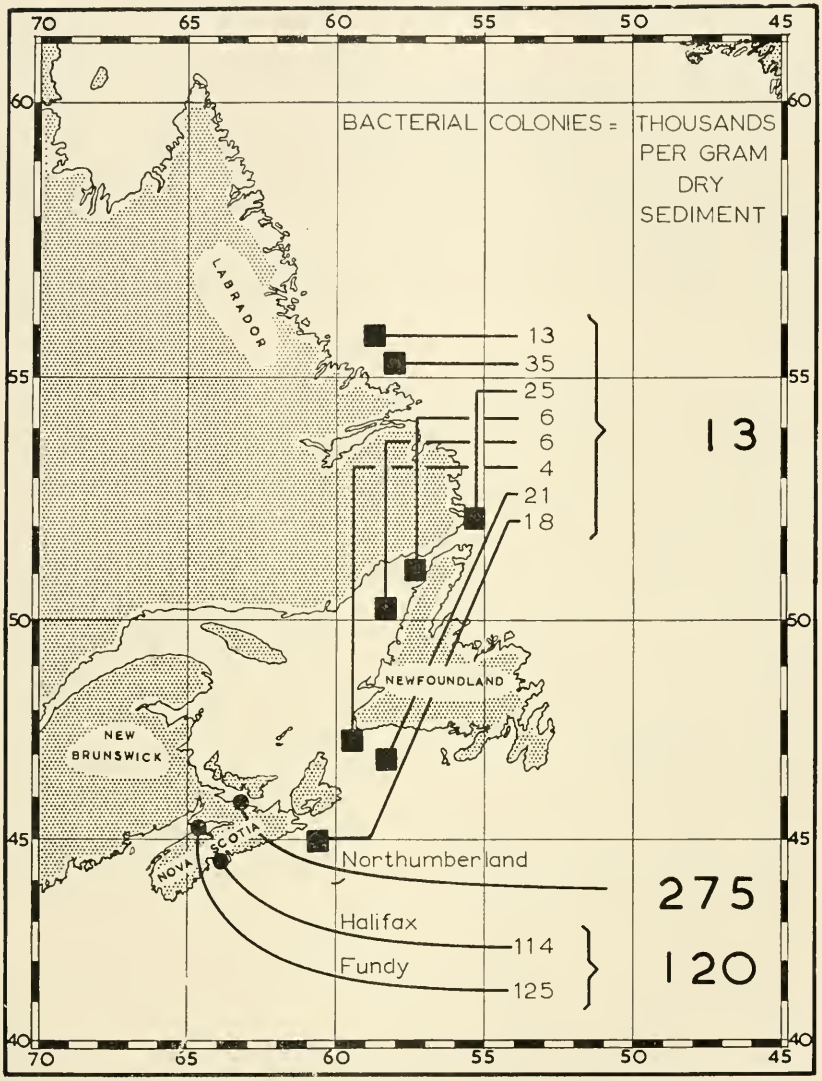

Fig. 2. Regions from which marine sediment was collected. The counts are based upon the following number of samples per region: Fundy 13; Halifax 23; Northumberland 41; Shelf 32. Mean counts are shown for individual parts of the shelf region. Each is based upon counts of four samples or less, hence they do not provide a basis for distinguishing one part of the shelf from another and are grouped together as eight trips to one region. The trip means were averaged via their logarithms to produce the final results that are recorded in large numerals.

Regional Variability

The three sides of the Nova Scotian coast that were sampled have the following characteristics:

1. Fundy: a region of very high tides and associated turbulence that results in considerable scouring of the bottom and makes it difficult to obtain cores.

2. Halifax: this region borders immediately upon the Atlantic Ocean and receives the drainage of an infertile granite-quartzite area.

3. Northumberland Strait: this region separates Nova Scotia from Prince Edward Island and is adjacent to the Gulf of St. Lawrence. The water is warmed to the bottom in summer and good farm land is found on either side of the Strait.

4. A fourth set of sampling stations runs from the Labrador Shelf, through the Strait of Belle Isle, and on to the Scotian Shelf.

The number of trips and stations devoted to collecting sediment samples are shown in Table 1. It should be emphasized that these samples - including the ones from coastal regions of high tides - were from sediments that were never exposed even by the lowest tidal level. Some liberty has been taken with the meaning of the word "station" in Region 4, which was covered once during a joint cruise of the vessels *Vema* and *Sackville*. On basis of the results, it was decided to treat the visit as eight trips to one region. Figure 2 shows the location of the regions and the mean results arising from one summer's observations.

It will be seen that the highest numbers were found in Northumberland sediments. The Fundy and Halifax areas can not be distinguished from one another on the basis of these counts, but both are higher by a factor of ten than the counts from the shelf. It would appear that various regions may be quantitatively distinguishable from one another on the basis of their microflora, although it remains to be seen whether there are seasonal effects that will modify these findings. A notable feature of the results is the extremely small number of bacteria in all the sediments as revealed by the membrane filter technique.

Comparison of Fluorescence Microscopy Counts with Membrane Filter Counts

The fluorescence technique of Strugger (6) differs from other direct counting methods in distinguishing between living and dead bacteria on the basis of differential absorption of acridine orange. Direct counts of living bacteria in 77 marine sediment samples were made by this method. In order that they might be compared with colony counts on the filters, the two methods were applied concurrently to duplicate samples from the three coastal regions.

In general, the direct counts exceeded filter counts by a factor of 10^4. The means of direct counts were subject to the same or smaller errors than corresponding filter counts, but the former did not follow a Poisson series. While it is possible to distinguish between the three coastal regions on basis of the fluorescence counts, it may be seen from Table 2 that the regions are ranked differently by the two methods, and it will be even more apparent from Figure 3 that no simple ratio exists between the two counts.

TABLE 2

A Comparison of Regional Variability in Bacterial Numbers as Observed by Two Methods of Counting: Membrane Filter and Fluorescence Microscopy. Fluorescence Counts were Higher than Filter Counts by a Factor of 10^4

		Relative Numbers of Bacteria in Sediments, Calling Highest Count for Each Method 100	
Number	*Region*	*Membrane Filter*	*Fluorescence Microscopy*
1	Fundy	40	44
2	Halifax	43	100
3	Northumberland	100	14

The working time required to obtain a trip average is about the same for these two techniques. The membrane filter method is readily carried out at sea, but it is doubtful that such is the case for fluorescence microscopy. Eye fatigue undoubtedly involves error that might be avoided by introducing photometric measurements in place of the visual counts of fluorescing particles.

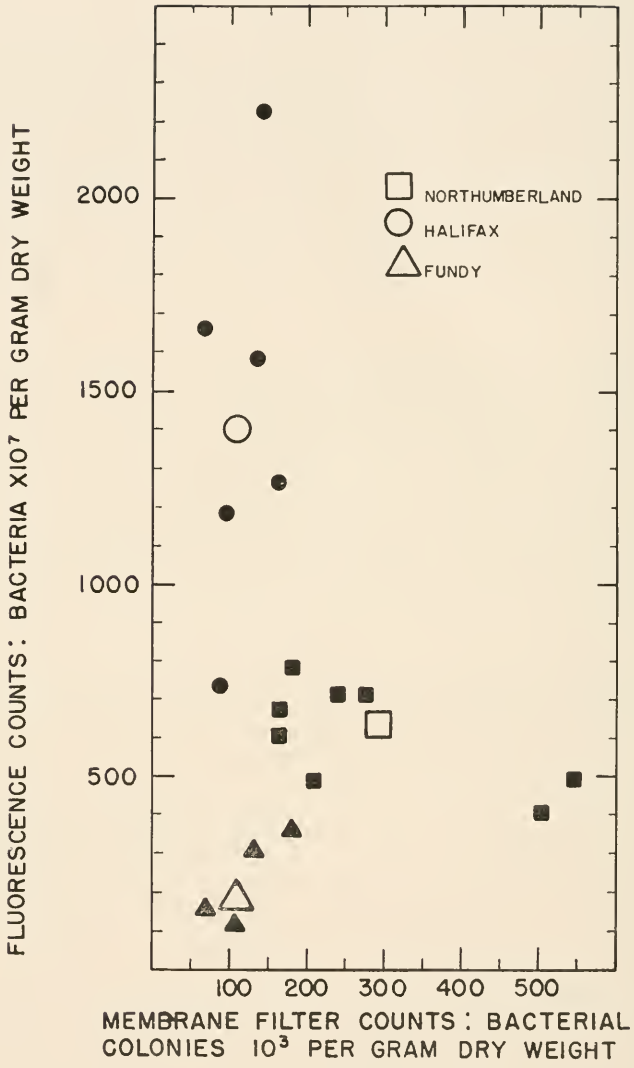

Fig. 3. The comparative abundance of bacteria in marine sediments as shown by membrane filter and fluorescence microscopy techniques, using duplicate sediment samples. Trip means are indicated by solid points. Results of averaging trip means by their logarithms are shown as open figures. There appears to be no simple relationship between results of these two methods.

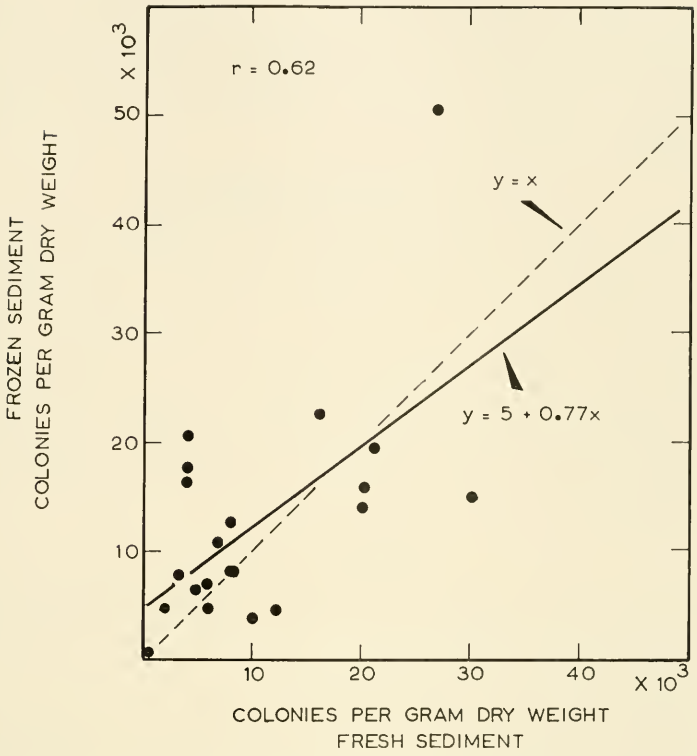

Fig. 4. A comparison of bacterial counts made immediately at sea with counts made six months later in the laboratory upon duplicate sediment samples at -20 C since collection. The points are means of four filter counts upon each of the duplicates. The solid line is calculated by method of least squares; it does not differ greatly from the broken line indicating complete agreement between the two counts. The correlation co-efficient (r) indicates a probability of 0.01 for the twenty-one samples.

Comparison of Counts Upon Fresh and Frozen Marine Sediments

A simple method of bringing sediments back to the laboratory without changing their bacterial count would be most useful. To check the possibility that freezing might serve this purpose, duplicates of most of the samples from the Labrador-Scotian shelf were frozen and held at -20°C for over six months before counting. Figure 4 shows that these duplicate filter counts parallel one another surprisingly well. Freezing and thawing of terrestrial

soil generally brings about an increase in the colony count that is attributed to dispersal of the organisms by such treatment (7). The apparent absence of this effect from the present results may indicate that bacteria in marine sediments are more dispersed than those in ordinary soil.

ACKNOWLEDGMENTS

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Of several students who took part in this work, three deserve particular mention, Misses: Heather Crowdis, Jill Garrard, and Gail Mattinson.

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Chapter 49

Bacterial Habitats in the Antarctic Environment * †

John McNeill Sieburth

The microflora of the Antarctic environment is of interest due to this continent's isolation from other land masses, its low temperatures, highly productive marine basins, and its comparative freedom from human habitation. The early marine studies have been reviewed by ZoBell (28) while those dealing with Antarctic animals have been reviewed by Sieburth (19, 22). Despite the renewed interest in Antarctica since World War II and the opportunities afforded by the International Geophysical Year, few studies have been conducted during the last decade. Sladen (25) has reviewed the few papers dealing with medical microbiology. Bunt has made some preliminary studies on the fecal (1), littoral (2) and soil microflora (3) of subantarctic Macquarie Island. During operation Deep Freeze II to McMurdo Sound McBee (11) culturing the intestinal contents of several Antarctic animals, showed the presence of anaerobic bacteria and later obtained *Clostridium* species in enrichment culture of a frozen specimen. Straka and Stokes (26) used frozen samples of fecal and soil material of undescribed nature from non-designated areas to demonstrate that taxonomically undescribed psychrotolerant organisms could be cultivated. A section made from the Antarctic to the mouth of the Ganges by Lebedeva (9) indicated that in

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† Contribution No. 38 from the Narragansett Marine Laboratory, University of Rhode Island.

40 ml aliquots of Antarctic sea water heterotrophic bacteria were usually absent.

The purpose of this paper is to show that in certain Antarctic habitats an inability to cultivate bacteria may be due to a lack of knowledge of natural nutrient-temperature interrelationships and regulating mechanisms rather than to the actual sparsity of bacteria. In an attempt to explain this opinion, data are presented in order to 1) verify the apparent sparsity of bacteria in the air and sea; 2) illustrate the role of an algal acid in the gastrointestinal antibiosis of penguins; 3) suggest the role of algal acrylic acid in nurturing and inhibiting marine bacteria associated with Phaeocystis blooms; and 4) illustrate the active decomposition of organic matter at ambient temperatures as indicated by bacteriological changes in the guano at penguin rookeries.

THE BACTERIAL CONTENT OF AIR AND SURFACE SEA WATER

An aerosol sampler for membrane filters (AA type, Millipore Filter Corp.) was used to sample the air from the foredeck of the ship. Samples ranging from 25 to 100 liters of air obtained in the Drake Passage in December 1957 failed to demonstrate the presence of bacteria when the membranes were cultivated on Eugonagar (-B.B.L.). While fecal and soil samples were being obtained from penguin rookeries at three islands in the South Shetland Group, air exposure plates were also made. Heart infusion agar plates (Difco) exposed on the ground between nests for periods up to eight hours failed to yield detectable organisms.

During the 1957 crossing of the Drake Passage, HA type Millipore filters were inoculated with aseptically collected surface water samples and cultivated on media made with varying amounts of Eugonbroth (-B.B.L.) and sea water. A few samples below the Antarctic convergence contained as many as a hundred orange pigmented mesophilic heterotrophs per ml, however, most of the samples contained fewer than ten organisms per ml. Sea water agar pour plates (heart infusion agar base) prepared by Paul R. Burkholder during the 1958-1959 phytoplankton studies in the Bransfield and Gerlache Straits (5) also detected only several organisms per ml in surface water samples. Burkholder (4) found

that low counts of bottom sediments were markedly increased when the medium was supplemented with partially decomposed phytoplankton net hauls before autoclaving. These fragmentary observations on the bacterial content of Antarctic air and sea water confirm the apparent sparsity of bacteria in these habitats when conventional media and procedures are used for cultivation.

MODIFICATION OF THE ENTERIC MICROFLORA OF PYGOSCELID PENGUINS BY PHAEOCYSTIS PHYTOPLANKTON BLOOMS

The failure to detect a sizeable air or sea water microflora as well as the numerous reports on the frequent occurrence of "bacteriologically sterile" Antarctic animals focused attention on the gastrointestinal microflora of Antarctic birds (16). The presence of 10^4 to 10^7 organisms per gram in all gastrointestinal segments of some birds while some of the pygoscelid penguins did not contain a detectable microflora in their anterior segments, indicated that either the dietary materials contained an inhibitory substance or that the diet had a negligible inoculum. Unlike the other birds studied, the pygoscelid penguins did not contain typical lactose-fermenting strains of *Escherichia coli*. Direct microscopic examinations revealed an abundant dietary microflora which could not be cultivated. Antibacterial assays of the euphausid diet of the pygoscelid penguins indicated that the lack of growth was associated with inhibitory activity. An example of the correlation of the number of cultivable bacteria (mainly lactose-fermenting gram-negative organisms) with the antibiotic activity of the ingesta is given in Figure 1, taken from Sieburth (18). The euphausid stomach contents of this gentoo penguin, which were highly inhibitory, failed to yield a detectable microflora. The central intestinal contents contained a considerable bacterial population and failed to inhibit the test organisms. Although the terminal intestinal contents also contained a large bacterial population, they were somewhat inhibitory, presumably as a result of bacterial by-products.

The antibiotic activity of the euphausids *(Euphausia superba)* was traced to their stomach contents and then to phytoplankton blooms in which a green mucilaginous colonial alga dominated

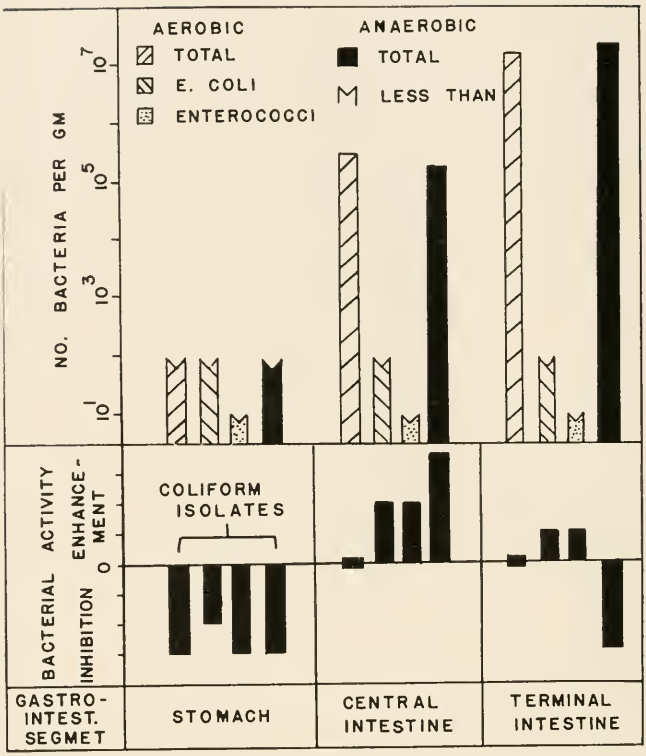

Fig. 1.

(17). These observations were confirmed by a second field study during which the alga was identified as *Phaeocystis pouchetii* and algal concentrates were collected for laboratory studies (24).

The antibiotic principle of *Phaeocystis pouchetti* was found to be a volatile acid which was isolated as the sodium salt (20) and later identified chemically, physically and biologically as acrylic acid, $CH_2=CH-COOH$ (21). The effect of synthetic sodium acrylate on the coliform flora of chicks is shown in Figure 2. The acrylate level of 0.1 per cent which approximates that estimated to be naturally ingested by penguins had a marked effect on the suppression of *Escherichia coli*. Further antibiotic properties of acrylic acid have been studied in vitro and in vivo (23).

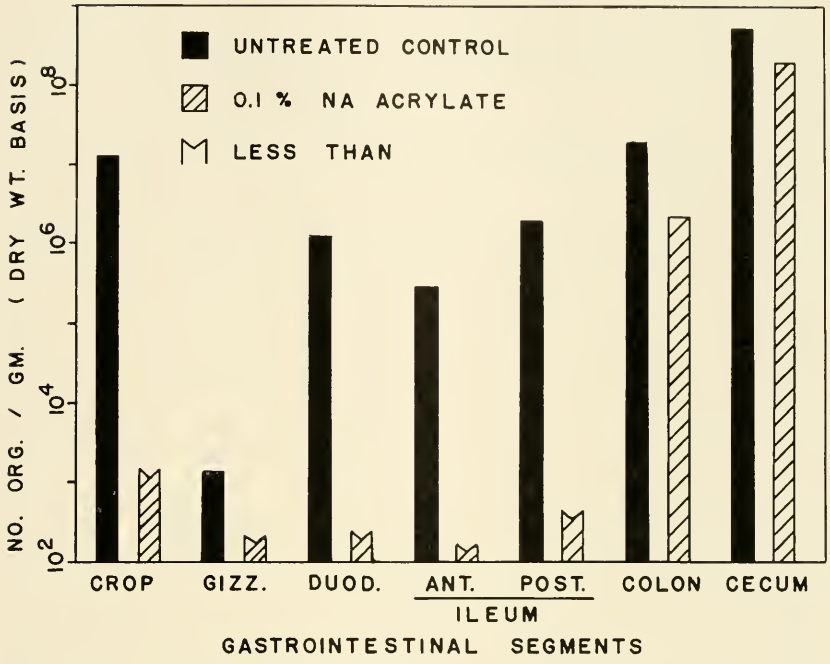

Fig. 2.

ROLE OF ALGAL VOLATILE ACIDS IN THE NUTRITION AND INHIBITION OF PLANKTONIC MARINE BACTERIA

The sparsity of bacteria in the euphotic zone of Antarctic marine waters, which are usually highly productive in phytoplankton, has been noted by the earlier Antarctic expeditions as well as by Lebedeva (9) and Kriss *et al.* (7), and in the observations made earlier in this paper. The finding that certain phytoplankton blooms contain an antibacterial acid suggests that the well recognized bactericidal property of sea water reviewed by Carlucci and Pramer (6), may be due to substances of biological origin (12, 13). However these "ectocrines" (10) have been poorly defined and many phenomena such as the dominance and succession of algal blooms have been ascribed to such substances without adequate substantiation.

An immediate objection to attributing the sparsity of marine bacteria in Antarctic waters to the high acrylic acid content (8% dry weight) of the Phaeocystis blooms (21) is that an acidic

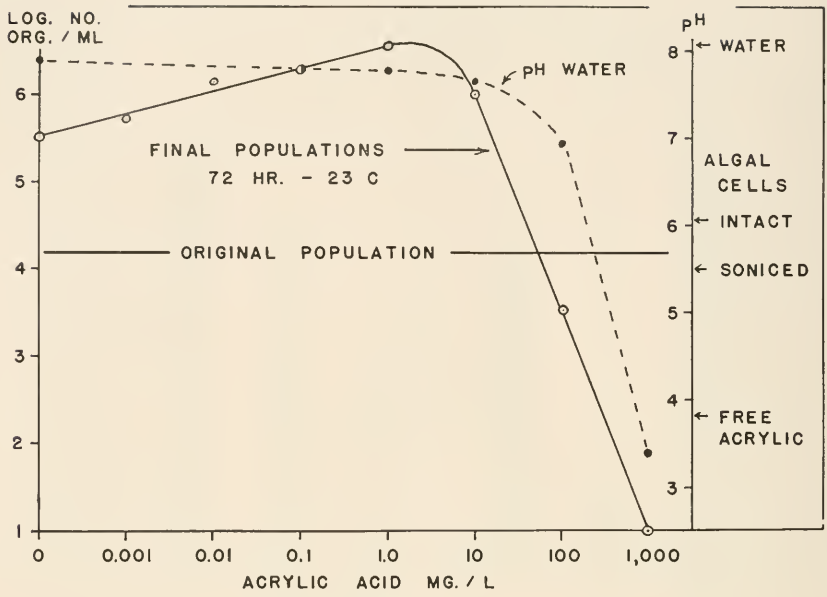

Fig. 3.

medium such as the avian gut enhances the antibacterial activity, while sea water is highly buffered and has an alkaline pH (8.0 + .5). In an attempt to determine the effect of free acrylic acid upon a natural population of marine bacteria, sea water dilutions of acrylic acid were added to aliquots of aseptically collected samples of water from Narragansett Bay (R.I.) and counts were made on the original population and after three days incubation at 23 C. The original and final populations of marine bacteria at the various acrylic acid concentrations as well as the original pH values of the sea water of a representative trial are given in Figure 3. At very small concentrations, 1-100 μg/liter, acrylic acid appeared to enhance growth. Only at concentrations above 10 mg/liter where the acid decreased the pH of the water did acrylic acid suppress growth. Mean pH values of Antarctic sea water, intact algal cells, sonic disrupted cells, and the free acrylic acid fraction of Phaeocystis net hauls are also given in Figure 3. Since the pH of the fresh intact cells was acidic (pH 6.05) it is very possible that the microinterface between the algal cell and the sea water is also acidic and antibacterial as a result of acid excre-

tion by actively metabolizing cells. Therefore it is possible that algal acids may provide a nutrient source for marine bacteria as well as serve as a protective mechanism against bacterial decay of the metabolizing algal cell.

A procedure similar to that used to isolate acrylic acid (21) has been used to obtain the volatile fatty acid fraction from sea water. Preliminary studies during winter phytoplankton blooms in Narragansett Bay indicate that most of this fraction is acetic acid and that values of 30 mg/liter of sea water are not uncommon. Supplementation of artificial sea water with this level of acetic acid and ammonium salts as a nitrogen source is sufficient to support the growth of marine bacteria contained in natural sea water inocula. Sea water media containing acetate, ammonium salts and water soluble vitamins are capable of yielding counts of 10^4 organisms/ml which equals or at low temperatures exceeds the counts on ZoBell's medium (14). It is possible that further studies will reveal that the apparent absence of marine bacteria in the higher latitudes is due to a requirement for nutrients of algal origin rather than of animal origin which support good growth in tropic and sub-tropic areas (7).

BACTERIAL CHANGES IN PENGUIN GUANO UNDER NATURAL CONDITIONS

Penguin feces which form guano deposits at rookeries apparently undergo bacterial decomposition at ambient temperatures (<5 C) to yield a "humus" that supports a bryophytic flora. In an attempt to characterize bacterial changes which occur in penquin fecal material under natural conditions, penguin guano in various stages of decomposition as well as soil samples were studied at penguin rookeries on three islands in the South Shetland group. The samples were aseptically collected and immediately brought back to the ship for pH determinations, for cup antibacterial assays against *Staphylococcus aureus* and *Sarcina lutea*, and for the enumeration of bacterial types. Tenth ml aliquots of serial decimal dilutions in tryptose broth were used to surface inoculate slants of heart infusion agar (Difco) (incubated both aerobically and anaerobically by the method of Parker (15) for total counts), tergitol-7 agar (for coliforms) and azide agar

TABLE 1

THE pH, ANTIBACTERIAL ACTIVITY, BACTERIAL CONTENT, BIOCHEMICAL ACTIVITY AND DOMINANT MICROFLORA OF FECAL MATERIAL FROM A RINGED PENGUIN ROOKERY ON HALF MOON ISLAND, SOUTH SHETLAND ISLANDS

					No. org. per gram (wet weight)				*Biochemical Activity of Dominant Microflora*					
			Antibacterial Activity mm		*Total*			*Bacilli*	*Saccharolytic*		*Proteolytic*		*No.*	
Sample	*Material*	*pH*	*S. aureus*	*S. lutea*	*Aerobic*	*Coliforms*	*Cocci*	*(Apparent)*	*CHO'S*	*Milk*	*Gelatin*	*Milk*	*Isolates*	*Tentative Identification*
A	Adult feces	5.7	1.0	3.0	8.0×10^5	3.6×10^5	5×10^2	$<10^2$	6	1	1	0	7	Pseudomonads (6) *Sarcina aurantica* (1)
B	Chick feces	6.0	1.0	5.0	6.8×10^4	$<10^2$	$<10^2$	$<10^2$	2	5	0	0	7	Diphtheroids (6) *Escherichia coli* (atyp.-1)
C	Nest guano	9.1	9.0	6.5	1.2×10^5	1.8×10^3	3.5×10^4	$<10^2$	0	5	0	0	6	Diphtheroids (6)
D	Nest run-off	9.2	17.0	8.0	1.1×10^5	3.5×10^3	9.0×10^4	$<10^2$	0	6	0	0	6	Diphtheroids (6)
E	Guano pool	8.8	8.0	5.0	1.0×10^5	1.0×10^2	2.0×10^3	1.3×10^4	2	4	1	2	6	Diphtheroids (2), Pseudomonad (1), Bacillus (1), *E. coli* (typ.-1) *Sarcina ureae* (1)
F	Guano "Humus"	5.8	0	0	6.2×10^4	1.5×10^4	1.3×10^4	7.0×10^3	5	5	5	5	5	*Bacillus tinakiensis* (5)

TABLE 2

THE pH, ANTIBACTERIAL ACTIVITY, BACTERIAL CONTENT, BIOCHEMICAL ACTIVITY AND DOMINANT MICROFLORA OF FECAL MATERIAL FROM A GENTOO PENGUIN ROOKERY ON KING GEORGE ISLAND, SOUTH SHETLAND ISLANDS

			Antibacterial Activity mm		*No. org. per gram (wet weight)*					*Biochemical Activities of Dominant Microflora*					
					Total Counts				*Bacilli*	*Saccharolytic*		*Proteolytic*		*No.*	*Tentative*
Sample	*Material*	*pH*	*S. aureus*	*S. lutea*	*Aerobic*	*Anaerobic*	*Coliforms*	*Cocci*	*(Apparent)*	*CHO'S*	*Milk*	*Gelatin*	*Milk*	*Isolates*	*Identification*
G	Chick feces	7.1	0	0	2.1×10^{6}	1.8×10^{6}	2.1×10^{6}	$<10^{2}$	$<10^{2}$	3	5	1	1	6	Diphtheroids (3), Providence group (2), Pseudomonad (1)
H	Vacant nest	8.6	8.0	6.0	2.5×10^{4}	1.0×10^{4}	$<10^{2}$	$<10^{2}$	$<10^{2}$	7	7	7	7	7	*Bacillus pumilus* (7)
J	Guano pool	8.1	7.0	5.0	4.3×10^{4}	1.8×10^{3}	9.5×10^{3}	1.5×10^{3}	$<10^{2}$	8	5	2	0	8	Micrococcus (6), *Achromobacter guttatus* (2)
L	Dry feces	6.3	0	0	1.3×10^{4}	7.0×10^{2}	$<10^{2}$	$<10^{2}$	$<10^{2}$	5	0	0	0	8	*Mycococcus albus* subsp. *lactis* (5), *Bacillus sphaericus* (3)
I	Old nest guano	4.1	0	0	1.0×10^{4}	1.4×10^{5}	$<10^{2}$	1.8×10^{3}	6.0×10^{2}	3	3	3	3	3	*Bacillus tinakiensis* (3)
K	Guano "Humus"	6.6	0	0	1.3×10^{6}	7.0×10^{4}	4.8×10^{5}	1.4×10^{4}	1.0×10^{3}	–	–	–	–	–	No viable isolates

TABLE 3

THE pH, ANTIBACTERIAL ACTIVITY, BACTERIAL CONTENT, BIOCHEMICAL ACTIVITY AND DOMINANT MICROFLORA OF FECAL AND "SOIL" MATERIAL FROM A RINGED PENGUIN ROOKERY ON THE VOLCANIC ASH OF DECEPTION ISLAND, SOUTH SHETLAND ISLANDS

			Antibacterial Activity mm		*No. org. per gram (wet weight)* *Total Counts*					*Biochemical Activities of Dominant Microflora*					
Sample	*Material*	*pH*	*S. aureus*	*S. lutea*	*Aerobic*	*Anaerobic*	*Coliforms*	*Cocci*	*Bacilli (Apparent)*	*Saccharolytic CHO'S*	*Proteolytic Milk*	*Gelatin*	*Milk*	*No. Isolates*	*Tentative Identification*
M	Fresh feces	6.5	0	0	4.0×10^{7}	4.4×10^{7}	3.9×10^{7}	$<10^{2}$	$<10^{2}$	9	9	0	0	9	*Escherichia coli* (Typical 9)
N	Vacant nest	8.4	0	0	1.5×10^{5}	1.8×10^{4}	$<10^{2}$	$<10^{2}$	3.0×10^{4}	6	2	2	2	9	Sarcina (5), *Bacillus tinakiensis* (2), *Micrococcus conglomeratus* (1), Mycococcus (1)
O	"Soil" above rookery	7.1	0	0	9.6×10^{4}	1.2×10^{5}	6.0×10^{2}	3.0×10^{4}	$<10^{2}$	5	5	0	0	5	Mycococcus (5)
P	"Soil" between nests	5.6	0	0	3.0×10^{2}	1.0×10^{3}	$<10^{2}$	3.0×10^{2}	$<10^{2}$	–	–	–	–	–	None viable
Q	"Soil" between nesting areas	8.5	0	0	9.9×10^{4}	1.5×10^{4}	1.3×10^{3}	1.6×10^{3}	$<10^{2}$	7	7	0	0	7	Mycococcus (3), *E coli* (Typ.-2) *Streptococcus faecalis* (2), *Sarcina ureae* (1)
R	Pond between rookeries	7.1	0	0	$<10^{2}$	$<10^{2}$	$<10^{2}$	$<10^{2}$	$<10^{2}$	–	–	–	–	–	None detected

(for enterococci). Counts were made after three days incubation at 25 C. Isolates from the countable slants were used to determine the taxonomic and physiological types in the dominant flora. A summary of the results for each of the three rookeries studied is given in Tables 1, 2 and 3.

The ringed penguin rookery studied at Half Moon Island (62°36′07″ S; 58°52′20″ W on 4 February 1959) was situated on a rocky bluff and contained late nesting chicks and adults. The data are shown in Table 1. The freshly voided feces were acidic (samples A and B) while the fresh nest guano, guano from around the nests and a pool of fecal material (samples C, D and E) were alkaline and quite inhibitory to the test organisms. The guano "humus" (sample F), which probably resulted from protracted bacterial action and weathering, was acidic. Despite the differing age and condition of the materials, the total bacterial counts were somewhat constant (10^4 to 10^5 organisms per gm). The coliform and enterococci counts of the decomposing material indicated a surprising persistence and even increase in these "fecal" types. Their presence in the soil "humus" may indicate preservation by the cold temperatures rather than a continuing development during the decomposition of fecal material. The isolates from the dominant flora indicated that there was a transition from a fecal flora (pseudomonads and diphtheroids) to a guano flora (diphtheroids) to a highly mixed flora in the guano pool to a Bacillus flora in the guano "humus." The fecal and guano flora was saccharolytic while the proteolytic activity of the "humus" was due to *Bacillus tinkiensis* (8).

A gentoo penguin rookery located in a somewhat sheltered rocky area of King George Island (62°09′ S; 58°07′ W), which consisted mainly of chicks past the nesting stage, was studied 9 February 1959. The results are given in Table 2. Desiccated feces from the vertical slope of a rock (sample L), old nest guano (sample I) and guano "humus" (sample K) were acidic and devoid of antibacterial activity. Material from a vacated nest and a guano pool (samples H and J) were alkaline and antibacterial to the test organisms. As in the previous rookery, the total aerobic counts were similar (10^4 to 10^6 organisms per gm) while the coliform and enterococci populations of the various samples

were highly variable. The dominant flora of freshly voided chick feces consisted of diphtheroids and gram-negative rods in contrast to the desiccated feces which contained a "soil-like" flora of *Micrococcus albus* subsp. *lactis* and *Bacillus sphaericus*. The guano pool contained *Micrococcus* species and *Achromobacter guttatus* while a recently vacated nest and old nest guano were dominated by *Bacillus pumilus* and *Bacillus tinakiensis* respectively. These latter two organisms were proteolytic while most of the other isolates were only saccharolytic.

A second ringed penguin rookery containing birds past the nesting stage was studied at Deception Island (63°00′ S; 60°45′ W on 14 February 1959). The data are summarized in Table 3. The volcanic ash which covers most of this crater island apparently prevents the accumulation of guano which did not exceed a quarter inch in depth in most areas of the rookery. In contrast to the other rookeries, the alkaline samples were devoid of antibacterial activity, and the total bacterial counts of the different samples varied considerably. Unlike most pygoscelid penguins studied, the pooled sample (M) of adult and chick feces was dominated by lactose-fermenting strains of *E. coli*. Material from the vacant nest (sample N) contained a mixed flora of Sarcina, Micrococci and Mycococcus species as well as the proteolytic organism, *Bacillus tinakiensis*. The "soil" on a steep incline of a ridge high above the rookery (sample O), which was free of direct penquin contamination, contained a dominant Mycococcus flora (10^5 organisms per gm.) and a minor population of *E. coli* ($6x10^2$/gm). However the "soil" between nests (sample P) had only a small population (10^3/gm) of fastidious organisms. The "soil" in a ravine between nesting areas (sample Q) contained a mixed "soil" and "fecal" flora of 10^5 organisms per gm. An adjacent pond which was green with an algal bloom was free of detectable bacteria.

DISCUSSION

The nutritional and temperature requirements of marine bacteria in the phytoplankton rich waters of Antarctica require further study. The ability of marine bacteria to use algal acids such as acrylic and acetic as a carbon source indicates that the ap-

parent sparsity of marine bacteria may be due to the use of animal peptone media as well as elevated temperatures and short incubation periods commonly used in shipboard surveys (7), rather than to a lack of a bacteria.

Waksman (27) has pointed out that under natural conditions antibiotics play no significant role except when the habitat is relatively simplified such as within the gastrointestinal tract of animals. The acidic gut of the pygoscelid penguins was optimal for the detection of the acid potentiated antibiotic activity of acrylic acid from the alga *Phaeocystis pouchetii*. However the acidic nature of phytoplankton net hauls and the concentration of small carbon acids produced by marine algae indicated that the micro-interface of the algal cells may have a sufficiently acidic reaction even in sea water to permit these acids to act as bacteriostatic compounds. Such a phenomenon might protect metabolizing algal cells against bacterial attachment and decay.

The studies on fecal and "soil" material from penguin rookeries on three different islands in the South Shetland group indicate that bacterial decomposition does occur at the cool ambient summer temperatures. Studies to date, such as that by Straka and Stokes (26), report the use of frozen samples of undetermined age and nature to enumerate and isolate psychrotolerant microorganisms. The acidic feces which contained a variable fecal flora apparently underwent rapid bacterial decomposition as indicated by the alkaline reaction and antibacterial activity (presumably as a result of ammonia formation) of fresh guano in occupied nests. Nest guano had a "fecal," mixed "fecal and soil," or "soil" flora depending upon age and nesting activity in the rookery. Various organisms such as *Sarcina* and *Micrococci* which are minor components of the fecal flora became dominant. Old nest guano, soil and soil "humus" which was acidic in nature contained organisms similar to *Mycococcus albus* subsp. *lactis* and *Bacillus tinakiensis* (8). Many of the isolates required incubation temperatures below 20 C after the initial subculture. Changes in pH and antibacterial activity and a transition from a "fecal" to a "soil" flora in material of various ages and states of decay indicated that at least certain components of the bacterial flora

were active and able to decompose the fecal material at near freezing temperatures.

SUMMARY

Studies on the gastrointestinal microflora of Antarctic birds indicated a modification and suppression of the microflora of pygoscelid penguins when their euphausid diet was grazing on *Phaeocystis pouchetii* blooms. The "antibiotic" principle of this alga was isolated, identified as acrylic acid, and its anti-coliform activity in the acidic avian gut was verified. Acrylic acid, which both enhanced and inhibited the growth of marine bacteria in natural sea water, depending upon concentration, may serve as both a nutrient for planktonic bacteria and as a protective mechanism against bacterial decay of the metabolizing algal cells. The apparent sparsity of marine bacteria in the euphotic zone may reflect a lack of knowledge of the nutrient-temperature requirements of bacteria associated with the phytoplankton. Penguin feces apparently underwent bacterial decomposition at ambient temperatures as shown by changes in pH, antibacterial activity and a transition from a fecal to a soil microflora in material at various stages of decomposition.

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Chapter 50

Distribution of Heterotrophic Bacteria in Some Seas of the Mediterranean Basin

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and J. A. Gorbenko

The Mediterranean Sea is one of the most poorly explored basins in regard to microbiological research, although investigations were begun there at the end of the last century. The Adriatic Sea has been studied most completely. Researches being carried out by some scientists in Italy, France and Spain mainly concern the study of microflora of the coastal regions of the west part of the basin. The literature does not reveal any microbiological study of the Aegean Sea, the Sea of Crete, the Ionian Sea and the Levant Sea. Except for the Adriatic Sea, such microbiological counts as exist are single observations made many years ago; they do not give an adequate picture of the development of bacterial life in the open regions of the Mediterranean Basin.

MATERIAL AND METHODS

During the oceanographic researches carried out for the International Geophysical Year from July 17 to October 9, 1959, in the Mediterranean Sea by the Sevastopol Biological Station of the Academy of Sciences of the U.S.S.R. on board the ships ACADEMICIAN KOVALEVSKY and ACADEMICIAN VAVILOV, Institute of Oceanology, Academy of Sciences of the U.S.S.R., the quantitative distribution of heterotrophic bacteria at different depths of the water mass was studied. Stations were in the open regions of the following seas: The Sea of Crete (3 stations), the Levant Sea (21), the central part of the Mediterranean (13), the Ionian Sea (7), the Tyrrhenian Sea (10), the Otranto Strait (3) and in the Strait of Tunis (6). (See Fig. 1.) Samples were obtained with a bathometer from standard hydrological horizons (0, 10, 25, 50, 75, 100, 150, 200, 250, 300, 400, 500, 750, 1000,

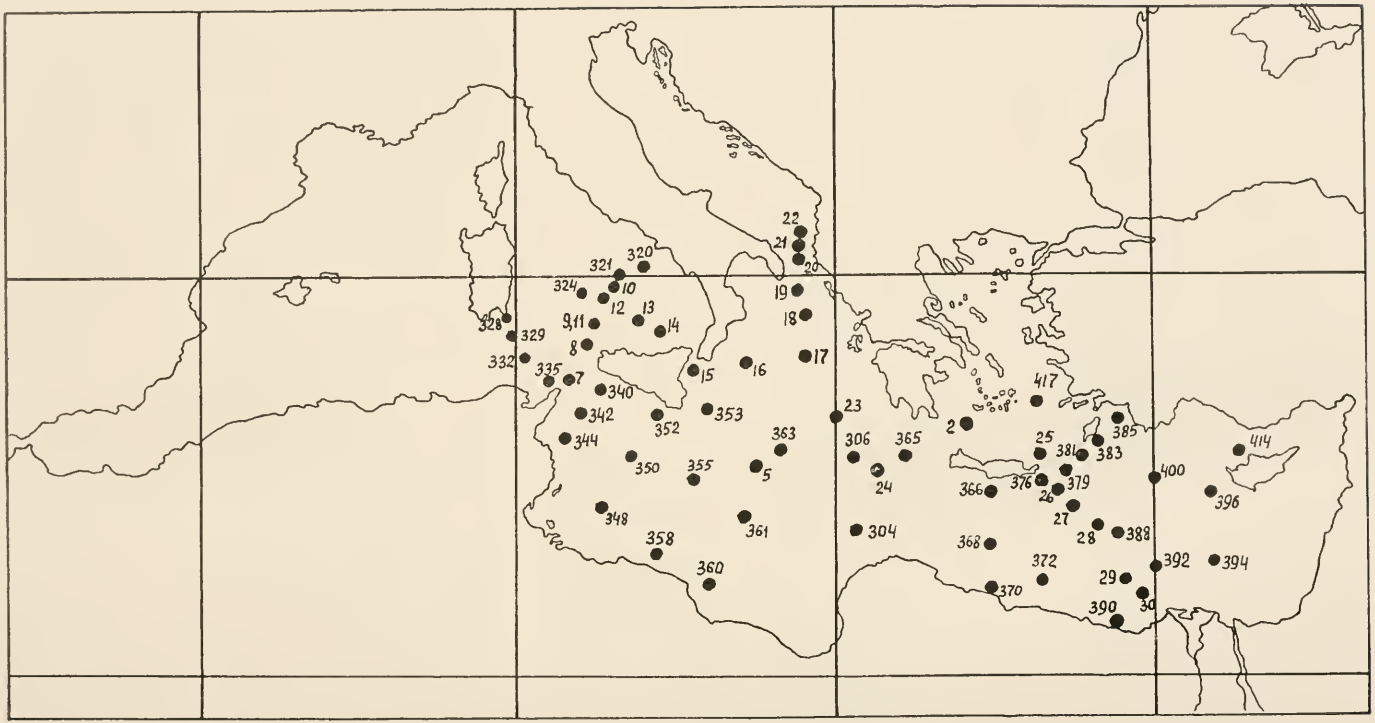
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Fig. 1

1250, 1500, 2000, 2500, 3000, 3500, 4000 m.). A 40 ml sample was passed through a membrane ultrafilter no. 2 and was grown on a medium of dry nutrient agar* (50 g per liter of sea water).

RESULTS AND DISCUSSION

A comparison of the Mediterranean regions investigated, shows that the richest, according to the development of albuminate-decomposing bacteria, are straits. The highest counts came from the Otranto Strait and the next from the Strait of Tunis. The largest numbers of bacteria were recorded in these two places; as Table 1 shows, the sum of stations having 100 colonies and over amounts of 81.7 per cent and 66 per cent respectively. This is attributed to the strong current of the straits, since it is known that where whirlpools form at the edge of currents, various organic residues are readily accumulated, and such residues provide the basis for the development of saprophytic bacteria (5-6-7-9-13). The stations of the Otranto Strait show a higher number of bacteria at almost all depths, the maximum quantities of heterotrophs (from 1000 to 2904) are found over the greater part of the depths sampled. In the Strait of Tunis, bacterial numbers are usually from 101 to 500; samples containing 501-1000 bacteria being found relatively seldom, and only in one case were more than 1000 (1100) bacteria counted.

It should be noted that the highest counts of bacteria were obtained at the 300 m horizon, next in order were the 100 m, 25 m, and the 0 m levels. It is noteworthy that the greater development of phytoplankton (from 1000 to 4000 Coccolithophorideae per ml) near Sicily was found in July 1955 between 300 and 400 m (4).

The Levant Sea and the Tyrrhenian Sea are approximately equal as regards the quantitative development of heterotrophic bacteria: in both seas half of the samples (40% and 49.3%) gave from 51 to 500 bacteria; 3.1 per cent and 3.4 per cent gave from 501 to 1000, and about 3 per cent gave more than 1000 (to 1550). In an equal number of cases (43.6 per cent and 44 per cent) the minimum number of bacteria was recorded. In the Levant Sea,

* The hydrolysate of fish meal obtained by working up with ferment trypsin (60% hydrolysate, 40% agar-agar).

TABLE 1

Frequency of Occurrence of Heterotrophic Bacteria (in Percentage) in Different Regions of the World Ocean (in 30-50 ml of Water)

No.	Seas	The Quantity of Filtered Water	Date of the Expedition	Stations (in All)	Samples (in All)	M Depth 0
1	The Greenland Sea (Kriss, 1959, table 125-126)	40	July-August 1956	34	451	44.8
2	The deep-water stations in the Sea of Okhotsk and in the northwest part of the Pacific Ocean (Kriss, 1959, table 10)*	35	1951	3	61	52.5
3	The north-west part of the Pacific Ocean (Kriss, 1959, table II)*	30-40	1953	9	184	55.4
4	The central part of the Pacific Ocean (Kriss, 1959, table 112-118)*	50	1957 1958	55	1027	6.5
5	The Antarctic sector of the Indian Ocean (our own material)	40	Feb.-Apr. 1957	30	536	46.5
6	The Subtropics and tropics of the Indian Ocean (our own material)	40	Apr.-May 1957	31	581	12.8
7	The Black Sea (Kriss, 1959, table 19)	20**	June 1950	6	85	
8	The Otranto strait (our own material)	40	Aug. 25-29 1959	3	38	2.6
9	The strait of Tunis (our own material)	40	July 26-Aug. 14 1959	6	53	3.8
10	The Levant Sea (our own material)	40	Sept. 2-Oct. 7 1959	18	261	2.3
11	The Tyrrhenian Sea (our own material)	40	July 30-Aug. 16 1959	10	146	4.1
12	The central part of the Mediterranean Sea (our own material)	40	July 19 to Sept. 15 1959	13	160	6.9
13	The Ionian Sea (our own material)	40	Aug. 22-Sept. 12 1959	7	107	7.5
14	The Sea of Crete (our own material)	40	July 17, Sept. 23, Oct. 9, 1959	3	35	14.3

*The estimations have been made by us from tabular data of Kriss's researches (1959) and on the grounds of our own figures obtained during the cruise of diesel electric ship the "Ob" over the Indian Ocean.

**Recomputation has been made for 40 ml.

TABLE 1

Number of Colonies									
		M Depth							
1	*2-10*	*11-50*	*51-100*	*101-500*	*501-1000*	*Over 1000 (to 3000)*	*Continuous growth*	*A lot of colonies*	*Merging growth*
11.3	23.9	13.3	3.1	2.2	0.2	0.4	0.7	0	0
24.6	19.7	1.6	0	1.6	0	0	0	0	0
17.4	19.5	4.4	2.2	0	0	0	1.1	0	0
5.0	15.7	23.7	16.1	33.1	0	0	0	0	0
11.9	20.3	8.8	2.1	6.3	3.5	0.6	0	0	0
5.7	13.4	9.1	6.6	19.8	19.1	12.1	1.4	0	0
0	1.2	10.6	16.5	41.2	0	0	0	10.6	20.0
2.6	2.6	5.3	5.3	21.1	23.7	36.9	0	0	0
1.9	5.7	15.1	7.5	52.8	9.4	1.9	1.9	0	0
0.4	10.4	30.6	12.3	37.5	3.1	3.1	0	0	0
2.1	15.1	22.9	13.8	35.8	3.4	1.4	1.4	0	0
5.6	21.2	18.1	13.1	31.2	1.9	0	1.9	0	0
6.5	18.5	30.8	2.8	24.3	6.5	2.8	0	0	0
5.7	14.3	31.4	5.7	28.6	0	0	0	0	0

our attention was drawn to stations 29 and 30, lying close to the mouth of the Nile. Here the largest numbers of heterothrophs were obtained and large numbers were observed over the whole water mass. It leads one to expect that the Nile waters penetrate into the open sea for at least 40 miles, and that the main stream of water goes in a north-west direction. If one takes into consideration that the stations were occupied in September when the Nile was in high flood (14) and as a consequence, the maximum run-off is taking place, a considerable development of bacterial life is possible, perhaps indicating its influence upon almost all the horizons (except 150 and 750 m) at the deep-water station 388 (123 miles from the mouth of the Nile). The central part of the Mediterranean Sea and the Ionian Sea are deficient in saprophytic bacteria compared with the above mentioned seas, judging by the frequence of occurrence of samples giving low counts. Thus the colony count from 0 to 10 occurs in 33.7 per cent and 32.5 per cent of the samples; 31.2 per cent and 33.6 per cent had counts from 11 to 100 bacteria; and approximately the same percentage (33% and 33.6%) had from 100 to 2500. Thus these seas are approximately equal in numbers of heterotrophic bacteria. In the Ionian Sea, stations 18 and 19, situated close to the Otranto Strait, gave the highest bacterial counts; in the central part of the Mediterranean, the shallow stations 342, 350 and 335, lying in the regions where the Atlantic waters make their influence felt, yielded the largest number of colonies.

In the deep water stations a sharp increase in bacterial counts at great depths, up to 2500-3500 m, have been observed. In some regions of the Mediterranean Sea, the number of heterotrophic bacteria in the depths often considerably exceeds the number found in the layer of photosynthesis. According to Buljan's hypothesis, submerged eruptions of volcanoes in the Tyrrhenian Sea are of great importance to the enrichment of deep waters. From these sources, nutrients are carried by currents all over the Mediterranean Sea, and this is partly the reason for the greater development of phytoplankton at great depths (15). Hence the richness of bacterial life in the deep water regions of the Mediterranean basin becomes clear. As far as it is possible to judge from the three stations occupied, the Sea of Crete has the

smallest bacterial population. Here, sterile samples of water have been found most often (14.3%), while 20 per cent of the samples contained single numbers of bacteria (from 1 to 10), and 37.1 per cent contained from 11 to 100. The maximum figure was 382 colonies.

A comparison of the quantitative distribution of saprophytic bacteria at various depths with the vertical distribution of temperature and salinity does not show any direct relation with these factors; this confirms previous observations by us for other sea basins (11, 12). On the whole, for the vertical distribution of heterotrophs in the Mediterranean Sea, as for other seas and oceans, microzones are typical. It reflects the distribution in the water of forms of organic matter available for these organisms (9, 11). However, there is at the same time a general scheme; that is, the presence at any horizon of more or less sharp increases in bacterial numbers in comparison with adjacent horizons. The minimum most often occurs on 10-50 or 50-75, 250, 400 or 500 (seldom 750), 1250 or 1500, 2000 or 2500, 3000 or 4000 m. The maximum is usually observed at 0, 25, 100 or 100-150 (seldom 200), 300 or 400, 500 or 750, 1000 or 1250, 1500 or 2000, 2500 m.

One may observe a certain lack of maximum and minimum zones relative to each other on the horizons of some stations deeper than 250 m. The minimum at 10, 50 and 250 m; and the maximum at 0, 25 and 100 m are observed with the highest accuracy and highest frequency.

As is known, the quantitative distribution of heterotrophic microorganisms gives a picture of the distribution of available forms of suspended and dissolved organic matter, which, in turn, are determined by the hydrological regime of the basin (9). Therefore, one can say with confidence that the typical regularity of the quantitative distribution of heterotrophic bacteria, as here reported, is a reflection of the hydrological structure of the regions of the Mediterranean Sea under investigation. In Table 1, the Mediterranean Sea is compared with other seas and oceans with respect to the quantitative distribution of saprophytic bacteria as studied by the filter method. It is seen that the number of heterotrophs in the Mediterranean basin considerably exceeds those found in the regions of the North Pole, the Greenland Sea,

the deep-water stations of the Sea of Okhotsk and the north-west part of the Indian Ocean. Except for the Sea of Crete they are not inferior to the central part of the Pacific Ocean and are only a little inferior (excluding the stations of the Otranto Strait) to the subtropics and the tropics of the Indian Ocean and the Black Sea.

Taking into consideration that the quantitative development of heterotrophs is determined by the presence of the forms of organic matter assimilated by this group, the main source of which is phytoplankton (1, 2, 8, 10), it can be inferred that the Mediterranean Sea is not poor in this form of life.

SUMMARY

Regions of the Mediterranean Sea studied by us differ from one another with respect to the quantitative development of saprophytic bacteria. The richest parts are straits: first, the Otranto Strait and then, the Strait of Tunis. In decreasing order of the number of bacteria present are: the Levant Sea, the Tyrrhenian Sea, the central part of the Mediterranean Sea, the Ionian Sea and the Sea of Crete. As in other marine basins which have been investigated, microzonation is observed in the distribution of heterotrophs, and no relation has been found between their quantitative distribution at different depths and the vertical distribution of temperature and salinity. The character of distribution of maxima and minima in bacterial numbers at different depths is similar in different stations, and suggests a general stratification of the whole mass in the Mediterranean Sea. As regards the quantity of heterotrophs, the parts of the Meriterraneon basin studied by us usually exceed considerably the Arctic and the Antarctic regions of the world ocean. With the exception of the Otranto Strait, they are only a little less numerous than in the subtropics and tropics of the Indian Ocean and the Black Sea. The riverflow into the Mediterranean is relatively small so that the main source of organic matter for bacteria is apparently phytoplankton. Thus, taking into consideration the considerable development of saprophytic bacteria, it may be inferred that the Mediterranean Sea is not very poor in plant life. This is in accordance with the data of Bernard (3) who has found that the southern parts of the Mediterranean Sea (from Algeria to Libya)

are, on the average, equal to or higher than the oceans in the productivity of phytoplankton.

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Chapter 51

Studies on the Ecology of a Marine Spirillum in the Chemostat

Holger W. Jannasch

Dynamic phenomena of turnover processes in the sea are closely connected with the dynamic nature of growth of the organisms involved. In the present paper, kinetic studies of bacterial growth under conditions approaching those of the marine environment were made, and the results suggested to be quantitatively comparable to bacterial activities in the sea.

Because of technical difficulties, little is known about the growth of bacteria in extremely dilute media like seawater. In some earlier studies, glucose and peptone were found to become growth limiting below concentrations ranging from 0.1 to 10 mg/L (1, 2, 15). More recently, "threshold" concentrations of different nitrogen sources for growth of bacteria ranging from 0.001 to 0.5 mg/L were obtained by Jannasch (8). In all those studies, however, the quantitative data cannot be expected to represent absolute requirements, but depend on the varying growth conditions in batch culture and on the particular criteria of growth. Recently, the advantages of the continuous culture technique for the study of growth relationships have been demonstrated and discussed in several papers. Its application in microbial ecology seems most promising.

In the chemostat (11) or bactogen (10) one nutrient is, in principle, growth limiting. A continuous flow of medium through the culture vessel results in a self-adjusting steady state in which the dilution rate is equal to the growth rate; conditions of growth being kept constant. This principle allows separate adjustment of two factors at will: the concentration of the growth-limiting substrate in the inflowing medium (S_R) which determines the population density, and the dilution rate (D) which determines the

specific growth rate of the organisms. Although there are considerable data about high substrate concentrations and growth rates, little has been done to assess possible effects which may occur at low population densities in dilute media.

In the present experiments, the growth-limiting substrate has been supplied in decreasing concentrations, the dilution rate being kept unchanged. Ecological approaches were made by applying the known growth constants to experiments using natural seawater as the medium, the total effect of nutrients being the unknown factor.

EXPERIMENTAL

As a suitable organism for continuous culture, a marine Spirillum was selected which was found not to grow on the walls of the flask. It was isolated from decaying seaweed by enrichment with Ca-lactate in liquid culture and final streaking on a corresponding agar medium. It closely resembles *Sp. curvatum* as described by Williams and Rittenberg (14), differing, however, in not being able to use hexoses and such simple nitrogen sources as ammonia, nitrate, and urea.

The base medium was a 3 per cent solution of re-crystallized commercial sea salt at pH 7.8 with the addition of 0.005 per cent phosphate from a buffer solution. Using Na-lactate as the carbon source and l-asparagine as the nitrogen source, the C/N ratio was set at 60, a level at which the nitrogen source was found to be definitely growth limiting. In its highest concentration (starting concentration) the medium contained 46 mMol Na-lactate/L and 1 mMol l-asparagine/L. The absolute concentrations of asparagine are given below. Decreasing nutrient concentrations were prepared by a stepwise dilution of the initial medium by alternating factors of 2 and 5, i.e., 1.0 - 0.5 - 0.1 etc. mMol asparagine/L (respectively 28 - 14 - 2.8 etc., mg asparagine-N/L).

Continuous culture took place in an apparatus similar to that of Novick and Szilard (11). Technical details will be reported elsewhere. The same apparatus was used for measuring the growth constants μ_m (maximal growth rate) and K_s (substrate concentration at which the specific growth rate reaches half of its

maximal value) in batch culture. When used for continuous culture, the flow rate was set and time allowed for the adjustment of a constant population density indicating a steady state. Since the cell concentration was too low for measuring the turbidity, cells were counted microscopically and the growth rate given as division rate. To avoid errors due to varying cell size, two full spiral curves as a unit cell have been counted. The size of such a standard cell varied within a range of 6-8 μ in length and 1-1.5 μ in width. When the cell count decreased below 10^7/ml, counting on membrane filters (7) was employed. When a steady state was definitely established, the culture vessel was connected to another storage bottle containing the next lower nutrient concentration.

Corresponding experiments were conducted using natural, sterile-filtered sea water as medium. The samples taken from two different sources (highly contaminated water from Naples Harbour, and offshore seawater) clearly showed differences in the contents of dissolved nutrients as proved by bioassay. The Spirillum was grown in batch culture and inoculated from the exponential phase into the chemostat. When wash-out was evident, the dilution rate was lowered with the intention of establishing the concentration of the unknown growth limiting factor which would result in a steady state.

RESULTS

The growth constants measured in batch culture at 18 C. were: $\mu_m = 0.37$ hr^{-1} and $K_s = 0.06$ mg asparagine-N/L. The latter has been graphically obtained by plotting reciprocals of four determinations of μ and may be subject to considerable error. With the starting concentration of 28 mg asparagine-N/L in the inflowing medium (S_R) at the set dilution rate (D) of 0.185 hr^{-1} ($=\mu_m/2$), the steady state was established after 6 hr. at a cell concentration of 86 x 10^7/ml (yield determinations showed that both nitrogens of asparagine had been used).

The steady state was maintained for 60 hours and then the culture shifted to the next lower concentration of S_R. The subsequent decrease of the cell count is shown in Figure 1 for the four lowest concentrations of S_R (2.8 - 0.14 mg asparagine-N/L). Values represent means of a triplicate experiment. Re-adjustment

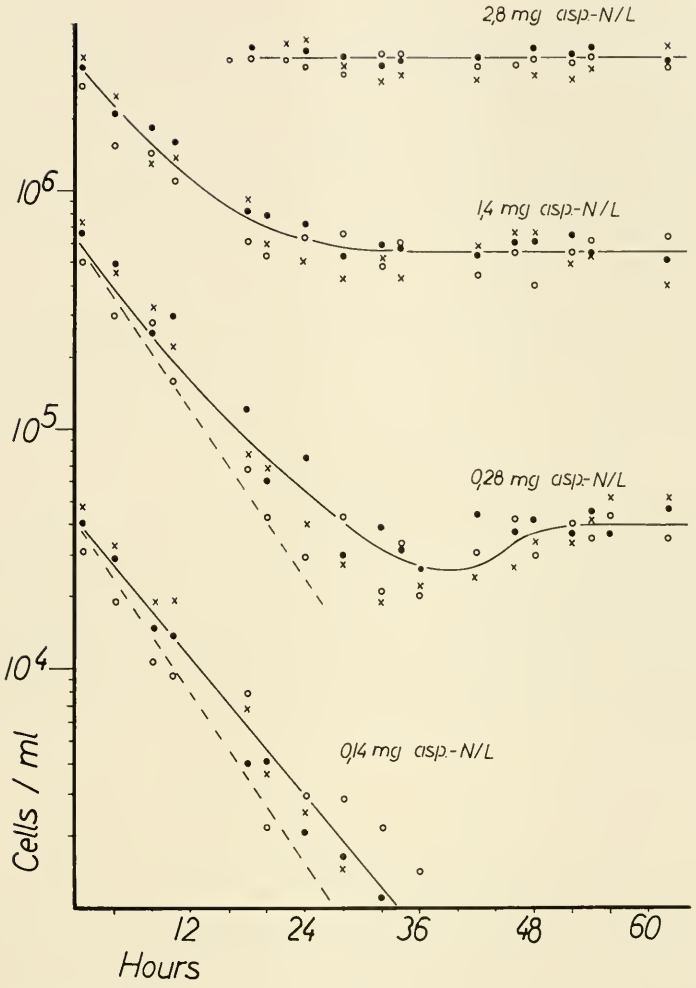

Fig. 1. Readjustment of steady state of the population of *Spirillum sp.* in the chemostat at three different concentrations of growth limiting substrate (given as mg. asparagine-N/L) and final washout at a constant dilution rate of 0.185 hr^{-1} (broken line).

of steady states at higher substrate concentrations indicated no perceptible change of the growth rate. At an S_R of 0.28 mg asparagine-N/L, however, initially the cell count fell almost as fast as the dilution rate. The growth rate apparently did not recover until the cell count had fallen below the value which

finally indicated re-establishment of a steady state. This undershoot phenomenon may be explained by growth rate hysteresis (12) occurring at transient state. It may be attributed to technical properties of open culture systems and will not be discussed in the present paper. In the presence of a substrate concentration of 0.14 mg asparagine-N/L, slow wash-out appeared. In these experiments, wallgrowth seemed unlikely, although complete wash-out never resulted in sterility of the culture vessel.

In experiments using natural seawater as culture medium, no steady state was observed in the range of dilutions tested, which went down to a rate of 0.03 hr^{-1} (a theoretical doubling time of ca. 24 hr). The corresponding batch culture showed appreciable growth only when supplemented with 1 mg asparagine-N/L.

DISCUSSION

Monod's (9) fundamental description of the relationship between growth rate and substrate concentration and its mathematical treatment by Herbert *et al.* (4) has been adopted here to compute the theoretical value of the critical dilution rate (D_c) at which wash-out appears. Under steady state conditions, the relationship between D_c and S_R depends on the growth constants μ_m and K_s:

$$D_c = \mu_m \frac{S_R}{K_s + S_R} \quad (1)$$

From this equation the importance of the ratio S_R/K_s is evident. When S_R is lowered stepwise - enough time being allowed for readjustment of a new steady state - the concentration of the substrate consumed in the culture, S_R-S (S being the substrate concentration in the effluent) also decreases, because S is nearly independent of S_R. As a result, the cell concentration decreases, compensating for the lower substrate concentration available for consumption. This continues until S_R becomes too low to maintain any cell concentration at the given growth rate. At that point, D becomes D_c and wash-out appears.

In the present experiment, D is deliberately set at $\mu_m/2$, i.e. it becomes D_c when S_R is equal to K_s. This simple relationship is derived from rearranged formula (1):

$$S_R = K_s \frac{D_c}{\mu_m - D_c} \quad (2)$$

Assessing D_c by lowering S_R, theoretically represents a new means of evaluating K_s. As the experiments show, however, the exact position of D_c is difficult to determine, due to large fluctuations in the cell count when wash-out approaches.

From formula 2) the theoretical value of S_R, causing wash-out at the given dilution rate, is 0.06 mg asparagine-N/L. The experimental value, however, must be somewhat above 0.14 mg asparagine-N/L (Fig. 1). In other words, as regards a decreasing S_R, wash-out appears earlier than one would expect from the growth constants measured in batch culture. According to Perret (12), there may be several reasons for this deviation. Powell (13) supposes a decrease of viability with decreasing substrate concentration and dilution rate, a phenomenon which contributes to Perret's growth rate hysteresis. In general, interpretation of results obtained with the chemostat at low values of S_R as well as of D involves inherent difficulties as indicated by the frequency function of generation time. Moreover, at growth rates as low as $\mu_m/2$, the cell number may be influenced by an inconstancy of the yield as observed by Holme (6) and Herbert (5).

When the substrate concentration was shifted from 0.28 to 0.14 mg asparagine-N/L, the cell count did not decrease in proportion to the dilution rate, thus still indicating growth. Nevertheless after 60 hours (ca. 11 volume changes of the culture vessel), cell count was practically zero. Large fluctuations of the cell count below 5 000/ml must also be attributed to the increasing statistical error.

Contois (3) in a study of the relation between specific growth rate and population density showed that D_c was independent of S_R, thus making K_s a function of S_R. Although D_c was not determined with numerical exactitude in the present work, the dependence of its decrease on S_R was clearly observed.

The threshold concentrations of the growth-limiting nutrient, as here reported, naturally depend on the given dilution rate. If the dilution rate, beginning at D_c, is lowered at a constant S_R, the substrate concentration in the effluent (S) decreases. This

means an increase of that fraction of S_R available for consumption (S_R-S). Thus, growth occurs again, and a new steady state establishes. Now S_R can be lowered until a new value for D_c is reached. In the sea, a factor similar to the experimental dilution rate must be anticipated which - along with S_R - also determines the relative minimal requirement of a nutrient for bacterial growth.

When the experiments were modified, using natural seawater as the medium and raising the dilution rate, the resulting D_c is believed to characterize the growth relationships between the organisms tested and the total effect of nutrients present. The Spirillum did not show the ability of multiplying at a rate of one generation per ca. 24 hours; even in the sample of extremely polluted sea water from Naples Harbour. Presence of growth inhibiting material is not likely, because growth is easily stimulated by adding nutrients in concentrations derived from the experiments above.

Considering the experimental conditions, it can be suggested that growth of the particular organism used in this study is restricted to environments of higher substrate concentrations and probably spatially limited to a zone surrounding decaying particulate plant material. In support of this assumption, all enrichments from sea water without addition of pieces of decaying seaweed proved unsuccessful.

The communication of these preliminary data is intended to show that experimental reproduction of growth conditions in the chemostat similar to those in sea water may allow close approach to true ecological relationships between marine bacteria and environmental factors.

ACKNOWLEDGMENT

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SUMMARY

Threshold concentrations of the nutrients limiting growth of marine bacteria in seawater are determined by internal factors (growth constants) and external (i.e., environmental) factors.

The present study was confined to the growth of a heterotrophic marine *Spirillum* in a chemostat at low concentrations of the growth-limiting nitrogen source. Complete wash-out of the population, believed to indicate the lower limit of quantitative requirements, occurred in concentrations of 0.14 mg asparagine-N/L or less. This value depends on the arbitrary choice of the dilution rate ($D = \frac{1}{2}\ \mu_m = 0.185\ hr^{-1}$). The experimental value was found to be considerably higher than the theoretical value. This suggests the existence of an absolute threshold concentration of the growth-limiting nutrient at the lowest possible dilution rate.

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Chapter 52

The Bactericidal Action of Sea Water*

David Pramer, A. F. Carlucci and P. V. Scarpino

Great numbers of bacteria enter the oceans by way of surface drainage and sewage outfalls, but as one proceeds outward from land a rapid decrease in numbers of bacteria in the water is observed. ZoBell (10) estimated that the daily flow of sewage into the Pacific Ocean along the west coast of North America carries approximately 2×10^{18} coliform bacteria. This number, if distributed uniformly to a depth of 100 m in all offshore water along the continental slope, is sufficient to give a concentration of coliform bacteria of about 50 per ml. However, few if any are found except in the vicinity of the source material. Dilution, adsorption, aggregation, and sedimentation contribute to the decrease in bacterial counts, whereas death of the coliform organisms is attributed to predation, starvation, and the bactericidal action of sea water.

Ketchum, Ayers, and Vaccaro (8) determined the relative significance of dilution, predation, and the bactericidal action of sea water in reducing the number of coliform bacteria in a tidal estuary. More than 99 per cent of the decrease in coliform count was accounted for, and the bactericidal action of the water was the most important factor contributing to the result.

The bactericidal action of sea water is measured as the difference in survival of bacterial cells in untreated and autoclaved portions of the same water sample, and it has been observed repeatedly that bacteria survive to a greater extent in heat-sterilized sea water than in untreated sea water. Although the benefi-

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cial effect of sterilization by heat has been known for more than 75 years and was reported periodically by various investigators located throughout the world (see review by Carlucci and Pramer, (2)) the identity of the factors responsible for the phenomenon has not been established. Numerous suggestions have been made, but few were tested experimentally. Therefore, studies were initiated in this laboratory to evaluate the contribution of various physical, chemical, and biological factors to the death of *Escherichia coli* in sea water. The majority of the results have been published (1, 3, 4, 5, 6, 7, 9). Although the cause of the bactericidal action of sea water has not as yet been identified, the work has provided some insight into the complex interactions that influence growth and reproduction, as well as those that control survival and cause death of coliform bacteria in sea water.

The reaction, salinity, and low organic matter content of sea water contribute significantly to the death of cells of *E. coli* in a marine environment. Development of the bacterium is limited also by a lack of adequate amounts of nitrogen, phosphorus, and iron. The significance of biological factors has been emphasized by various investigators, who postulated that competitors, predators, phages, and heat-labile toxic substances of biological origin (antibiotics) contribute to the death of bacteria in sea water. Studies in this laboratory showed that *E. coli* was not able to compete effectively with the indigenous marine microflora for organic matter added to sea water. The importance of predation has not as yet been evaluated, but it was demonstrated that reasonable levels of phages and antibiotics do not significantly influence the survival of *E. coli* in sea water unless the water is supplemented with nutrients that support growth and multiplication of the bacterium.

Although one can visualize microhabitats and localized conditions in which biological factors may be of considerable importance, it is doubtful that they contribute significantly to the bactericidal action of sea water. It does not appear that the bactericidal action of sea water can be explained completely or satisfactorily in terms of the destruction or inactivation by heat, of competitors, predators, phages, or antibiotics. Moreover, the suggestion that the increased survival of *E. coli* in autoclaved water

was due to the ability of heat to disrupt and degrade microbial cells and thermolabile compounds, and thereby, to cause an increase in concentration of available nutrients in sea water was tested. Although added organic matter favored the survival of *E. coli* in sea water, the minimum nutrient level required to offset the bactericidal action was greater than that which would be provided by the heat treatment.

Artificial sea water formulated in the laboratory to contain no competitors, predators, phages, antibiotics, or organic nutrients exerted a bactericidal action comparable to that of natural sea water. However, it is not possible at this time to conclude that the factors responsible for the bactericidal action of artificial sea water are identical with those responsible in natural sea water. Present experience indicates that the bactericidal action of sea water is a complex phenomenon. It appears to be due to a combination of factors and the contribution of each to the overall effect may vary with the time and site of sampling. This is illustrated by the results of an experiment in which the survival of cells of *E. coli* in untreated, filtered, and autoclaved portions of each of six different water samples was determined (Table 1). In each of the six samples *E. coli* died more rapidly in untreated than in autoclaved water, but the magnitude of the bactericidal action varied greatly. Likewise, the influence of filtration was not constant. In four of the six samples, survival of the test organism was significantly greater in filtered than in untreated water. If it is assumed that the beneficial effect of filtration resulted from removal of competitors and predators, then their numbers and activities varied from sample to sample. The decreased survival of *E. coli* in the filtered portion of water sample 5 (Table 1) can be explained by the possible but improbable assumption that a beneficial substance or organism was removed by filtration. In two of the six samples (numbers 1 and 6) survival of *E. coli* was the same in filtered and autoclaved water and the bactericidal action may be fully accounted for by competitors and predators that were eliminated by filtration. In two other samples (numbers 3 and 5) filtration did not influence survival and it appeared unlikely that competition and predation contributed significantly to the death of cells in the untreated water. In four of the six samples

(numbers 2, 3, 4, and 5) competition and predation were not adequate to explain the rapid death of *E. coli* in untreated water, since survival in autoclaved water was considerably greater than survival in filtered water.

TABLE 1

THE SURVIVAL OF ESCHERICHIA COLI IN UNTREATED, FILTERED AND AUTOCLAVED PORTIONS OF SIX SAMPLES OF SEA WATER

	Survival After 48 hr. for 6 Samples					
Treatment	1	2	3	4	5	6
	%	%	%	%	%	%
None	2.2	0.7	4.6	22.8	4.4	2.7
Filtered*	8.3	3.8	4.8	30.1	0.6	39.6
Autoclaved	8.4	30.9	64.6	53.6	69.6	38.5

* Using type HA membrane, Millipore Filter Corp., Watertown, Mass.

The beneficial effects of autoclaving over and above those resulting from filtration may have been due to any one or combination of factors that require further study. The significance of biological factors has been overemphasized and the results suggest that greater attention should be focused on the influence of temperature on the physico-chemical characteristics of sea water. The beneficial effect of autoclaving on the survival of bacteria in sea water may be due in part to the influence of heat on such factors as redox-potential, concentration of dissolved gases, and ionic equilibria.

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Chapter 53

Suppression of Bacterial Growth by Sea Water*

Galen E. Jones

INTRODUCTION

The populations of viable bacteria in the sea range from 10^0 to 10^6 cells per ml (21, 22), and are usually less than 10^2 per ml of open ocean water, particularly in high latitudes (4, 7). However, the natural organic matter in sea water will support considerably larger populations, as is indicated by bacterial proliferation in sea-water samples kept in clean containers for a few days (23). These observations suggest that bacterial populations in the ocean are limited by a factor or factors other than the energy source.

There is abundant evidence that viable bacteria which enter the oceans from land drainage and sewage outfalls are soon drastically reduced in numbers (2, 3, 11). Pramer, Carlucci, and Scarpino (13) attribute greater bactericidal significance to physico-chemical than to biological factors in sea water.

The dynamic nature of physical, chemical, and biological balances in the sea was emphasized by the external metabolite theory of Lucas (8), who proposed that organisms may influence the activities of other living things by producing essential micronutrients, by removing inhibitory compounds, or by excreting inhibitory substances. There are now numerous indications that both stimulatory and inhibitory organic compounds are present in sea water (1, 5, 6, 17, 18); see also reviews by Vallentyne (19), Saunders (15), Provasoli *et al.* (14).

The purpose of this investigation was to obtain direct evidence for the effect of sea water from different sources on the growth of certain freshwater and marine bacteria. Previous investigators have studied mortality of pollution bacteria in sea

* Contribution from the Scripps Institution of Oceanography.

water, rather than bacterial growth. In the present study, careful control of all growth parameters, with the source of sea waters as a variable, demonstrates the sensitivity of the test organisms to differences in the water.

MATERIALS AND METHODS

Samples of pelagic sea water were collected at various depths with plastic samplers having a capacity of three to four liters (20). Bottom water samples were obtained by pouring off the water above the sediment collected by a Phleger Bottom Sampler (12), care being taken not to disturb the sediment or to allow any sediment particles to pass into the sample. Surface sea water was obtained off the end of the Scripps Institution Pier (more than 1000 feet beyond high-tide level) with a clean plastic bucket.

The samples were dispensed immediately into sterile, Pyrex reagent bottles, and were examined in the laboratory within four hrs after collection. They were filtered through Whatman No. 2 paper in a Buchner funnel to remove large organisms and particulate matter, and were then filter-sterilized by passage through sterile Morton (UF) fritted-glass filters. Autoclaving was found to diminish the inhibitory properties of sea water.

The test bacteria, *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis,* and *Micrococcus lysodeikticus* (ATCC 4698), have been maintained in the laboratory of Dr. C. E. ZoBell for a period of ten years or longer. *Escherichia coli* was isolated by Mrs. L. R. Berger in 1957 while working in this laboratory, and its identity was confirmed on Levine's EMB agar medium. *Serratia marinorubra* was isolated and maintained in this laboratory (24).

The sea water was tested in three ways with artificial sea water (9) used as the control:

(a) A sea-water concentration of 18 per cent (two ml added to nine ml of stock nutrient Medium I) was tested with the five test bacteria (0.15 ml inocula of twelve hour nutrient broth cultures grown at 30 C). The final concentrations per liter of nutrients were approximately: yeast extract (Difco), 0.82 g; proteose peptone (Difco), 1.64 g; glucose, 8.2 g; K_2HPO_4, 0.82

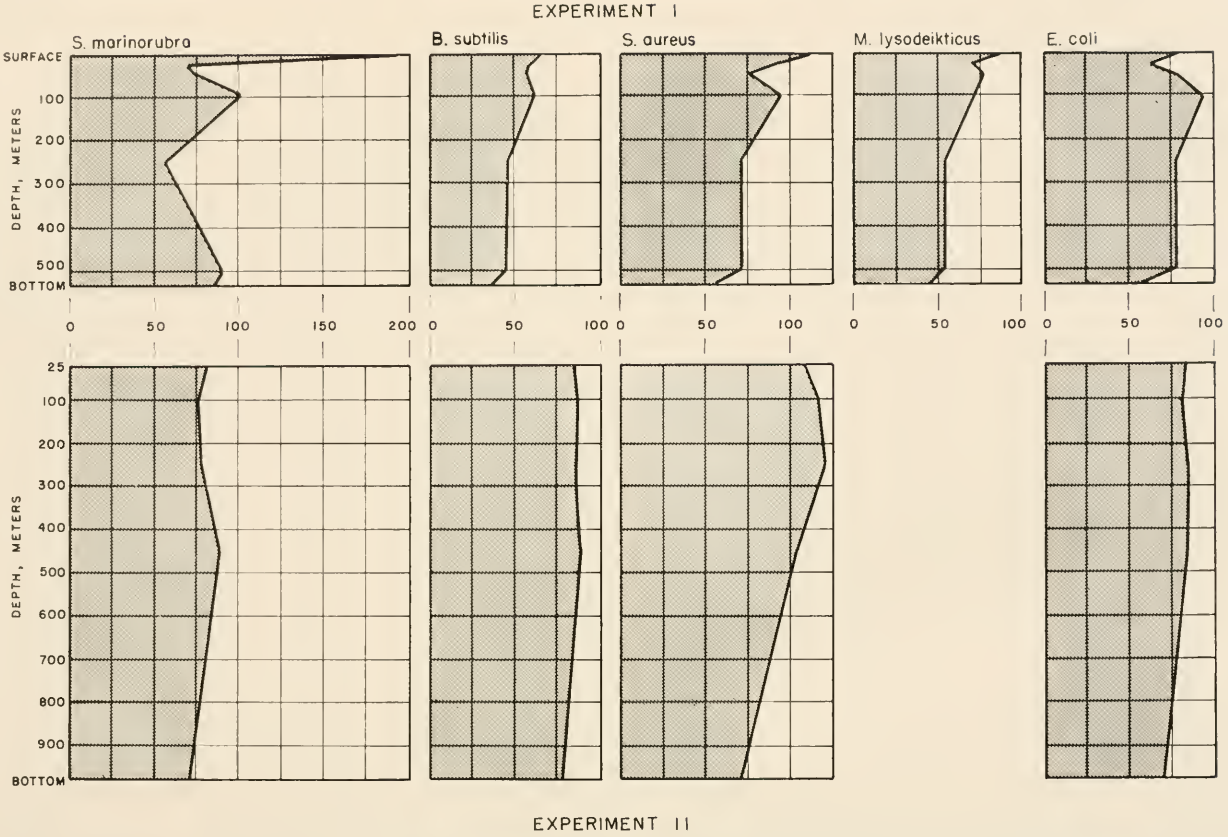

Fig. 1. Ratios (%) between the optical density at 420 $m\mu$ of cultures in media prepared with natural sea water, and the optical density of control cultures in artificial sea-water media. Values less than 100 per cent denote relative inhibition, and values greater than 100 per cent indicate relative stimulation. In Experiment I, method (a) was used and the cultures were incubated for twenty-four hours at 30 C. In Experiment II, method (b) was used and the cultures were incubated for 10 hr. at 30 C, or, in the case of S. *aureus*, at 37 C.

g; Tween 80, 8.2 ppm (v/v); and pH 7.0. The sea-water samples were collected from surface, 25, 50, 100, 250, 500, and 535 (bottom) meters. The cultures were grown in 20 x 150 mm tubes, and turbidity recorded at 420 mμ with a Bausch and Lomb "Spectronic 20" spectrophotometer.

(b) A sea water concentration of 90 per cent (nine ml added to one ml of stock nutrient Medium II) was tested with four test bacteria (0.15 ml inocula of 18 hr nutrient broth cultures grown at 30 C). The final concentrations per liter of nutrients were approximately: glucose, 1 g; casein hydrolyzate (NBC), 0.02 g; K_2HPO_4, 0.01 g; NH_4NO_3, 0.01 g; pH, 8.0. The sea-water samples were collected from 25, 100, 250, 450, and 980 (bottom) meters. The cultures were grown in tubes and turbidity recorded at 420 mμ with a Beckman DU spectrophotometer.

(c) A growth curve was obtained using S. *marinorubra* (0.15 ml inoculum of a sixteen hour culture grown at 37 C in nutrient broth) in 90 per cent sea water supplemented with Medium II. Fifty tubes of each type of sea-water medium were inoculated; three tubes for each treatment were sacrificed at hourly intervals for optical density determinations with the Beckman DU spectrophotometer.

RESULTS

The effect of natural sea water samples, collected ten miles west of San Diego, California (32° 44′ N, 117° 25′ W) on March 1, 1960, compared with artificial sea water using methods (a) and (b) is shown in Figure 1. The values presented are averages of duplicate readings. In Experiment I, growth of all the freshwater bacteria was suppressed in natural sea water, except for the case of S. *aureus*, grown in the surface sample. *B. subtilis* was the most susceptible to inhibition. Growth of S. *marinorubra* was greatly stimulated by surface sea water, but suppressed by samples from 25, 50, and 250 meters. All of the freshwater species were inhibited most by the bottom sample.

In Experiment II (Fig. 1), the complex organic additions were reduced and the sea-water concentrations increased, in sea-water samples collected 12 miles west of San Diego, California (32° 38′ N, 117° 29′ W) on May 17, 1960. These sea-water

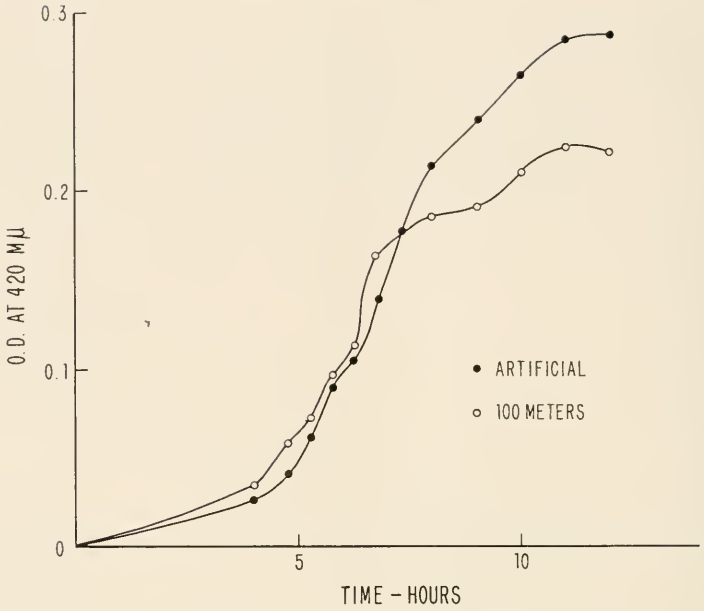

Fig. 2. Growth of S. *marinorubra* at 37 C in artificial sea water, and sea water collected from a depth of 100 meters. The 100-meter sea water was filter-sterilized and stored for one week at 4 C before being tested. The test was carried out in 90 per cent sea water supplemented with Medium II.

samples also were found to contain properties suppressing growth of *B. subtilis, E. coli,* and S. *marinorubra. S. aureus,* however, was slightly stimulated by all of the pelagic seawater samples. All of the test bacteria were inhibited to a greater extent by the bottom water than by any of the pelagic samples. In Experiment II, there was no appreciable increase of the inhibitory effect with the depth of the pelagic samples.

Although not shown in Figure 1, 16 units of penicillin G per ml of artificial sea water were added as an additional comparison in Experiment II. The natural sea-water samples were more inhibitory to *B. subtilis, E. coli,* and S. *marinorubra* than was penicillin at this concentration.

Terminal growth was determined by the experimental procedures described, prior to evidence for autolysis. Figure 2 shows a comparison between growth curves of S. *marinorubra* in media prepared with artificial sea water, and in media prepared with

natural sea water from 100 meters (32° 41′ N, 117° 25′ W) collected on May 17, 1960. Although the lag phase was slightly shorter, the final turbidity in the natural sea-water medium was less than in artificial sea water. This result has been confirmed many times in similar experiments.

Other data indicated that samples of natural sea water stored at 4 C gradually lost their inhibitory effect, presumably due to adsorption of toxic substances on the walls of the glass containers. The observations of ZoBell and Anderson (23), which indicated an increase of marine bacteria stored in containers, may have involved not only the concentration of naturally occurring organic matter, but also the removal of inhibitory substances by the addition of a glass-water surface.

DISCUSSION

The results indicate that sea water varies in its ability to support the growth of bacteria, depending upon where and when it is collected, and on the species concerned. Natural sea water, in comparison to artificial sea water, usually suppressed the growth of freshwater bacteria, and sometimes even that of the marine bacterium *S. marinorubra*. These growth studies confirm previous observations on the generally adverse effect of natural sea water on the survival of pollution and other freshwater bacteria, e.g., see Scarpino, Carlucci, and Pramer (16).

Artificial sea water may not be a suitable standard for comparison of growth with natural sea water. Although not stressed by the authors, it was shown by MacLeod and Onofrey (10) that in most cases marine bacteria developed better in artificial sea water than in natural sea water under similar experimental conditions. The major constituents of artificial sea water are similar to natural sea water, but the trace elements and growth factors are not identical. Growth suppression by natural sea water may be caused by traces of toxic ions, or slight shifts in redox potential.

SUMMARY

Terminal growth of various freshwater and marine bacteria (*Bacillus subtilis, Escherichia coli, Micrococcus lysodeikticus,*

Staphylococcus aureus, and *Serratia marinorubra)* was generally less in filter-sterilized natural sea-water samples, supplemented with essential minerals, glucose, and complex organic additions such as yeast extract and casein hydrolyzate, than in similar media prepared with artificial sea water. The inhibitory effect of pelagic sea-water samples varied with depth and location: samples obtained adjacent to the sea bottom were more inhibitory than any of the pelagic samples tested. These findings indicate that natural sea water contains factors inhibitory to bacteria.

On the other hand, growth curves of the test bacteria showed a lag period in natural sea-water samples slightly shorter than that in enriched synthetic sea-water media.

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Chapter 54

Notes on the Natural History of *Rhodopseudomonas palustris* *

Stanley Scher, Barbara Scher and S. H. Hutner

From van Niel's (11) classical studies it is known that no specific substrate selects with certainty any of the five facultative aerobic species of Athiorhodaceae (purple nonsulfur photosynthetic bacteria). Each species had a different set of vitamin requirements (2); *R. palustris* was set off from the others by requiring *p*-aminobenzoic acid (PABA). The findings that many, perhaps most, fresh water and marine phytoplankton organisms require vitamin B_{12}, thiamine and biotin (in decreasing order of frequency) (6) emphasized the seemingly unique PABA auxotrophy of *R. palustris* among photosynthetic organisms.

The present paper describes details of the enrichment and isolation procedure for obtaining benzoic acid-utilizing strains, and growth and manometric studies with such strains. We also outline some observations on the appearance of a vitamin B_{12} requirement for growth at supra-optimal temperature.

MATERIALS AND METHODS

Enrichment and Isolation

Purple bacteria utilizing aromatic substrates for anaerobic growth were isolated from soil or mud collected from fresh-water streams or marine intertidal flats along the northern California coast. Table 1 gives the medium used. Purple bacteria developed in anaerobic bottle cultures in light at room temperature. At pH 7.2-7.4 growth was proportional to benzoate concentration with the range 1-100 mg per 100 ml culture medium. Pure cultures

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TABLE 1

AROMATIC ENRICHMENT MEDIUM FOR NON-SULPHUR PURPLE BACTERIA
(WEIGHT PER 100 ML FINAL MEDIUM)

KH_2PO_4	0.05 g	Mo	0.005 mg
$MgCl_2$	0.02 g	V	0.001 mg
$MgSO_4 \cdot 7H_2O$	0.02 g	$CaCl_2$	0.002 g
Fe	0.2 mg	NH_4Cl	0.1 g
Zn	0.1 mg	Na_2CO_3	0.2 g
Mn	0.005 mg	Na benzoate	0.05 g
Cu	0.008 mg	Yeast extract	0.01 g
Co	0.01 mg	pH	7.2-7.4
B	0.01 mg		

were obtained by shake-culture isolation. When green algae interfered, the tubes were incubated under Bausch & Lomb interference filters transmitting far-red light (700-900 mμ). Selective filters within this range were used to discriminate between purple and green photosynthetic bacteria. In selecting for Athiorhodaceae, sulfate was kept minimal. After development of colonies, the agar column was ejected into a sterile Petri dish.

Growth and Manometric Experiments

Stock cultures were maintained as stabs on the enrichment medium or yeast extract 0.3 per cent supplemented with 1.5-2.0 per cent agar. Cells were grown in completely filled bottles, or in screw-capped culture tubes or micro-fernbach flasks under aerobic conditions (3). Growth was measured as optical density or dry weight. For manometry, cells were incubated at 25-30 C in the light, harvested by centrifugation and washed twice in 0.05 M phosphate buffer at pH 7.0. Oxygen consumption by resting cells suspended in buffer was determined at 30.0 C in the dark.

Several strains of purple bacteria typical of the genus *Rhodopseudomonas* and occasionally of *Rhodospirillum rubrum* were isolated from benzoate enrichment cultures. Chemically defined media in which yeast extract was replaced by PABA (10 μg%) proved more selective and invariably yielded *R. palustris* by the second transfer. Of more than 40 strains of *R. palustris*, all grew well with PABA as sole growth factor, as compared with ten out of fifteen strains isolated from non-aromatic substrate enrichments.

Inocula from mesophilic (15-30 C) and "thermophilic" (40-60 C) sites gave rise to typical rhodopseudomonads on incubation < 40 C in light. Parallel cultures > 40 C did not grow. It is known that there is a sharp increase in the B_{12} requirement of *Euglena gracilis* and *Ochromonas malhamensis* as the environmental temperature is raised above optimum (4). Addition of 0.1 μg% of vitamin B_{12} to pure cultures of purple bacteria isolated from various sources also permitted some strains to grow at 40-45 C. Studies in procress indicate that most strains of Athiorhodaceae produce one or more factors with vitamin B_{12} activity when cultivated at 25-30 C (5, 7).

Experiments with *R. palustris* indicate that the PABA requirement is greater at 40 C than at 35 C. We have also observed that the PABA requirement is not satisfied by folic acid as judged by the excessive concentrations required and the known instability of folic acid. The PABA requirement is spared by products of folic acid and metabolism, e.g., adenine and thymine. The addition of B_{12} and Ca pantothenate further reduces the PABA requirement (Table 2). These results are reminiscent of sulfanilamide inhibition of growth. But when *R. palustris* was grown in the presence of low concentrations of sulfanilamide without PABA, growth, surprisingly, was proportional to sulfanilamide concentration with the range of *ca.* 1.0 μg-10 mg%, suggesting that sulfanilamide could replace PABA as a growth factor. No competition was observed between PABA and sulfanilamide when the latter was supplied in growth-promoting concentrations. At higher concentrations of sulfanilamide (*ca* 0.1-0.4%) growth was inhibited; under these conditions, supra-optimal concentrations of PABA were required to restore growth. The requirement for vitamin B_{12} which emerges in *R. palustris* at elevated temperatures may be related to its PABA requirement, since less PABA was required when vitamin B_{12} was supplied.

Aromatic Compounds as Substrates for Purple Bacteria

Growth of anaerobic *R. palustris* cultures containing benzoic acid as sole substrate was strictly light-dependent. At limiting substrate concentrations of benzoate, growth was proportional to concentration. Growth experiments with strains of the five species

TABLE 2

EFFECT OF PRODUCTS OF FOLIC-CATALYZED REACTIONS ON THE p-AMINOBENZOIC ACID REQUIREMENT OF *Rhodopseudomonas palustris*

	Additions[1] *to Basal Medium*[2]		
PABA	*A*	*B*	C
0	0	0.10	0.06
0.002 μg %	0	0.19	0.28
0.006 μg %	0.02	0.56	1.00
0.020 μg %	0.1	1.24	1.54
0.100 μg %	0.5	1.48	1.80

[1] A: No addition
B: Adenine 1.0 mg %, Thymine 1.0 mg %
C: B + Ca pantothenate 0.1 mg %, vitamin B_{12} 1.0 μg %.

[2] Basal medium (per 100 ml final medium):

K_2PO_4	0.02 g
$(NH_4)_2SO_4$	0.06 g
$MgCO_3$ (basic)	0.025 g
$MgSO_4 \cdot 7H_2O$	0.025 g
$CaCl_2$	0.002 g
DI-Malic acid	0.06 g
Succinic acid	0.1 g
L-Glutamic acid	0.05 g
Glycerol	0.4 g
L-Leucine	0.004 g
Metals as in Table 1	

of facultative aerobic purple bacteria showed that utilization of aromatic substrates such as benzoate is widespread among the Athiorhodaceae.

Manometric experiments were done with resting cells of *R. palustris* and *R. rubrum* grown on benzoate-containing media under anerobic conditions in light oxidized benzoate. The O_2 consumed was proportional to the amount of aromatic substrate (Fig. 1). There was no induction period, which can be taken as evidence that these cells adapted to benzoate during anaerobic photosynthesis. Cells previously grown on substrates such as acetate or glucose require a period of adaptation before attacking benzoate.

For benzoate to be completely oxidized to CO_2 and H_2O, each μM of substrate requires 168 ml or 7.5 μM of O_2. Only 112 μl or 5 μM of O_2/μM of benzoate were consumed, which suggested that some additional substrate was assimilated.

DISCUSSION

Anaerobic enrichment cultures with benzoate, instead of

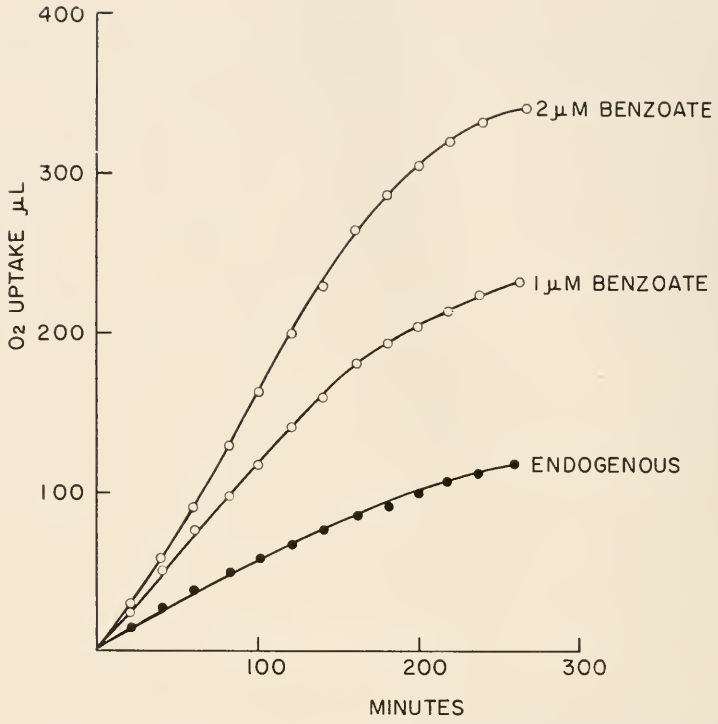

Fig. 1 Oxygen consumption by cells of *Rhodopseudomonas palustris* or *Rhodospirillum rubrum* grown on Na benzoate as sole substrate under anaerobic conditions in the light.

PABA, seemed highly selective in excluding purple bacteria other than *R. palustris.* By analogy, a specific enrichment for *R. rubrum* might be devised by taking advantage of its requirement for biotin. Similarly, the requirement for vitamin B_{12} which emerges at elevated temperatures may represent an additional selective factor to be exploited in searching for thermal auxotrophs among the purple bacteria.

Preliminary studies with single strains of the obligate anaerobes *Rhodospirillum photometricum* and *Rhodospirillum molischianum* indicate that these purple bacteria can be cultivated in synthetic media when given B vitamins such as biotin or nicotinic acid along with PABA. The frequent requirement for vitamin B_{12} in other photosynthetic microorganisms and the absence of B_{12} requirements in photosynthetic bacteria had appeared to set

the latter apart as a group. The present occurrence of B_{12} as a "temperature factor" for some members of the Athiorhodaceae and the recent report (8) that Thiorhodaceae such as *Chromatium okenii* and *Thiospirillum jenense* have absolute requirements for B_{12} help bridge this gap.

In nature, anaerobic growth of photosynthetic bacteria is limited by the light requirement. Bacterial chlorophylls absorb maximally in the far red at 800-900 mμ where algal and higher plant chlorophylls no longer absorb light effectively. When competing for light with photosynthetic organisms that utilize visible and near-red light, the purple bacteria absorb far-red light without interference. Since water absorbs strongly in the red to infra-red, photosynthetic bacteria are largely confined to the shallow bays, tidal marshes, and mud flats where sufficient red light penetrates to satisfy their requirements. Schlegel and Pfennig (9) found photosynthetic sulfur bacteria rich in ponds thickly covered over with duckweed, and used near infra-red for enrichments to avoid overgrowth by green and blue-green algae. As a consequence of their dependence upon light, photosynthetic bacteria are largely restricted to shallow aquatic environments. These may be subjected to extremes of temperature. We have frequently observed purple bacteria in hot springs at temperatures of 40-50 C; there are reports of their occurrence as high as 70 C at Searles Lake, California (1). Although knowledge of the basis of thermal tolerance is still fragmentary, the enhanced requirements for vitamins at elevated temperatures suggest that such induced growth-factor requirements set limits to the development of photosynthetic forms in nature.

Anaerobic use of organic substrates by photosynthetic bacteria is strictly light-dependent. There has been an idea that hydrogen acceptors other than molecular oxygen would not suffice for aromatic oxidations, derived from demonstrations that molecular oxygen was an obligatory oxidant for enzymes that cleave the benzene ring (10). On general grounds however, one would think that substrates for microorganisms under aerobic conditions should serve in the same capacity anaerobically if a suitable oxidant is available. Aromatic compounds such as benzoate no longer appear to be an exception to the rule: their utilization by purple

bacteria under anaerobic conditions provides evidence that a hydrogen acceptor—in this instance, a photochemical oxidant—can substitute for molecular oxygen.

The present enrichment-isolation procedure for *R. palustris* combines selective illumination, selective substrate, and a selective growth factor, with superposition of vitamin B_{12} and temperature representing a fourth selective factor of sorts. Quite likely this procedure selects for only one set of strains of *R. palustris*—those utilizing benzoate; the species-specific substrate, if existing, remains unknown.

SUMMARY

An anaerobic enrichment procedure for isolating *Rhodopseudomonas palustris* is described in which the specific requirement for *p*-aminobenzoic acid and the photosynthetic utilization of benzoic acid exclude other members of the Athiorhodaceae. Nutritional experiments with elective cultures and pure cultures of *R. palustris* indicate an increased requirement for *p*-aminobenzoic acid when incubated > 40 C. In addition, a requirement for vitamin B_{12} is superimposed at elevated temperatures. The requirement for PABA was not satisfied by folic acid and was spared by certain products of folic-catalyzed reactions. Low concentrations of sulfanilamide also satisfy the PABA requirement. Aromatic substrates such as benzoic acid can be utilized by representative species of rhodopseudomonads and at least one rhodospirillum.

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Chapter 55

Proposals on the Classification of Microorganisms Which Utilize the Polysaccharides of Marine Algae and a Definition for Agar

W. Yaphe

One of the problems involved in the study of the bacteria which utilize the polysaccharides of marine algae is the characterization of the algal preparations. The terms agar, carrageenan, funorin are used to describe extracts obtained from the Rhodophyceae and alginic acid; fucoidin, those obtained from the Phaeophyseae. Recent chemical studies have shown that these words do not refer to single substances but rather to mixtures of polysaccharides.

These polysaccharides are only found in marine plants. It is thus not surprising that the bacteria which can decompose them are present in relatively high numbers in the sea and are only occasionally isolated from the soil. The bacteria which decompose agar and alginic acid have been extensively investigated. This work can now be interpreted with reference to the new information, now available, on the structure of these polysaccharides.

Agar

The word agar-agar is used to describe any gelatinous material obtained from a number of different red algae. In 1881, the gel from *Eucheuma sp.* was used to prepare bacteriological media. A few years later, a material of lower viscosity and higher gel strength was obtained from Japan. This agar was prepared mainly from *Gelidium sp.* but it also contained the extracts from as many as 34 different species of red algae (12). The Japanese product was the main world source of agar up until 1939. Since then, agars have been prepared in a number of different countries. In most cases, the commercial products are sold under a trade

name and it is impossible to determine the botanical or geographical source. One can only assume that in North America the major source is an agar prepared from *Gelidium cartilagineum* (16). In New Zealand, Davis agar, is prepared from *Pterocladia capillaceae* and *P. lucida*. Some agar in the U.S.S.R. is obtained from *Ahnfeldtia plicata*.

Many attempts have been made to define agar. The Society for General Microbiology (13) reviewed the preparation and properties of different agars and found that it was not possible to lay down any standard specifications. The definition suggested to the American Society for Microbiology in the Manual of Microbiological Methods (9) was that "Agar is any phycocolloid derived from the Rhodophyceae which gels at 33 to 39 C and melts at 70 C." This definition will include preparations which contain different polysaccharides.

The agar from *Gelidium amansii* was shown to be a mixture of a major component agarose and a minor component agaropectin (2). Agarose consists of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose residues and is known to occur in a number of different species of red algae (16). As a result of these studies it is now possible to suggest the following definition.

Agar is a mixture of polysaccharides which is derived from certain species of the Rhodophyceae and which contains the polysaccharide agarose. The preparations which are supplied for bacteriological purposes should meet the specifications suggested by the committee on Bacteriological Technic of the American Society for Microbiology.

It is highly desirable that commercial preparations should indicate the botanical and geographical source of the algae used to prepare the agar.

Species of the Rhodophyceae which contain agarose are listed in Table 1. The concentration of agaropectin or other polysaccharides in the extracts obtained from these algae is not known. The species in which carrageenan is found are listed in Table 2. This polysaccharide yields a gel which may be used under certain conditions as a substitute for agar.

TABLE 1

SPECIES OF RHODOPHYCEAE WHICH CONTAIN AGAROSE

	Source
Gelidium amansii	Japan (2)
G. cartilagineum	U. S. A. (7)
Pterocladia lucida	New Zealand (16)
P. capillaceae	
Ahnfeldtia plicata	U. S. S. R. (15)
Gracilaria confervoides	Australia (4)
	South Africa

TABLE 2

SPECIES OF RHODOPHYCEAE WHICH CONTAIN κ CARRAGEENAN

		Source
Chondrus crispus	(British agar)	England (11)
Gigartina stellata		
Hypnea musciformis	(Hypnea agar)	U. S. A. (3)
Furcellaria fastigiata	(Danagar)	Denmark (8)

Alginic Acid

Alginic acid was separated into two fractions (5); with a high concentration of mannuronic acid in one fraction and guluronic acid in the other.

Carrageenan

This sulphated polysaccharide was fractionated with potassium chloride into the κ and λ fractions (11). A third component (10) may be present in the λ fraction.

Microorganisms

In most studies on the utilization of these algal polysaccharides, the object has been to isolate the bacteria which would degrade either agar or alginic acid. This has led to two viewpoints on the classification of these bacteria.

Thjøtta and Kåss (14) suggested that "the production of alginase must be a rare property in bacteria -.-.- well fitted to characterize a genus." The opposite viewpoint was expressed by Humm (6). He described twenty species of agardigesters and of these 70 per cent attacked alginic acid, 25 per cent attacked chitin and 20 per cent attacked cellulose. All species utilized the extract from *Chondrus crispus* (carrageenan) and none attacked the extract from *Gigartina acicularis*. Preliminary evidence sug-

gests (17) that the latter contains a high concentration of λ carrageenan. Humm concluded that these bacteria form a physiological rather than a natural group and could be designated as "seaweed-gel-digesters."

The approach used in the seventh edition of Bergey's Manual of Determinative Bacteriology emphasizes the utilization of a particular polysaccharide (Table 3). Thus only the species which utilize alginic acid are placed in *Alginomonas*. In this genus the three marine species also utilize agar. It is proposed that the description of this genus should be broadened so as to include the bacteria which can use other algal polysaccharides. Thus the first agar-digester to be isolated *Bacillus gelaticus* Gran at present classified as *Pseudomonas gelatica* should be renamed *Alginomonas gelatica*.

TABLE 3

Bacteria which Utilize Agar and Alginic Acid as Listed in Bergey's Manual of Determinative Bacteriology Seventh Edition

Order I.—Pseudomonadales
Suborder—Pseudomonadineae
Family IV—Pseudomonadaceae
Genus I—*Pseudomonas*
Ps. gelatica
Genus IX—Alginomonas
(soil and water bacteria that are known to dissimilate alginic acid)
5 species
Family VII—Spirillaceae
Genus I—*Vibrio*
5 species

Order IV.—Eubacteriales
Family III—Achromobacteraceae
Genus IV—*Agarbacterium*
Family IV—Enterobacteriaceae
Tribe I—Escherichieae
Genus 5—*Alginobacter*
Order V.—Actinomycetales
Family II—Actinomycetaceae
Genus 1—*Nocardia*

In the key to the Achromobacteraceae, the bacteria which can attack agar and/or alginic acid are placed in the genus *Agarbacterium*. The description of this genus states that agar is digested. The hydrolysis of alginic acid was not determined for any of the species. This genus should include bacteria which can use either agar or alginic acid. Thus Adams *et al.* (1) are justified in suggesting that *Agarbacterium alginicum* is the appropriate name for *Alginomonas alginica*, an organism which hydrolyzes alginates and is not known to utilize agar.

Similarly, the description of the genus *Alginobacter* should be extended to include the bacteria which hydrolyze agar and other algal polysaccharides.

The system proposed in Bergey's manual becomes more complex when the bacteria which use polysaccharides other than agar and alginic acid are investigated. Recently bacteria have been isolated that use κ and λ carrageenan (17). Most of these bacteria could use agar and/or alginic acid but a few strains were isolated which did not hydrolyze these polysaccharides. It is undesirable to establish a new genus to classify these bacteria. Therefore, it is proposed that the specifications for the above named genera be broadened so as to include bacteria which attack carrageenan. Consideration also should be given to change the prefix Algin and Agar to a word such as Phycus which would indicate that the bacteria in these genera can use a number of different algal polysaccharides. The names of the genera would thus be changed to *Phycomonas, Phycobacterium* and *Phycobacter.*

If the use of these algal polysaccharides is not considered a suitable property to characterize a genus, then the bacteria should be classified as sub groups of *Pseudomonas* and *Achromobacter.*

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Chapter 56

Occurrence and Significance of Chitinoclastic Bacteria in Pelagic Waters and Zooplankton

Donald W. Lear, Jr.*

INTRODUCTION

A primary function ascribed to marine microbes is their participation in cycling nutrients in the biological economy of the sea. Much of this concept has been adapted from analogy with terrestrial and limnological studies, as there are few field studies of this cycling in pelagic areas.

For this study, two indicators of microbial activity were selected: the aerobic heterotrophic marine microorganisms, and chitin-digesting bacteria.

The properties of chitin make it particularly suitable for the study of microbial participation in the cycling of organic matter. First, chitin is extremely resistant to ordinary chemical or physical degradation under conditions found in the marine environment, requiring the enzyme (or enzymes) chitinase to catalyze its breakdown. Second, few organisms in the sea other than fungi are reported as producing chitinase, and the few reported (20), are not comparable in numbers with the microflora. Third, chitin occurs in the sea in significant amounts in many animals and some plants, and particularly in the abundant crustacean zooplankton.

No investigations of pelagic chitinoclastic microflora have been reported in the literature, although there are several papers concerned with nearshore and benthic marine chitinoclasts (2, 6, 11, 12, 13, 25, 27, 29, 30).

Since the concept of cycling of organic matter is less compli-

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cated in pelagic areas, which are less affected by such factors as terrestrial runoff, upwelling, relatively rapid currents and countercurrents, etc., this work was done as much as practicable aboard ship in pelagic areas.

Preliminary to work at sea, efforts were made to evaluate media and culturing conditions of pelagic chitinoclastic microorganisms.

EXPERIMENTAL

Water samples were taken from shipboard casts, using sterile bacteriological J-Z water samplers, (28). The water samples were transferred aseptically from the J-Z bulbs to sterile glass bottles, and samples withdrawn from these for laboratory work aboard ship.

Zooplankton was taken in standard net tows (00 bolting silk, one meter nets, 200 meters wire out) on Cruises 3 and 5 (Fig. 1), and from an Isaacs-Kidd six-foot midwater trawl on Cruise 4.

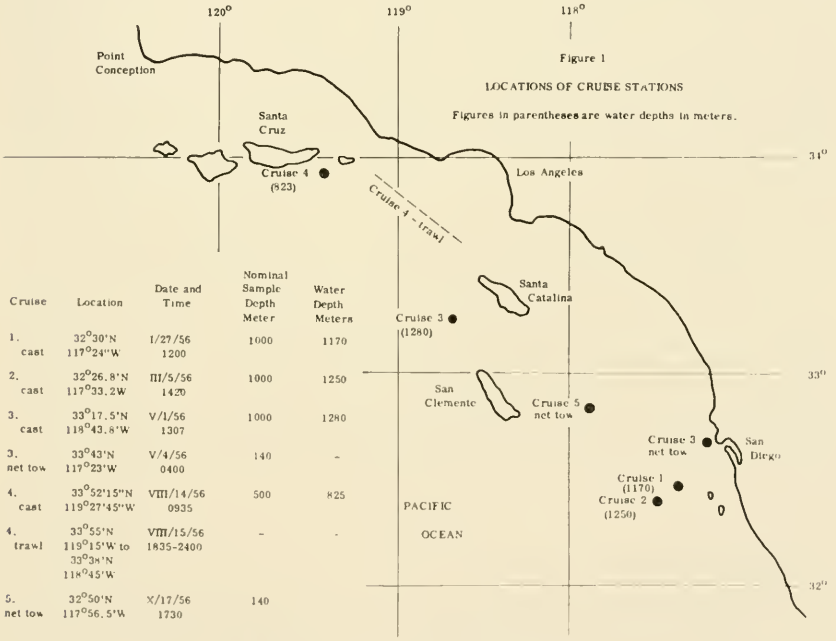

Figure 1

LOCATIONS OF CRUISE STATIONS

Figures in parentheses are water depths in meters.

Cruise	Location	Date and Time	Nominal Sample Depth Meter	Water Depth Meters
1. cast	32°30'N 117°24'W	I/27/56 1200	1000	1170
2. cast	32°26.8'N 117°33.2W	III/5/56 1420	1000	1250
3. cast	33°17.5'N 118°43.8'W	V/1/56 1307	1000	1280
3. net tow	33°43'N 117°23'W	V/4/56 0400	140	-
4. cast	33°52'15"N 119°27'45"W	VIII/14/56 0935	500	825
4. trawl	33°55'N 119°15'W to 33°39'N 118°45'W	VIII/15/56 1835-2400	-	-
5. net tow	32°50'N 117°56.5'W	X/17/56 1730	140	

Fig. 1. Locations of cruise stations.

Plankters were fished individually from the cod ends of the nets with sterile forceps and placed in 100 ml sterile sea water blanks. On Cruises 3 and 4, the intact plankters in the water blanks were thoroughly shaken and 1.0 ml water samples withdrawn for bacteriological plating. The animals in the water blanks were then aseptically crushed with a sterile wooden applicator stick, the bottle shaken thoroughly, then sampled again for bacteria.

Some myctophid fish were taken in net hauls and in the mid-water trawl. Sterile instruments were used to dissect out the viscera, which were placed in 100 ml sterile sea water blanks and aseptically crushed with the sterile applicator sticks, and sampled. Saury *(Cololabis saira)* were taken with dipnets under a night light, and the viscera sampled similarly.

Figure 1 shows the locations and data of the stations in the pelagic waters off Southern California. To realize pelagic conditions, as remote from terrestrial influence as practicable, effort was made to sample in locations with at least 1000m water depth.

Bacteriological manipulations were all done aboard ship, insofar as was possible. To minimize the difficulties of making pour plates aboard ship in a rolling sea, a tray was devised, suspended from a single point overhead and counterweighted below with a six-pound sash weight. This proved suitable under sea conditions with winds of 15 knots and three-foot swells, but developed too much oscillation for practical use with 22 knot winds and eight-foot swells.

Attempts were made to preserve bacteriological samples at sea by rapid freezing, using dry ice, then returning the samples to the laboratory for study. By plating aliquots at sea and similar frozen aliquots in the laboratory ashore, it was found that less than 1 per cent of the microorganisms survived freezing and thawing. After two attempts, this procedure was abandoned.

MEDIA

Total Counts

Aerobic heterotrophic microorganisms (or the "total" counts) were enumerated on agar pour plates, using ZoBell's 2216e medium:

peptone	0.5%
yeast extract	0.1%
$FePO_4$	0.01%
agar	1.5%
aged sea water	q.s.
pH 7.5-7.8	

Chitin Media

Before embarking on the pelagic investigations, some preliminary attention was given to evaluating culturing conditions for marine chitinoclasts on agar pour plate media.

Chitin was obtained from the spiny rock lobster *Panulirus interruptus*, and purified according to the method of Benton (3), which, briefly, involves decalcification in 1% HCl, extraction in hot 2% KOH, and in boiling ethanol. The criterion for chitin degradation by microbial action was the visible clearing of dispersed chitin from chitin agar plates. Fine particles of chitin were obtained by "dissolving" purified chitin in 50 per cent sulfuric acid, precipitating by dilution with distilled water, then washing and neutralizing (6). The resultant particles were readily dispersable in agar media, imparting a whitish cloudiness.

This "precipitated" chitin was compared with ground purified chitin, sieved through 60 mesh, in agar media. Growth and chitin degradation on agar media containing both types of chitin were comparable.

Media Constituents

Various modifications of chitin-sea water agar media were

TABLE 1

Growth and Chitinase Production by Marine Bacteria on Various Media

Inoculum	*Chitin + Agar*		*Chitin + Agar + 0.1% Yeast Ext.*		*Chitin + Agar + 0.1% Peptone*	
	Total Colonies	*Chitin Degraders*	*Total Colonies*	*Chitin Degraders*	*Total Colonies*	*Chitin Degraders*
Surface sea water, Scripps pier, La Jolla, Calif. (one ml.)	34	3	255	36	46	4
Enrichment culture of pelagic zooplankters (10^{-5} ml.)	9304	3	23,842	15	15,747	14

tried to determine the necessary nutrients for optimal growth and chitinase production. Table 1 shows the relative effects obtained by adding 0.1 per cent yeast extract (Difco) and 0.1 per cent peptone (Difco) to chitin-sea water agar (0.1% chitin, 1.5% agar). Incubation was at room temperature for twenty three days.

Oxygen Tension

Several experiments were run to determine oxygen tension preferences of marine chitinoclasts. Pour plates with sea water and with pelagic zooplankton inocula were incubated aerobically and anaerobically. Lack of oxygen inhibited growth and chitinoclastic activity. Another approach made was to inoculate semi-solid chitin agar tubes with natural inocula. In all cases, growth and chitinoclastic activity were greater with aerobic conditions.

Incubation

Extended incubation periods at 22-25 C. were found necessary for the development of chitinoclasts on chitin-yeast extract agar. The maximum number of colonies developed on the plates before clear zones around the colonies became evident under a

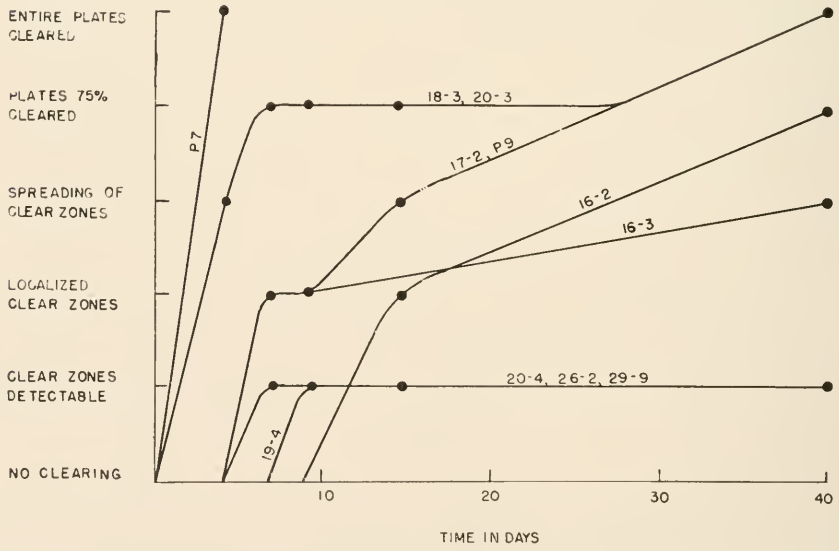

Fig. 2. Rate of chitin degradation by pure cultures of marine chitinoclastic bacteria.

microscope at 27X magnification. An exception to this was found in the very high chitinoclast populations in certain zooplankters, which showed chitin degradation within five days. Ten to 40 days incubation was generally necessary for the development of clear zones around colonies on chitin-yeast extract agar.

When pure cultures of chitinoclasts from pelagic sources became available, they were tested individually on the media used for enumeration in the pelagic work. Twenty-seven cultures were transferred to 2216e agar slants, incubated for three days, then streaks made from each on chitin-yeast agar plates. All streak plates showed good growth in twenty four hours, and the estimations of chitin degradation are shown in Figure 2. Representative responses, including the extremes, are shown in this graph.

The medium finally selected for the enumeration of marine pelagic chitinoclasts was of the following composition:

"precipitated" chitin	0.1%
yeast extract	0.1%
agar	1.5%
aged sea water	q.s.
pH 7.5-7.8	

RESULTS

Distribution of Marine Microorganisms in Pelagic Waters

Vertical Distribution of Aerobic Heterotrophs

Agar plate counts of water samples from the J-Z bottle casts of Cruises 1 to 4 are shown in Figure 3. The abscissa, showing the average number of bacteria from duplicate plates, is exaggerated decimally.

The greatest number of pelagic heterotrophs was generally found in the upper 50 meters; however, relatively low counts were found in the upper layers on Cruises 3 and 4.

Vertical Distribution of Chitinoclastic Marine Microorganisms

Figure 4 shows the average number of colonies appearing on chitin-yeast extract-sea water agar plates, and the number of chitin-degrading microbes (those producing clear zones in the chitin agar around the colonies) encircled at the appropriate

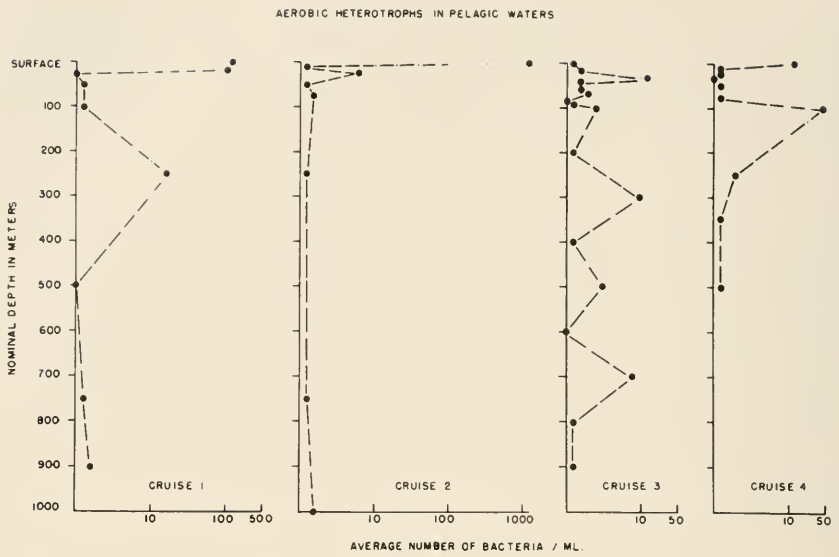

Fig. 3. Aerobic heterotrophs in pelagic waters.

depths. 1,241 colonies were examined on chitin-yeast extract-sea water agar, and of these, four showed evidence of de-grading chitin, or 0.32 per cent. All chitin plates were incubated for at least 30 days.

The average number of colonies that appeared on chitin agar plates seemed to be generally less than on the corresponding 2216e plates, but showed the same relative trends of population size.

In the evaluation of culturing conditions for chitinoclasts, mentioned above, samples of surface sea water from just beyond the surf zone, at the end of Scripps Institution Pier, La Jolla, California, were plated. On the same media as used in the pelagic studies, 18.9 per cent of the colonies on chitin-yeast extract sea water agar showed clearing zones around the colonies, compared to the 0.32 per cent in pelagic waters.

Chitinoclasts in Pelagic Zooplankton

Since so few chitinclasts were found in the water samples from hydrographic casts, efforts were made to examine zooplankters. The procedures described above were devised to determine

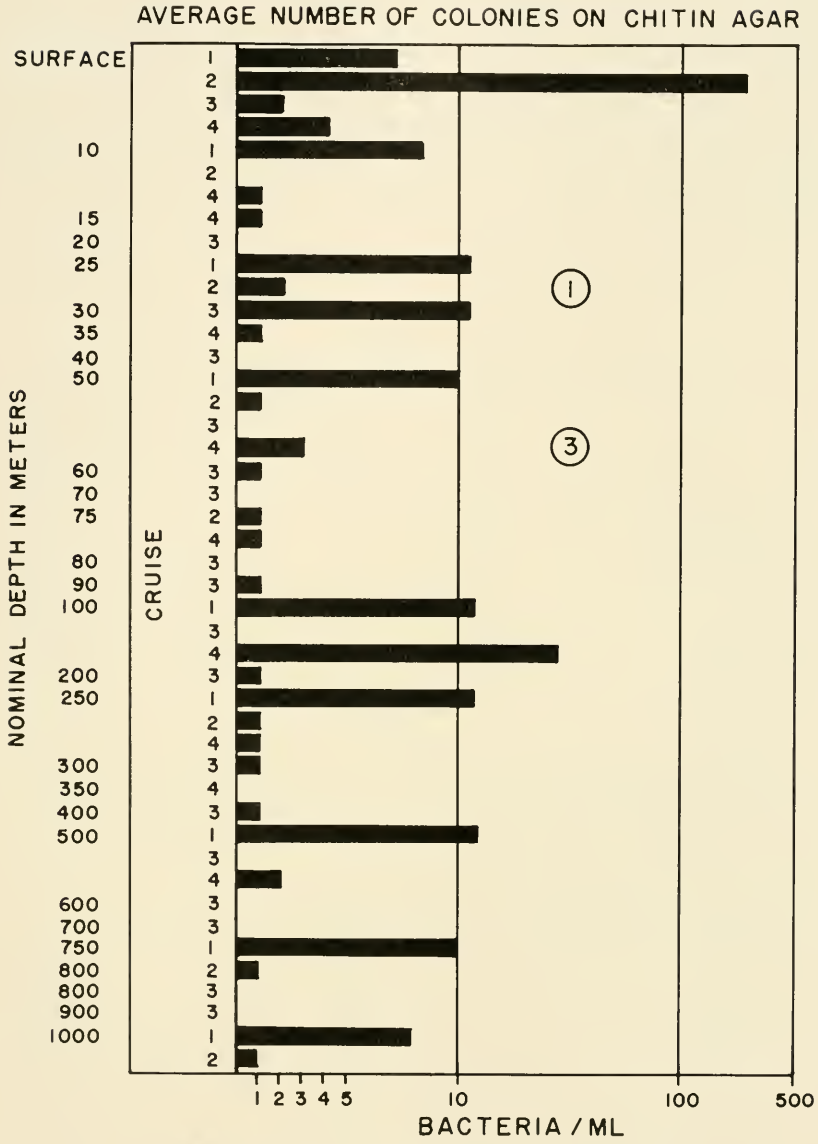

Fig. 4. Average number of colonies on chitin agar.

roughly whether a chitinoclastic flora was associated with zooplankters, and, by sampling before and after crushing the plankter, whether the microflora was associated internally or externally.

Table 2 shows the results from Cruises 3, 4, and 5. The chitin agar plates from Cruise 3 showed no chitin degradation by the flora sampled before crushing, while in several cases the microflora sampled after crushing completely hydrolyzed the chitin from the agar plates. There were no instances of discrete clear zones around colonies, but complete clearing of the plates occurred.

On Cruise 5 the procedure was repeated, and an especially

TABLE 2

CHITINOCLASTIC MICROORGANISMS IN PELAGIC ZOOPLANKTON

	Organisms	*Before Crushing, Bacteria/ml.* Total Colonies	Clear Zones	*After Crushing, Bacteria/ml.* Total Colonies	Clear Zones
Cruise 3 Plankton tow	Ctenophore		—		—
	Juvenile flatfish		—		—
	Radiolarian		—		—
	Juvenile fish		—		—
	Euphausiid		—		—
	Euphausiid		—		—
	Radiolarian		—		—
	Juvenile fish		—		—
	Copepod, chaetognath		—		—
	Decapod		—		—
	Amphipod, copepod		—		—
	Radiolarian, chaetognath, copepod, euphausiid		—		—
	Radiolarian, chaetognath, copepod, squid larva		—		—
	Radiolarian, chaetognath, copepod		—		—
	Chaetognath, copepod, euphausiid (gravid)		—		—
	Chaetognath, copepod, euphausiid		—		+
	Copepod, mysid		—		+
	Copepod, chaetognath		—		+
	Chaetognath, fish larva		—		+
	Copepod, chaetognath, fish larva		—		+
	Chaetognath, copepod, euphausiid		—		—
	Copepod, chaetognath, fish larva		—		—
	Copepod, chaetognath, fish larva		—		—
	Copepod, chaetognath, radiolarian		—		+
	Euphausiid		—		+
	Copepod, chaetognath, coelenterate (?)		—		+
	Copepod, chaetognath		—		+
	Chaetognath		—		+

Cruise 5 Plankton tow	Amphipod			1	0
	Euphausiid			3	0
	Amphipod, *Phronima*			70	1
	Amphipod			6	3
	Euphausiid			25	0
	Euphausiid			10	0
	Copepod			5,000	cleared
	Amphipod			5,000	cleared
	Decapod			7	1
	Amphipod			17	0
	Amphipod			11	0
	Amphipod			11	0
	Myctophid fish			15	1
Cruise 4 Midwater Trawl	Mysid, *Gnathophausia ingens*	1,000	0	10,000	0
	Euphausiid, *Nematoscelis sp.*	100	0	920	1
	Decapod, *Sergestes sp.*	1,120	0	576	cleared
	Galatheid decapod	172	12	200	50
	4″ squid	5,000	0	5,000	20
	Decapod, *Sergestes sp.*, 4″	10,000	cleared	10,000	cleared
	Pasiphaeid decapod, 4″	10,000	0	10,000	0
	Decapod, *Benthesicymus sp.*, 2.5″	10,000	cleared	10,000	cleared
	Decapod, *Acanthephyra sp.*, 2.5″	10,000	14	10,000	cleared
	Decapod, *Sergestes sp.*, 3″	10,000	0	10,000	0
	Mysid, *Gnathophausia ingens*, 2.5″	5,000	0	5,000	cleared

TABLE 3

SEMIQUANTITATIVE ESTIMATION OF NUMBER OF BACTERIA ASSOCIATED WITH ZOOPLANKTERS AND IN THE GUT OF FISH. Cruise 5

Organism	*Bacteria/Organism*	
	Total (2216e)	*Chitin-Decomposing (Chitin-Yeast Extract Agar)*
Amphipod	800—	<100
Euphausiid	300—	<100
Amphipod, *Phronima*	10,700—30,000	100
Amphipod	1,500—80,000	300
Euphausiid	1,900	<100
Euphausiid	2,900—10,000	<100
Copepod	70,400	>500,000
Amphipod	16,200—60,000	>500,000
Decapod	—	100
Amphipod	—	<100
Amphipod	20,000	<100
Amphipod	—	<100
Myctophid gut	—	100

large chitinoclastic microflora was found associated with a copepod and an amphipod, with fewer or none found in others, even of morphologically similar (same species?) plankters.

It should be noted, in comparing the three zooplankton samplings, that the plankters in Cruise 4 were taken in a midwater trawl, and were generally much larger specimens than the plankters of Cruise 3 and 5, which were taken in standard net tows.

Table 3 shows the semiquantitative estimation of the microflora of the zooplankters of Cruise 5. The data in Table 2 are the records of the numbers of colonies on plates, while these data have been recalculated, taking dilution into account, in Table 3 to indicate the approximate levels of the bacterial populations and the chitinoclastic microflora associated with the individual zooplankters. The counts range from 10^3 to 10^5 per organism, and with a method of sampling that would provide more homogeneity, would probably be considerable greater.

Eight fish were taken in the midwater trawl of Cruise 4, and the viscera aseptically removed and treated as described above. The chitin agar plates made on the cruise met misfortune, but the viscera were left to stand in the sea water blanks for 30 days at room temperature, then sampled and plated on chitin agar. Four specimens of the myctophid *Lampanyctus sp.* contained many chitinoclastic bacteria, while the viscera from three specimens of saury, *Cololabis saira,* showed few chitinoclasts. On Cruise 5 the viscera from one myctophid was sampled and showed the presence of chitinoclasts, while one saury showed none.

DISCUSSION

The distribution of heterotrophic bacteria in pelagic waters has been described by many investigators, among them Gazert (9), Reuszer (21), Russell (22), Otto and Neumann (19), Graf (10), Bertel (4, 5), Schmidt-Nielson (23), Foyn and Gran (8), Drew (7), ZoBell (28), Jannasch and Jones (14) and Kriss *et al.* (17). Most of the Russian endeavors in this field were recently summarized by Sieburth (24).

From the literature and from the data presented here, pela-

gic marine bacteria can be described as truly ubiquitous, but their numbers at a given locus unpredictable. In the vast realms of the sea they are nearly omnipresent, but in relatively low numbers, especially below the euphotic zone where there are but few bacteria per ml of water. There seems to be a tendency, but not a rule, for greater numbers to occur nearer the surface. The sparseness of the bacterial populations is demonstrated in Table 4, which indicates that in 82 per cent of one ml inocula of pelagic seawater, the population found was less than ten cells per ml, and in 93 per cent of the plates less than 50 cells per ml. As indicated, 32 per cent of the samples contained no bacteria, but rarely (four times) were replicates of a given water sample devoid of bacteria. These figures are results from pour plating techniques, and results from silica gel and molecular filter techniques generally show one to several orders of magnitude greater numbers (14, 17). The difference is probably due to the heat necessarily applied in the pour plating technique.

TABLE 4

Occurrence of Bacteria in Pelagic Waters, Irrespective of Depth. Ninety Plates, each with 1.0 ml. Inoculum

	No. of Plates	*%*
0 bacteria in 1 ml.	29	32
1— 10 bacteria in 1 ml.	45	50
10— 50 bacteria in 1 ml.	10	11
50— 100 bacteria in 1 ml.	2	2.3
100—1000 bacteria in 1 ml.	2	2.3
1000 bacteria in 1 ml.	2	2.3

The high bacterial counts found in a few of the water samples, generally from the surface layers, can be attributed to the periphytic association of bacteria on particulate matter as postulated by ZoBell (28) and others. Wide discrepancies between the counts on replicate plates were found in several instances, which would further indicate sorption phenomena. It should be pointed out, in this connection, that the limiting orifice of the J-Z water sampler was 3 mm, which might reduce numbers by selecting against those adsorbed on larger particulate matter.

Distribution of Pelagic Chitinoclastic Bacteria

Free chitinoclastic microbes in pelagic waters seem to be relatively scarce, as was indicated in Figure 4. Of 1,241 colonies on chitin agar plates with pelagic sea water inocula, only four colonies showed evidence of chitin degradation, even after extended incubation.

Adequacy of Technique

Unquestionably, optimal culture conditions for pelagic chitinoclasts were not discovered in the brief preliminary studies, as is indicated by the greater numbers of colonies that developed on replicate plates of 2216e medium than on chitin-yeast extract-sea water agar. However, the results obtained from zooplankters, in which clearing of chitin in many cases was quite rapid, indicate the medium was capable of detecting chitinoclastic activity. It is entirely possible, however, that materials carried in the inoculum from the zooplankters may have included factors promoting the production of chitinase.

When first examining culture conditions for marine chitinoclasts, inocula of sea water from the Scripps Institution's pier were used. After a few cruises to pelagic areas it became apparent the types of microorganisms were different, and evaluations of technique were checked with actual pelagic water inocula. In samples from just behind the surf zone, 18.9 per cent of the microbial population was chitinoclastic, while offshore populations were found to contain only 0.32 per cent. In addition, many fungi were encountered in nearshore samples, while they were rarely encountered away from the influence of land. This increased the difficulty of searching for optimal conditions. Work on shipboard is necessarily more limited in scope than in a laboratory ashore.

The preliminary culturing experiments indicate chitin, without supplementary factors, is not a suitable substrate for estimating the pelagic chitinoclastic microflora. The addition of 0.1 per cent peptone did not appear to be as suitable as the addition of 0.1 per cent yeast extract to the chitin medium. Further tests using 27 purified isolate of pelagic marine chitinoclasts showed that neither 0.1 per cent chitin, nor 0.1 per cent chitin and 0.1% NH_4NO_3 in sea water would support growth of these cultures.

Lingappa and Lockwood (18) have shown, in soils, that a chitin mineral agar supported actinomycetes well, but not bacteria. Chitin agar, with organic additives, on the other hand, supported chitinoclastic bacteria better than actinomycetes or fungi.

In the environmental situations examined, chitinoclasts seemed definitely aerobic in preference, although Aleshina (1) and Steiner (26) report anaerobic chitin degradation from bottom sediments.

An appreciable delay between the appearance of colonies on plates and the first appearance of clearing zones around them was noted in these studies. This delay may indicate these chitinases are endoenzymes and are released to the surrounding medium after autolysis, or that these enzymes are adaptive in nature. The differences in response of several pure cultures of pelagic chitinoclastic bacteria on the chitin-0.1 per cent yeast extract-sea water medium, shown in Figure 2, indicate this medium is not optimal for many strains, and possibly that different chitinase systems may be represented by the different organisms. This lag in chitinoclastic activity *in vitro* has been noted by other investigators (16).

Nature of the Microflora of Pelagic Zooplankton

The microflora of pelagic zooplankters seems to be similar to the pelagic microflora described by many authors (*cf.* ZoBell (28) for summary). Few or no fungi were encountered on plates from pelagic areas, and no actinomycetes. Of the microflora of zooplankters, Gram negative asporogenous rods were the most numerous (69%), Gram positive cocci next most abundant (23.5%), Gram positive rods amounted to 5.5 per cent and yeasts, 2 per cent. These figures are based on a random examination of 55 colonies. Approximately 50% of the colonies were chromogenic. These results will be reported in detail in a later paper.

Microbial Participation in the Bioeconomy of the Sea

In striking contrast to the relatively sparse microbial populations found in sea water samples, are the large, active populations found associated with zooplankters. The population levels of aerobic heterotrophs from water samples were generally less

than 50 cells per ml, while the populations found in individual zooplankters were frequently several to many orders of magnitude greater. Even more striking is the comparison of chitinoclastic bacteria, in which only four of 1241 colonies examined from water samples showed evidence of degrading chitin, while in zooplankters chitinoclasts frequently were extremely populous. In the zooplankters examined, the microbes seem to be intimately, possibly internally associated. No attempts were made in this study to establish any commensal or symbiotic relationships between the pelagic zooplankters and the microflora, nor are the data sufficient to establish any taxonomic relationships between the marine animals and the chitinoclastic microorganisms.

These data suggest the hypothesis that the primary loci of the microbial agents responsible for cycling animal matter in the sea are intimately associated with individual plankters. Jones (15) shows similar data with Radiolarians. Bacteria are much more effective physiologically in localized culture than as scattered individuals, and it would seem that a relatively high degree of efficiency in the cycling of nutrients would be maintained. "Dead" organic matter, which results from the death of an organism, or of ecdysis, or excreta, is already amply inoculated with the microbial agents of decay, and probably decay occurs near the original locus of the host. In the case of phytoplankton, the relationship between microbes and host would appear to be primarily sorptive in nature. The concepts of zones or layers of active decomposition would, under this hypothesis, be limited roughly to the degree of zonation of the plankton.

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Chapter 57

Host and Habitat Relationships of Marine Commensal Bacteria* †

J. Liston and R. R. Colwell

It is now generally accepted that free swimming fish normally carry a population of commensal bacteria, and the nature of this flora has been defined for a number of species of fish in particular habitats (mainly northern) (11, 5). Much less is known concerning the natural bacterial populations of marine invertebrate animals. A few observations concerning individual types of bacteria, such as *Achromobacter* and *Cristispira*, isolated from shellfish, have been recorded (1, 2, 8) and rather limited accounts of the bacterial flora of soft shelled clams *(Mya arenaria)* (9, 10) have been published. Scattered observations and descriptions of individual bacteria isolated from marine invertebrates have been published by other workers. Evidence has been presented (4) that a distinct commensal bacteria flora is associated with the Pacific oyster *(Crassostrea gigas)* and that habitat conditions may affect the balance of types in this population to a limited extent.

The dual importance of host and habitat in determining the bacterial flora of fish has been emphasized by Wood (12, 13, 14) who reported marked differences between the generic composition and physiological capabilities of teleost and elasmobranch fish caught off Australia. Liston (6) was unable to distinguish significant differences between teleost and elasmobranch fishes in the North Sea and concluded that habitat may be more important than host species in this respect.

A continued interest by the authors in the effects of the host-

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habitat relationship on the normal, non-pathogenic flora of fish, which appear to have yielded such contradictory results in previous studies, inspired them to carry out the studies reported here. These investigations were designed to provide information on the nature of the bacterial floras associated with vertebrate and invertebrate animals in the Pacific Ocean, north of the equator.* It was hoped that some information might be obtained on: (a) the differences between the floras of different animal types in the same area and the same animal types in different areas; and (b) what reasons exist for such differences, if they occur.

MATERIAL AND METHODS

The animals, both vertebrate and invertebrate, were sampled in the field by the sterile swab technique using cotton swabs, sterilized in separate 16 x 150 mm screw-capped culture tubes containing 1 to 2 cc of aged sea water plus 0.5 per cent peptone. The fish species caught in the northern areas of the Pacific Ocean were taken in Puget Sound, i.e., from the waters off Port Orchard, Gig Harbor and Golden Gardens, and from oceanic waters off the Washington Coast. The swabs were transported to the laboratory and streaked on seawater agar plates. The seawater agar consisted of 0.5 per cent yeast extract, 0.8 per cent nutrient broth (Difco), 1.5 per cent agar (Difco) and aged seawater (7).

Cultures of littoral zone animals and of floating tray-grown invertebrates were also obtained by the swab technique; after aseptically opening the shell in the case of molluscs. In no case did sampling and streaking on agar involve a greater delay than twenty-four hours.

The cultures from the southern Pacific Ocean areas were obtained by the swab technique; the vertebrate and invertebrate specimens were sampled directly in the field and the swab streaked onto the surface of slanted agar in small (1½ oz.) prescription bottles. The bottles were transported by air to the laboratory and restreaked onto the maintenance medium described.

The colonies appearing on the streak plates after five days at

* The terms northern Pacific and southern Pacific are used in this paper to designate areas more and less distant from the equator, respectively.

25 C (RT) and after 25 days at 0 C were picked off the agar into tubes of seawater broth. A random selection of colonies was made. The total number in the samples was governed by the maximum number which could be handled at one time. The

TABLE 1

CODED CHARACTERISTICS USED IN THE STUDY OF MARINE COMMENSAL BACTERIA

Morphology

rods	short (0.2 μ - 0.6 μ)	motile
curved rods	medium (0.6 μ - 1.2 μ)	polar flagellation
ovals (spheres)	slender (0.2 μ - 0.6μ)	Gram negative
filaments	stout (0.6 μ - 1.0 μ)	Gram variable
singles	rounded end	
pairs		
chains		

Culture

small colony (< 2mm)	heavy turbidity	
med. colony (2 - 5 mm)	moderate turbidity	
entire edge	slight turbidity	
convex	even turbidity	
white	granular sediment	
off-white	ring	
gray	pellicle	
green pigment (diffusible)	fluorescent	
opaque		
translucent		

Physiology

growth at 0 C
growth at 25 C (RT)
growth at 37 C
seawater required
catalase positive
penicillinase present
0/129 insensitive

Biochemistry

agar digested	glucose positive (Hugh and Leifson, 1953)
gelatin liquefied	glucose acid
litmus milk peptonized	glucose gas
litmus milk surface peptonized	maltose acid
litmus milk acid	maltose gas
litmus milk alkaline	sucrose acid
litmus milk reduced	sucrose gas
litmus milk acid→ alkaline	lactose acid
litmus milk alkaline→ acid	lactose gas
ammonia produced in peptone water	galactose acid
Voges-Proskauer positive	galactose gas
methyl red positive	mannitol acid
indole positive	mannitol gas
growth in Koser's citrate	starch hydrolyzed
urease production on Christianson's urea agar slants	gluconate oxidized
	nitrates reduced to nitrites
	nitrites reduced
cytochrome oxidase positive (Gaby)	hydrogen sulfide produced from cysteine
oxidase positive (Kovacs)	trimethylamine oxide reduced to trimethylamine

broths containing the selected colonies were incubated at 25 C (RT) or 0 C, depending upon the initial temperature of isolation.

Each isolate was streaked three times to check purity before the testing program was carried out. The pure cultures were studied for morphological, physiological and biochemical characteristics.

A full description of the methods and media used in testing the bacteria was given in a previous paper (3) and the tests are summarized in Table 1. Altogether 48 tests were carried out on each organism, and in most cases the tests were repeated at the end of six months.

Thus, there was obtained for analysis, a total of approximately 200 characteristics for each of 743 pure cultures taken from nineteen species of fish, representing twenty genera from

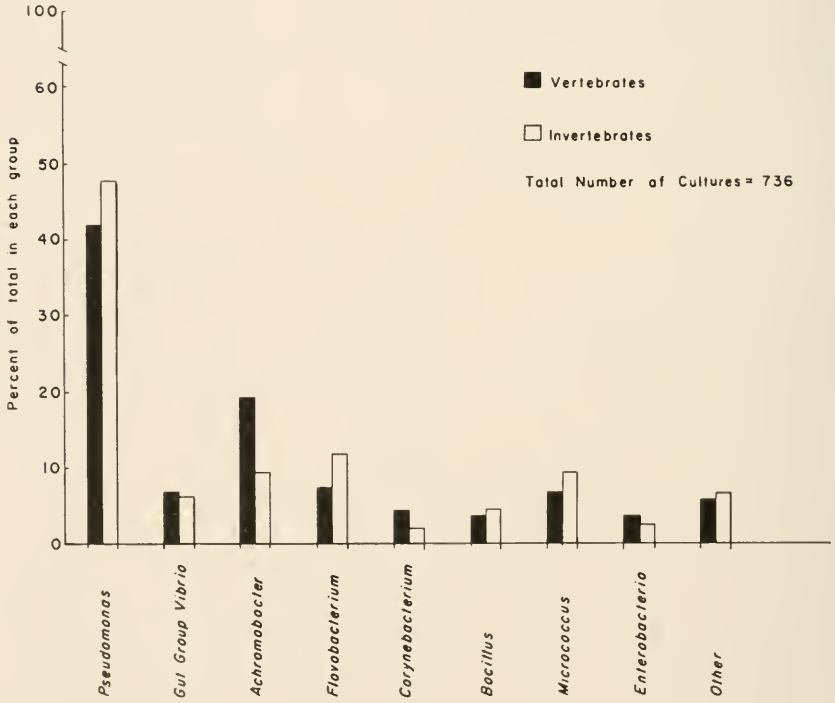

Fig. 1. Generic distribution of bacteria found on marine animals (expressed as per cent).

the classes Chondrichthys and Osteichthyes and 14 species of invertebrates, representing five phyla.

RESULTS AND DISCUSSION

The generic distribution of bacteria in the commensal floras of vertebrates and invertebrates from the Pacific Ocean are shown in Figure 1. Gram-negative organisms of the Pseudomonas/Achromobacter/Flavobacterium group were preponderant on both groups of animals. In both cases, representation of Gram-positive groups was very low. Generally, the populations of bacteria harbored by marine vertebrates and invertebrate animals are similar. However, there are detectable differences in the representation of the Pseudomonas/Achromobacter and the Flavobacterium groups. Vertebrate animals apparently carry greater numbers of *Pseudomonas* sp. and *Achromobacter* sp. and invertebrates greater numbers of *Flavobacterium* spp. Also, *Micrococcus* sp. are generally found in greater numbers on invertebrate animals.

In Figure 2, the results obtained from comparisons of the bacterial flora of vertebrate animals caught in Puget Sound and near Eniwetok Atoll are given, representing the possible effects of habitat between a cold water and a warm water environment. However, apparent differences, though detectable, were small. Gram-negative organisms of the general Pseudomonas/Achromobacter/Flavobacterium grouping dominated both floras. *Achromobacter* sp. were somewhat more plentiful in the Puget Sound samples, and so were *Flavobacterium* sp. On the other hand, enterobacterial types (non-fecal) were present in notably higher numbers in Eniwetok Atoll samples.

A similar comparison of the bacterial flora of invertebrate animals caught in Puget Sound and Eniwetok Atoll is shown in Figure 3. Gramnegative organisms again predominated, but greater differences in the balance of the populations were noted. *Achromobacter* sp. were much more abundant in the warm water samples and *Flavobacterium* sp. were completely absent. Surprisingly, perhaps, there was little difference in the numbers of enterobacterial types.

The overwhelming importance of Pseudomonas and Achro-

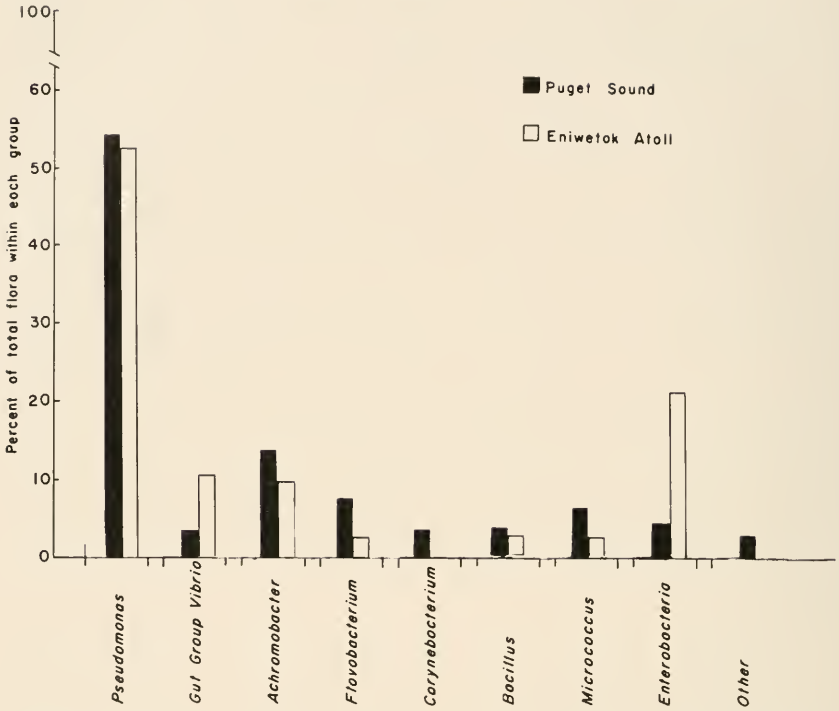

Fig. 2. A comparison of the distribution of generic types of bacteria isolated from vertebrate animals taken in Puget Sound, off the Washington Coast, on Eniwetok Atoll and in Rongelap Lagoon.

TABLE 2

DISTRIBUTION OF PREDOMINANT GENERA WITH RESPECT TO (A) HABITAT AND (B) HOST

A.

	North. Pacific	*South. Pacific*
Pseudomonas sp.	51%	29%
Achromobacter sp.	12%	24%
Total	63%	53%
(Number in sample)	(338)	(108)

B.

	Vertebrate Marine Animals	*Invertebrate Marine Animals*
Pseudomonas sp.	47%	45%
Achromobacter sp.	18%	9%
Flavobacterium sp.	7%	13%
Micrococcus sp.	5%	9%
Total	77%	76%
(Number in sample)	(276)	(170)

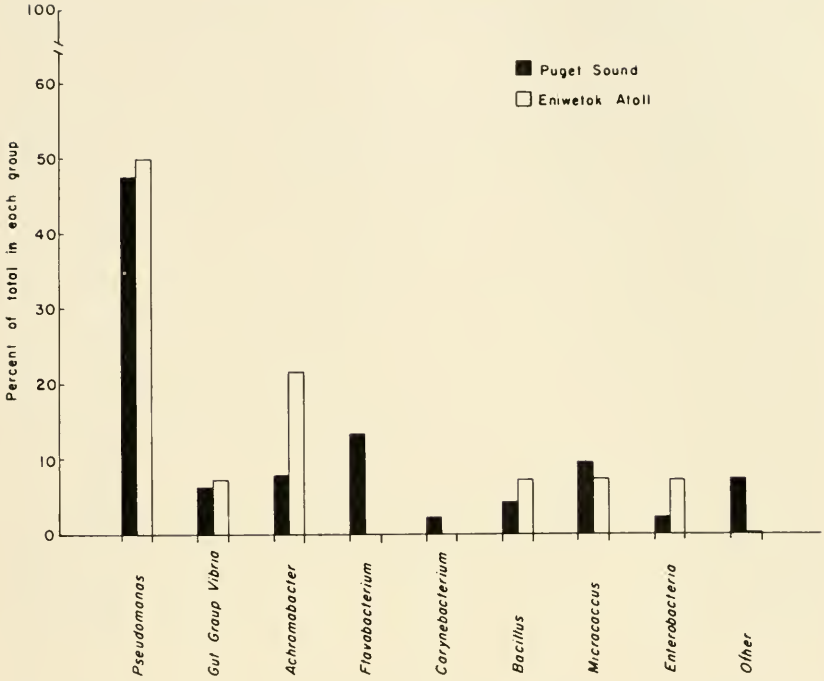

Fig. 3. Distribution of generic types of bacteria isolated from invertebrate animals taken in Puget Sound and in the Eniwetok area.

mobacter types in the marine animal floras is emphasized in Table 2A where the representation of these groups in northern and southern Pacific animals is summarized. Despite large differences in their relative abundance, indicating a habitat effect, the groups together make up 63 and 53 per cent of the total flora, respectively.

The situation with regard to the major bacterial genera in vertebrate and invertebrate animals is summarized in Table 2B. Here there is little difference in numbers of *Pseudomonas* sp., but a lesser representation of *Achromobacter* sp. in the flora of invertebrate animals which is apparently compensated for by increased occurrence of *Flavobacterium* and *Micrococcus* species. Thus, a minor effect of host (at the phylum level) is apparent.

The authors found little difference between the bacterial

floras of individual species within the vertebrates and invertebrates.

The theory of commensalism of bacteria assumes a selection of certain predominant physiological and biochemical types as a result of the physicochemical conditions of the environment. Such selection is implicit in the previously noted generic differences associated with host and habitat since physiological and biochemical characteristics constitute the main classification criteria. However, a wide range of such characteristics is, in fact, encompassed by species within any genus and this is particularly evident in nutritionally non-exacting groups such as Pseudomonas. The biochemical and physiological properties of all bacteria isolated from vertebrates and invertebrates in both areas of the Pacific were therefore studied.

The general biochemical capabilities of the two major groups isolated from northern Pacific and southern Pacific marine animals are listed in Table 3. Pseudomonas from the southern Pacific animals seemed to be more active in attacking milk and, in general, exhibited a more active nitrogen metabolism than those

TABLE 3

PHYSIOLOGICAL PROFILE OF PSEUDOMONAS AND ACHROMOBACTER FROM MARINE ANIMALS

	Pseudomonas		*Achromobacter*	
	North. Pacific	*South. Pacific*	*North. Pacific*	*South. Pacific*
Acid*	18**	32	31	46
Peptonized*	46	55	8	0
Negative*	40	13	51	35
Gelatin liquefied	61	61	36	46
Urease positive	35	35	36	15
H_2S produced	11	23	8	4
Citrate positive	37	52	28	19
Free NH_3 produced in peptone water	71	96	51	23
Nitrates reduced	26	61	31	31
Acid in glucose (aerobic)	71	35	69	19
Number in sample	174/338 (51%)	31/108 (29%)	39/338 (12%)	26/108 (24%)

* Litmus milk reactions.

** Expressed as per cent of the sample testing positive.

from the north Pacific, but the latter attack glucose more frequently. Somewhat paradoxically, a slightly larger percentage of southern Pacific *Pseudomonas* sp. were able to utilize citrate as sole carbon source. Differences seemed less between the Achromobacter groups, though northern Pacific strains were apparently more saccharolytic and greater numbers utilized citrate as sole carbon source than did those strains from the southern Pacific.

The most clear-cut difference between commensal bacteria of the northern and the southern Pacific Ocean areas was in temperature growth range. As can be seen from Table 4, in the northern waters psychrophilic bacteria predominated; in the southern areas the mesophils were the ascendant types. In both areas, a significant and consistent number of eurythermic bacteria (those able to grow at both temperature extremes) persisted. Thus, temperature, a long recognized determinant factor in bacterial growth, has a definite effect on the populations of bacteria found associated with marine animals from different climatological habitats.

TABLE 4

HABITAT AND TEMPERATURE RANGE RELATIONSHIPS FOR BACTERIA FROM MARINE ANIMALS

	Puget Sound	*Washington Coast*	*Eniwetok Atoll*	*Rongelap Lagoon*
Per cent showing growth at 0 C*	77.0	60.6	30.9	14.3
Per cent showing growth at 37 C*	24.0	59.0	92.4	97.1
Per cent showing growth at 0 C and 37 C*	23.0	30.8	30.4	12.8

* Expressed as per cent of total sample tested.

Figure 4 presents data obtained from studies of the biochemical and physiological activities of bacteria taken from vertebrates and from invertebrates. There are no explicit differences in the metabolic activities of the bacteria isolated from the two animal groups. However, bacteria isolated from vertebrates demonstrated greater reductive capacity, and, a greater tendency to produce acid in litmus milk and aerobic dissimilation of sugars possibly indicates a more active carbohydrate metabolism. This hypothesis is further supported by the greater preponderance of citrate

utilizers in the bacterial populations from the latter group of animals. On the other hand, organisms isolated from invertebrates showed greater and consistent incidence of anaerobic glucose dissimilation and polysaccharide (starch) hydrolysis.

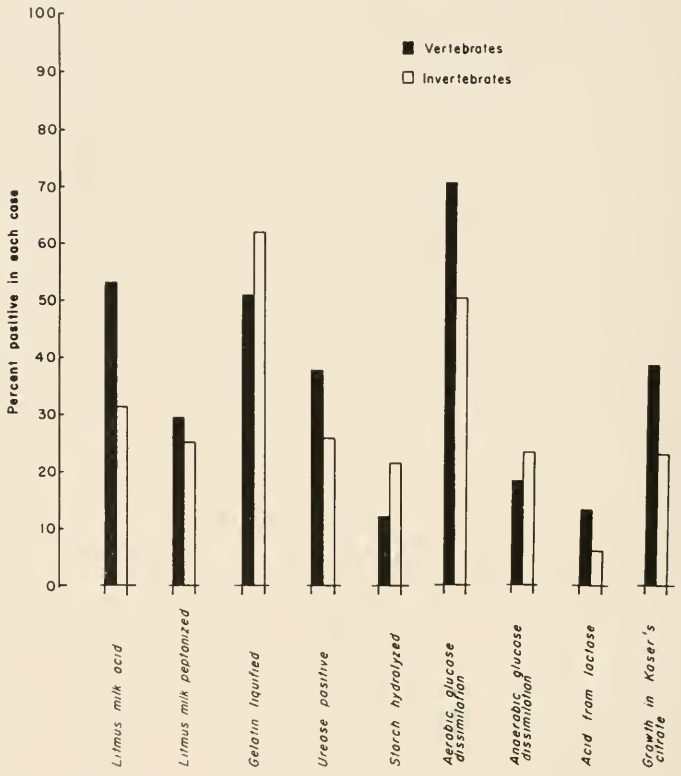

Fig. 4. A comparison of biochemical and physiological activities of bacteria associated with vertebrates and invertebrates.

The physiological properties of *Pseudomonas* and *Achromobacter* sp. isolated from vertebrate and invertebrate marine animals were studied in detail (see Table 5) in an effort to relate the general picture of metabolic functions of the bacterial populations to the major groups of bacteria isolated. The *Pseudomonas* sp. from both animal groups were relatively similar, but greater overall activity of *Pseudomonas* sp. from vertebrate animals was noted. *Achromobacter* sp. yielded no distinguishable pattern

as compared by animal host. However, gelatin liquefaction and glucose attack was more frequently encountered in *Achromobacter* sp. from vertebrates; ammonia-producing and nitrate-reducing *Achromobacter* sp. were more frequently isolated from invertebrate animals.

Table 5 presents the comparison of physiological and biochemical properties of *Micrococcus* and *Flavobacterium* sp. from vertebrate and invertebrate marine animals. *Micrococcus* sp. from vertebrates showed much greater general physiological and biochemical activity, particularly noticeable in the nitrogen metabolism reactions. Little apparent difference was noted in carbohydrate degradation by the two groups. The Flavobacterium population from the two types of marine animals demonstrated considerable carbohydrate utilization, but proved to be a more exacting population, with respect to nutrition, than any of the other three groups considered above: few *Flavobacterium* sp.

TABLE 5

Comparison of Physiological Properties of *Pseudomonas*, *Achromobacter*, *Micrococcus* and *Flavobacterium* sp. from Vertebrate and Invertebrate Marine Animals

Reaction	*Pseudomonas*		*Achromobacter*		*Micrococcus*		*Flavobacterium*	
	Vert.	*Inv.*	*Vert.*	*Inv.*	*Vert.*	*Inv.*	*Vert.*	*Inv.*
Acid*	26**	17	40	27	36	0	0	0
Peptonized*	53	37	8	7	82	31	46	14
Negative*	29	46	42	53	9	69	31	77
Gelatin Liquefied	62	59	46	20	64	19	23	68
Urease Positive	43	21	28	27	27	31	38	14
H_2S produced	14	12	10	7	9	31	15	9
Citrate positive	48	24	22	33	27	6	8	9
Free NH_3 produced in peptone water	67	80	32	67	55	12	77	5
Nitrates reduced	33	29	28	40	18	38	38	14
Acid in glucose (aerobic)	72	54	52	40	73	69	85	87
Number in sample	129/276 (47%)	76/170 (45%)	50/276 (18%)	15/170 (9%)	15/276 (5.4%)	16/170 (9.4%)	19/276 (6.9%)	22/170 (13.0%)

* Litmus milk reaction

** Expressed as per cent of the sample testing positive

Vert. = vertebrates

Inv. = invertebrates

utilized citrate as sole carbon source. Only a slightly greater overall proteolytic-activity can be ascribed to those species from vertebrate animals. *Flavobacterium* sp. isolated from invertebrate animals showed a pronounced ability to liquefy gelatin. Thus, with the exception of a few characteristics in *Achromobacter* and *Flavobacterium*, the physiological properties of each of the major groups corresponded well with the general picture for the total flora in vertebrates and invertebrates. Within the limitations of the normal range of properties of the different genera, species having similar physiological characteristics tend to predominate in the particular environment provided by the vertebrate or invertebrate body.

Unfortunately there is insufficient information presently available on the normal chemical, or even physical, conditions of the exposed surfaces of marine animal bodies to correlate the observations on bacterial physiology with the actual nutritional composition of the microenvironment. In view of the reasonably clear cut picture which emerged from the results of this study, it would seem that studies in this general area might be rewarding.

SUMMARY

An analysis was made of data obtained from a study of 743 bacterial cultures associated with nineteen species of marine vertebrates and *circa* fourteen species of invertebrates captured in several areas of Puget Sound and off the Washington Coast, in the northern Pacific Ocean, and in several areas near Eniwetok Atoll and in Rongelap Lagoon in the southern Pacific Ocean. Gram-negative, asporogenous, rod-like bacteria predominated in all populations of bacteria isolated from the marine animals sampled. There was little evidence of any major taxonomic differences between bacterial populations of vertebrates and invertebrates or among populations derived from different geographical areas. *Pseudomonas* and *Achromobacter* sp. predominated in all samples studied, but minor differences were observed in the proportions of these and other bacterial groups present. Populations of bacteria associated with sessile invertebrates generally included greater numbers of *Micrococcus* and *Flavobacterium* sp. than populations associated with vertebrates taken in the

same general area. All samples from Eniwetok and Rongelap showed greater numbers of *Micrococcus* and (non-fecal) Enterobacteriaceae sp. than samples from the northern Pacific Ocean areas.

Physiological and biochemical activities of the bacterial populations studied showed some differences do exist between populations taken in different geographical areas and from the different phyla of invertebrates and chordates. Organisms isolated from southern areas were preponderantly mesophilic and showed greater proteolytic and reductive activity associated with nitrogen metabolism. Northern areas of the Pacific Ocean yielded marine animal commensals which were psychrophilic and active in aerobic carbohydrate utilization. A consistently eurythermic portion of the commensal bacteria sampled in all areas was found to be present. Animal host differences were less explicit, but, in general, bacteria from vertebrates were more active biochemically, both in toto and by major groups, than those from invertebrates.

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Chapter 58

Phytoplankton Successions in the Canadian Arctic

Adam S. Bursa

The term "succession" is defined as the sequence of species in time and space. Broadly, phytoplankton is rare in the sea when metabolic activities decline, and increases when factors permit. The time sequence of growth from initial populations, cyst for mation and germination is therefore significant for the delimitation of the duration of individual cycles of plankton species building up to their collective maxima.

Cleve (5) classified early spring diatoms as "Arctic" and later spring species as "Boreal." Recent views include those of Braarud *et al.* (4), who conclude that successions which depend on the initial composition of the phytoplankton may vary from year to year. Margalef (9), on the other hand, has suggested that the annual regularity of successions probably cannot be distorted by tide and wind.

The vegetative or growth season in warm seas lasts twelve months, and in the high arctic, only two or three months. This latter short vegetative period induces development of the unimodal annual cycle characteristic for the high arctic, although a bimodal cycle occurs in low arctic seas (2).

The study of successions in the environment should be carried out with the microscopic sedimentation method to include ultraplankton normally lost in net samples. Sampling should be frequent; because of the rapid quantitative fluctuation of arctic plankton, maxima could be missed in samples which are separated by periods of a few days. However, regardless of technical preparedness, selective grazing and sudden changes of conditions of water and ice can obscure the picture of successions.

Phytoplankton collected 100 miles offshore in the Arctic

Ocean (Canadian Polar Shelf) consisted of 44 diatoms, thirteen dinoflagellates and six flagellates, the majority being Centricaceae. The populations are so small and so irregular in vertical distribution that the seasonal sequence of successions is hard to recognize.

The neritic, brackish phytoplankton of the Isachsen area, slightly to the south of the Arctic Ocean in the Canadian Arctic Archipelago, shows a bimodal annual cycle. In 1960 the spring maximum in this cycle was formed by Pennatae, mostly *Nitzschia pungens, N. lineola,* and *N. seriata,* with one peak on July 2 and a second on July 9, after which they declined. The summer Centriceae succession, formed mainly by *Chaetoceros ceratosporum,* also had two peaks, the first on July 9, and the second and largest on July 13. All cells by this latter date had formed cysts and their cycle was completed. Lesser cycles included a unimodal succession of freshwater green flagellates with a peak on July 2 and a unimodal dinoflagellate cycle with a peak on July 22. The Isachsen cycle, with 48 diatoms, 33 dinoflagellates and 20 flagellates also formed larger populations than found in the offshore waters of the Arctic Ocean. The definite maxima of Pennatae followed by Centriceae were separated by only a few days, but the annual cycle must be considered bimodal, although the area is certainly high arctic by Bogorov's (2) criteria.

Igloolik, Foxe Basin, 660 miles southward of Isachsen, shows clearly a bimodal cycle caused by the alternation of the spring Pennatae and the summer or open-water Centriceae, with maxima separated by 28 days. The spring maximum occurred in 1956 in mid July and the summer maximum on August 11, and the latter was followed by maxima of dinoflagellates and Coccolithineae. The phytoplankton cycle in the coastal location of Rowley Island, 60 miles to the south of Igloolik, was exposed to severe ice effect, which delayed the summer successions of Centriceae until mid September. The contrasts between the two environments are also reflected in the numbers of species: 34 diatoms, 5 dinoflagellates, 1 Coccolithineae at Rowley Island; 78 diatoms, 30 dinoflagellates, 9 Coccolithineae at Igloolik.

Further to the south in Hudson Bay, 235 phytoplankton species have been recorded. This marked increase is related to higher temperatures, light, less rigorous ice conditions, and the

hydrographical character of waters derived from different biogeographical regions, such as Polar and Atlantic, as well as fresh water. Similar diversity was found in phytoplankton at Point Barrow, Alaska, with 90 diatoms, 78 dinoflagellates, and 22 flagellates, compared to Isachsen, with 48 diatoms, 38 dinoflagellates and 20 flagellates. These localities, 990 miles apart, show different ecological conditions. Point Barrow plankton originates in different areas, such as the Pacific, Bering Sea and Arctic Ocean, while the Isachsen plankton is essentially polar autochthonous.

The general course of seasonal successions in the Canadian arctic is similar to that in Denmark Strait, according to Braarud (3), in the East Greenland Current, according to Steeman Nielsen (10), and East Greenland waters (6), although the taxonomic composition of the standing crops differs according to locality.

Certain pennate diatoms are exclusively arctic endemic, and their range seems to parallel the distribution of ice, which increases the capacity for attachment. Ice creates ecological niches for surface blooms, microflora of meltwater ponds on the ice surface, and for films of diatoms on the under surface. *Arctic neritism* is characterized by the abundance of neritic, freshwater and brackish forms permitted by the existence of the ice. The pennate diatoms, adapted to reduced light under the ice, initiate their growth probably as late as late May at Isachsen, April at Igloolik, possibly in March in James Bay and southern Hudson Bay, and in the winter in the Gulf of St. Lawrence. Arctic endemic diatoms are known in the Gulf of Maine (1, 8), and are common in the Gulf of St. Lawrence according to Gran (7). A brown-green film of diatoms taken from ice in the Gulf of St. Lawrence in April, 1957, consisted mostly of arctic endemic forms, in which Pennatae dominated in biomass. These vanish from the plankton when the ice melts in spring. This early-spring, ice-loving succession has its southern range limited by the duration of ice.

Freshwater green flagellates, *Euglena, Chlamydomonas, Polyblepharis, Chlorella,* and the chrysomonads, *Mallomonas* and *Ochromonas*, constitute an autotrophic, acclimatized arctic succession, abundant in brackish and high salinity waters. Although Peridineae are rare, fair numbers of them live in the arctic. Some holophytic Gymnodinioideae become abundant at the end of

summer and form large populations which are reduced by selective grazing. Evidence has been obtained that the large *Coscinodiscus* species require a biennial cycle to complete their development, which is retarded by the arctic environment.

In summary, the arctic phytoplankton shows a heterogeneous taxonomic composition, mostly cosmopolitan with less numerous endemic arctic forms. The universal rhythm of successions in cold seas involving spring Pennatae and summer Centriceae is in its main outline similar throughout the northern latitudes, with the respective maxima occurring much closer together in the shorter seasons of the far north. The absence of Pennatae in seas with no ice and the absence of Cyanophyceae in the arctic are fundamental biological characters of these seas.

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Chapter 59

Osmotic Regulation of the Growth Rate of Four Species of Marine Bacteria*

Darrell Pratt and Mary Austin

A number of marine bacteria have been reported to require relatively high concentrations of Na^+ for their optimal growth and metabolic activity; this requirement has been considered to be more than a simple expression of osmotic activity since the total replacement of Na^+ with nonspecific solutes has not been successful (4, 6, 8, 9, 10). The possibility that Na^+ plays some osmotic role has been demonstrated by the partial substitution of sucrose for NaCl in metabolic and growth experiments. The results of these latter experiments have been variable and the degree of replacement has been low; consequently, the osmotic function of Na^+ has been considered a minor one by some workers. However, failure to replace an effective solute by a nonspecific solute must be interpreted with caution since the technic has several hazards. The solutes selected, in addition to being osmotically effective, must also not have stimulatory or inhibitory effects on the test organism in the experimental circumstance. The stringent nature of these demands is evident since they require the substance to have little or no secondary biological activity at relatively high concentrations. Thus, the Na^+ requirement of a marine bacterium could be largely osmotic in character and still be relatively specific because of the cell's intolerance for other solutes at osmotically equivalent concentrations. This report deals with the results of a study of the osmotic function of Na^+ in the growth medium for a bacterial isolate of marine origin. One objective was to determine if osmotic equivalence of solutes could be observed with suboptimal concentrations, an equivalence

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which might be masked by inhibitions at higher concentrations. The results reported in this paper are consistent with the view expressed by Richter in 1928 (quoted by Flannery (1)), that Na^+ satisfies both a specific nutritional function and a non-specific osmotic function in the growth of marine bacteria.

METHODS

The organism employed was previously described under the designation MB 22 (8, 10). It was a gram-negative, polarly flagellated rod, which produced acid anaerobically from glucose, formed indole from tryptophan, and nitrite from nitrate. The cells were curved in older cultures, particularly in solid media. This isolate has been tentatively considered to belong to the genus *Vibrio* and has been designated *Vibrio* MB 22. Preliminary experiments were also made using three other marine isolates: MB 1, MB 21, and MB 29 (10).

The procedure was to study the effect of solute concentration on the growth rate and total growth in a basal medium consisting of 0.1 per cent trypticase (BBL), 0.05 M $MgCl_2$, 0.005 M K_2SO_4 and 0.0003 per cent $FeSO_4{:}7H_2O$. The medium was adjusted to pH 7 with dilute KOH. This medium containing 0.4 M NaCl was used to grow inocula; the inoculum cultures were centrifuged and resuspended in a salt solution containing 0.4 M NaCl, 0.05 $MgCl_2$, and 0.005 M K_2SO_4. The cell density of the inoculum was adjusted so that a one hundred fold dilution in the trial medium had a slight turbidity; the cell density after dilution was approximately 10^8 cells per ml. The introduction of salts from the inoculum was minimized by dilution and made no significant contribution to the osmotic activity of the medium. The volume of medium was 20 ml; the inoculum size was 0.2 ml; and the incubation temperature was 32 C. The cultures were grown in 250 ml Erlenmeyer flasks with an attached colorimeter tube and shaken constantly during incubation; the turbidities of the cultures were measured periodically. For the purpose of clarity in presenting results, growth rate was determined during five hours incubation. Examination of individual growth curves demonstrated that differences in growth lag did not make a ma-

jor contribution to the differential growth observed after five hours.

Comparisons between solutes were made on an osmolal basis. The basal medium was assumed to make a constant contribution to the total osmolality and not to interact appreciably with the added solutes; the osmolality of the medium was considered to be that produced by the added solutes (3). The media were prepared on a molal basis, i.e., moles of solute to be tested per 1000 g of solvent. The calculations of osmolal concentrations were made using the data given by Heilbrun (3) and by Harris (2).

RESULTS

Effect of NaCl on the Growth Rate

The growth rate of *Vibrio* MB 22 was regulated by the amount of NaCl added to the basal medium; the total growth yield was independent of this concentration (Fig. 1a). No growth occurred in the basal medium without the addition of NaCl. That this result was the reflection of the differential death of the inoculum cells resulting from osmotic shock was considered a possibility. However, when inoculum cells were resuspended for 30 minutes at 32 C in the basal salt solution (0.05 M $MgCl_2$, 0.005 M K_2SO_4) containing respectively 0.05 M, 0.1 M, and 0.14 M NaCl, no differences among the viable counts in the suspensions were observed; the effect of osmotic shock was probably minimal. The maintenance of these cells in the hypotonic solutions was probably largely due to the Mg^{++} content of the basal medium (7, 9). A second possibility was that the cells varied in their ability to grow at lower salt concentrations; however, the viable count of cells in the inoculum was equivalent in agar media containing 0.4 M, 0.1 M, or 0.05 M NaCl. The colonies growing in any particular concentration were equal in size; the colonies developed at least twenty-four hours earlier in the medium containing 0.4 M NaCl than in that containing 0.05 M NaCl. No adaptation of the cells to lower NaCl concentrations was observed; inocula grown in 0.05 M NaCl responded to NaCl in a manner indistinguishable from the usual inoculum (Figure 1b). From these observations it

was concluded that the effect of NaCl was to control the growth rate of the intact cells of MB 22.

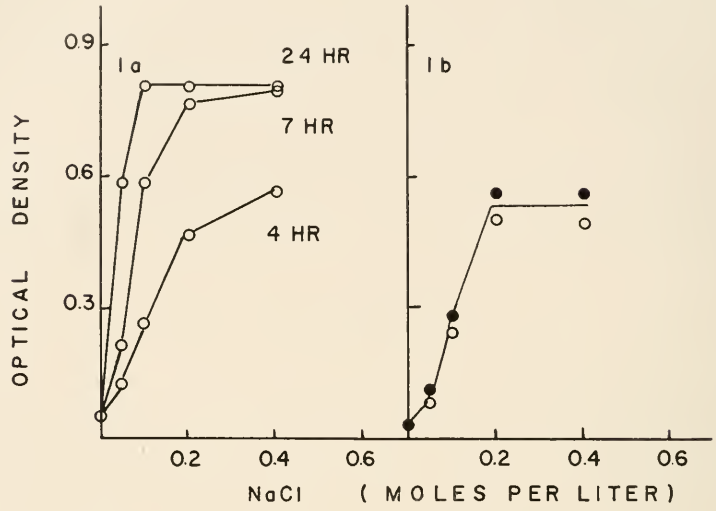

Fig. 1. 1a Effect of the concentration of NaCl on the growth rate and growth yield of *Vibrio* MB *22. 1b.* Effect of the concentration of NaCl. in the inoculum culture on the subsequent response of the cells to NaCl. Inoculum was grown in 0.4 M NaCl (open circles). Inoculum was grown in 0.05 M NaCl (closed circles). Incubation seven hours.

Replacement of NaCl with Na_2SO_4

Although Na_2SO_4 was not a suitable salt to test the specificity of the Na^+ requirement, it was useful in determining the character of the response since it differs from NaCl in both its colligative activities and ionic strength, allowing comparisons to be made on both bases. MacLeod and Hori (5) have reported media of suitable ionic strength to be essential to the activity of cell-free preparations of isocitric dehydrogenase and aconitase isolated from a marine bacterium. The Na^+ requirement for MB 22 was satisfied by Na_2SO_4; equivalence with NaCl was found when the results were compared on an osmolal basis (Fig. 2). Thus, 0.16 molal NaCl and 0.13 molal Na_2SO_4 produced an equivalent osmolality of 0.3. This equivalence suggested that at these concentrations the principal factor controlling the growth rate was the tonicity of the medium.

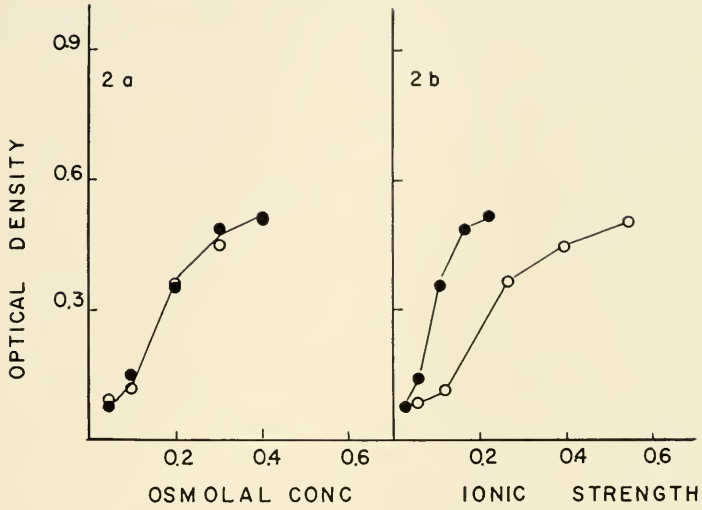

Fig. 2. 2a. Comparison on an osmolal basis of the effect of Na_2SO_4 and NaCl on the growth of *Vibrio* MB 22 after five hours incubation. Na_2SO_4 (open circles). NaCl (closed circles). *2b.* Comparison of the same data but with ionic strength plotted on the abcissa. Na_2SO_4 (open circles). NaCl (closed circles).

Partial Replacement of NaCl by KCl or $MgCl_2$

Neither KCl nor $MgCl_2$ wholly replaced the Na^+ requirement; however, in the presence of suboptimal concentrations of NaCl the growth rate of MB 22 was enhanced by either of these salts. Approximately 0.1 osmolal NaCl was required to produce the maximal growth rate in the basal medium containing 0.6 osmolal KCl. In the absence of the latter salt four or five times as much NaCl was required. The effectiveness of KCl was tested in the presence of 0.125 osmolal NaCl and was found to give growth responses equivalent to NaCl at all concentrations tested (Fig. 3a). The lower concentrations of $MgCl_2$ were as effective as NaCl when their activities were compared on an osmolal basis; however, higher concentrations depressed the growth rate (Fig. 3b). The effectiveness of $MgCl_2$ was of significance since it is a three ion salt whose colligative properties do not depart greatly from the ideal at the concentrations used; hence, the function of Na^+ was in part replaced by Cl^- when $MgCl_2$ was employed. The

partial replacement of NaCl by KCl and $MgCl_2$ demonstrated the non-specific nature of an appreciable component of the salt requirement for this organism.

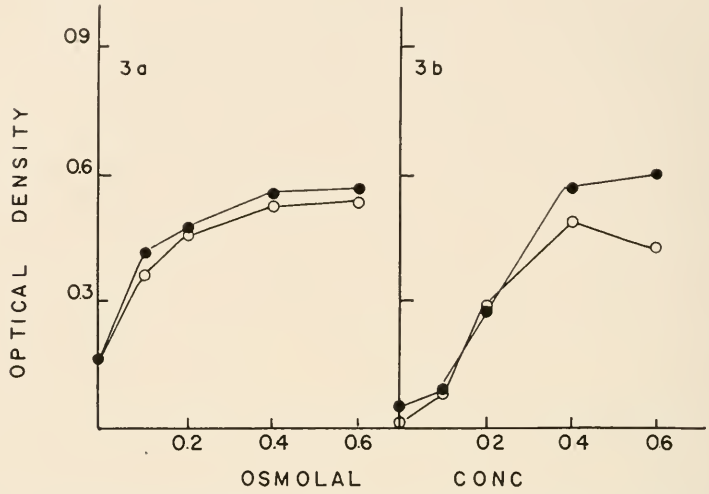

Fig. 3. 3a. Partial replacement of the growth for NaCl by KCl using *Vibrio* MB 22. NaCl (0.125 osmolal) was added to the basal medium. KCl (open circles). NaCl (closed circles). Incubation five hours. *3b.* Partial replacement of the growth requirement for NaCl by $MgCl_2$ using *Vibrio MB 22.* NaCl (0.1 osmolal) was added to the basal medium. $MgCl_2$ (open circles). NaCl (closed circles).

Partial Replacement of NaCl with Sucrose

Vibrio MB 22 fermented sucrose with the production of acid, which inhibited growth in the unbuffered medium; consequently, quantitative comparisons of growth rates were impossible. Periodic neutralization of the cultures with NH_4OH during incubation allowed good growth; neutralization was essential only during the early period of incubation, suggesting the accumulation of a buffering organic acid. Fumaric, succinic, citric and glutamic acids permitted growth in media containing 0.4 M NaCl and inhibitory amounts of sucrose (0.1%). The organic acids were neutralized with NH_4OH. Succinic acid was the most effective and its function as a buffer was apparently supplemented by its being metabolized, thus increasing the available base. The

addition of 0.2 per cent succinic acid resulted in growth and the maintenance of a neutral pH in the presence of sucrose; this concentration (0.017 M) did not contribute significantly to the osmolality of the medium. The growth rate of isolate MB 22 was enhanced by the addition of sucrose to a medium containing succinate and optimal amounts of NaCl. The concentration of sucrose required for maximal stimulation was 0.1 per cent, again an amount not contributing significantly to the osmotic activity of the medium. In view of these results 0.1 per cent sucrose and 0.2 per cent succinic acid were added to the basal medium; the addition of optimal amounts of NaCl to this medium resulted in a rate of growth which was not increased by the further addition of sucrose. Growth did not occur in this new basal medium without the addition of Na^+; however, sucrose caused an appreciable stimulating effect in the presence of 0.1 osmolal NaCl. The lower concentrations of sucrose were equivalent to NaCl and main-

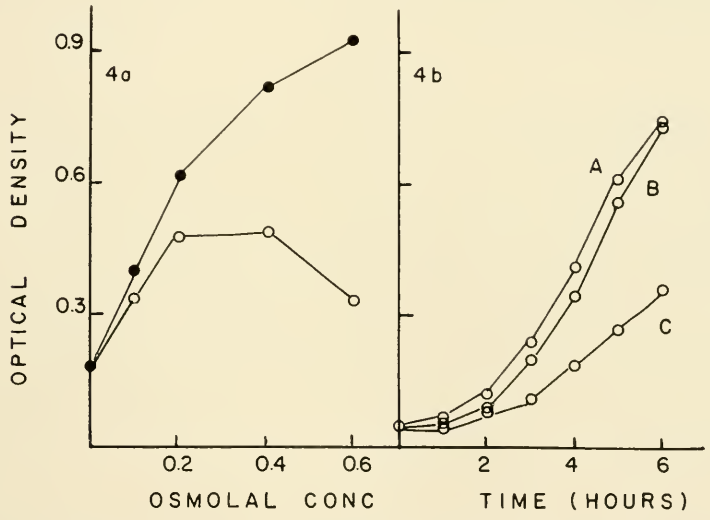

Fig. 4. Partial replacement of the growth requirement for NaCl by sucrose using *Vibrio* MB 22. NaCl, 0.1 osmolal; succinic acid, 0.2 per cent; and sucrose, 0.1 per cent were added to the basal medium. *4a.* Comparison of growth in sucrose and NaCl after five hours. Sucrose (open circles). NaCl (closed circles). *4b.* Comparison of growth rates in 0.2 osmolal sucrose and 0.2 osmolal NaCl (total osmolality, 0.3). A. 0.3 osmolal NaCl; B. 0.2 osmolal sucrose, 0.1 osmolal NaCl; C. 0.1 osmolal NaCl.

tained the growth rate through several hours; the higher concentrations were inhibitory (Fig. 4).

Preliminary Results with Other Isolates

Three other marine isolates, MB 1, MB 21, and MB 29 (10) were tested to determine if the findings with MB 22 were generally applicable. The solutes were tested by comparing the growth rate obtained in 0.3 osmolal NaCl with that obtained in a medium containing the solute, 0.2 osmolal, and NaCl, 0.1 osmolal. Under these conditions KCl was equivalent to NaCl in its effect for each organism. Sucrose and $MgCl_2$ exerted a sparing effect in only one isolate, MB 21, but inhibitory effects were observed using these solutes with the other two isolates. No growth occurred with these organisms in the absence of Na^+; Na_2SO_4, 0.3 osmolal, replaced and gave a growth response equal to that produced by 0.3 osmolal NaCl for each organism. These results indicated that species variability may have accounted for some of the divergent results reported by previous investigators.

SUMMARY

The growth rate of a marine bacterium, *Vibrio* MB 22 was found to be a function of the concentration of NaCl; the effect of NaCl was on the intact cells and was not the result of preventing cytolysis in the hypotonic solutions. The total growth yield was not regulated by concentration of NaCl; however, in the absence of Na^+, no growth occurred. The requirement was satisfied by Na_2SO_4, which allowed growth rates equivalent to those observed in osmotically equivalent concentrations of NaCl. Partial replacement of the Na^+ requirement was accomplished with KCl, $MgCl_2$ and sucrose; KCl was osmotically equivalent in its effect at all concentrations but $MgCl_2$ and sucrose were so equivalent only at lower concentrations. At higher concentrations these latter solutes were inhibitory and their osmotic effectiveness was masked. The magnitude of the osmotic component of the salt requirement of *Vibrio* MB 22 was shown by the occurrence of the maximal growth rate in 0.1 osmolal NaCl when in the presence of 0.6 osmolal KCl; in the absence of the latter salt, 0.4 to 0.6 osmolal NaCl was required. Preliminary experiments with three other iso-

lates, MB 1, MB 21 and MB 29, gave variable results; however, KCl partially replaced Na^+ in all; sucrose and $MgCl_2$ were effective in MB 21 but not in the other two.

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Part 6
Marine Bacteriology and the Problem of Mineralization

Chapter 60

Mineralization of Organic Matter in Santa Monica Bay, California

Charles G. Gunnerson

INTRODUCTION

Knowledge of the regeneration of nitrogen and phosphorus in the ocean has been obtained from field observations of the distribution of these nutrients over periods of time and from laboratory experiments. The data collected in Santa Monica Bay during oceanographic investigations of the effects of municipal sewage treatment plant effluent were examined. Local variations in nutrient concentrations suggested that mineralization rates could be computed. The effluent discharge thus provides a convenient opportunity for studying processes which normally take place over large, diffuse areas in the ocean, and which constitute part of what Emery (1) has defined as man-made oceanography.

Santa Monica Bay occupies a crescent-shaped indentation about 45 km long and 18 km wide on the Southern California Coast. The bathymetry is characterized by a broad shelf incised by two submarine canyons.

The oceanography of the bay reflects the influence of the California current, frequent coastal counter-currents, and the local diurnal wind regime. The afternoon sea breeze typically brings surface water from the southwest into the bay where it divides and subsequently leaves near Pt. Dume and Pt. Vicente (13). The net movement of near-shore water is generally up-coast or northerly. This corresponds with the geostrophic effect of the local density distribution. Littoral drift is down-coast due to the predominant swell pattern. Upwelling frequently is observed along the northern boundary of the bay, and cold spots or domes of well-mixed bottom and intermediate waters frequently result

from tidal currents along the break in slope at the 50-fathom contour.

The most significant discharge to the bay is the effluent from the City of Los Angeles' sewage treatment plant at Hyperion. During 1956-59, about 1 x 10^6 m^3/day of secondary effluent were disposed of through a submarine outfall about 1.5 km from shore at a depth of 15 m. The activated-sludge treatment included screening, grit removal, primary settling, aeration, final settling, chlorination, separate sludge digestion, and fertilizer production.

Oceanographic investigations of Santa Monica Bay were made by the Scripps Institution of Oceanography of the University of California, the Allan Hancock Foundation of the University of Southern California, and the City of Los Angeles during the period 1954-59. The sanitary, gross biological, and physical aspects of the discharge of Hyperion effluent have been previously reported (3, 7). Receiving water conditions in the bay have been subsequently changed by expansion and modification of the sewage treatment and disposal regimen; nevertheless, some of the results of earlier investigations are pertinent to the present study.

As the treatment plant effluent rose through the 15 m water depth, it was initially diluted with about 20 parts sea water per part effluent, and appeared on the surface as a "boil". Stirring processes continued for about four hours at which time the dilution was 100:1. Subsequent mixing took place slowly by means of lateral eddy diffusion and, after twenty-four hours in the water, the dilution was about 300:1. A restricted visible field and a larger in-shore water mass were formed.

The in-shore water mass was a typically bell-shaped, lenticular mass extending over about 50 km^2, which remained within the upper 10 m because of its high vertical stability. The boundary of this mass was characteristically established by the 300:1 dilution contour (the highest ratio which could be determined with any assurance), gradients in plankton, oxygen and turbidity concentrations, color changes at the surface, and convergences.

Coliform bacteria in surface waters were reduced by an order of magnitude every 6.5 hours during the first day after discharge in accordance with the relationships:

$$\frac{N_t}{N_o} = 10^{\frac{-t}{T\text{-}90}} \quad \text{and} \quad \frac{1}{T\text{-}90} = \frac{1}{T\text{-}90_m} + \frac{1}{T\text{-}90_d} + \frac{1}{T\text{-}90_s}$$

where N_t = number of survivors after t hours, N_o = initial population, T-90 = time in hours for 90 per cent reduction in population, and $T\text{-}90_m$, $T\text{-}90_d$, and $T\text{-}90_s$ = times for 90 per cent reductions due to mortality (17.8 hr.), dilution (20 hours), and sedimentation (21 hours), respectively.

The daily discharge contained about 20,000 kg ammonia nitrogen, 200 kg nitrate nitrogen, 22,000 kg organic nitrogen, 8,500 kg phosphate phosphorus, and 5,500 kg organic phosphorus. Sedimentation of organic material and mineral nutrients is dependent upon their distribution between the suspended and dissolved fractions, and may be assumed to be related to the reduction in surface coliforms due to sedimentation (4). On this basis, it is computed that 20,000 kg organic nitrogen, 5,000 kg organic phosphorus, and 460 kg PO_4-phosphorus settled to the bottom during the first day after discharge.

The present study is a further evaluation of the fate of the effluent in the receiving waters and is directed towards the time and space variations of nitrogen and phosphorus.

DATA AND CALCULATIONS

Figures 1 and 2 summarize observations of the effluent field along the 15 m contour between the outfall and 3 km downstream made January 13, 1956, on VELERO IV Cruise 235 (11). The dilutions are based upon chlorinities and define an effluent field floating within the upper 10 meters. The lower limit of this field corresponds with high O_2 and low NO_3-N values and with discontinuities in NH_3-N and PO_4-P concentrations. Water temperatures varied from 13.3 to 14.0 C at the surface and from 11.9 to 12.3 C at the bottom. A northerly current of about 15 cm/sec is estimated from surface dilution and surface coliform concentrations.

The total inorganic-N, assuming that nitrite is negligible, and PO_4-P within the upper 10 meters were markedly less than

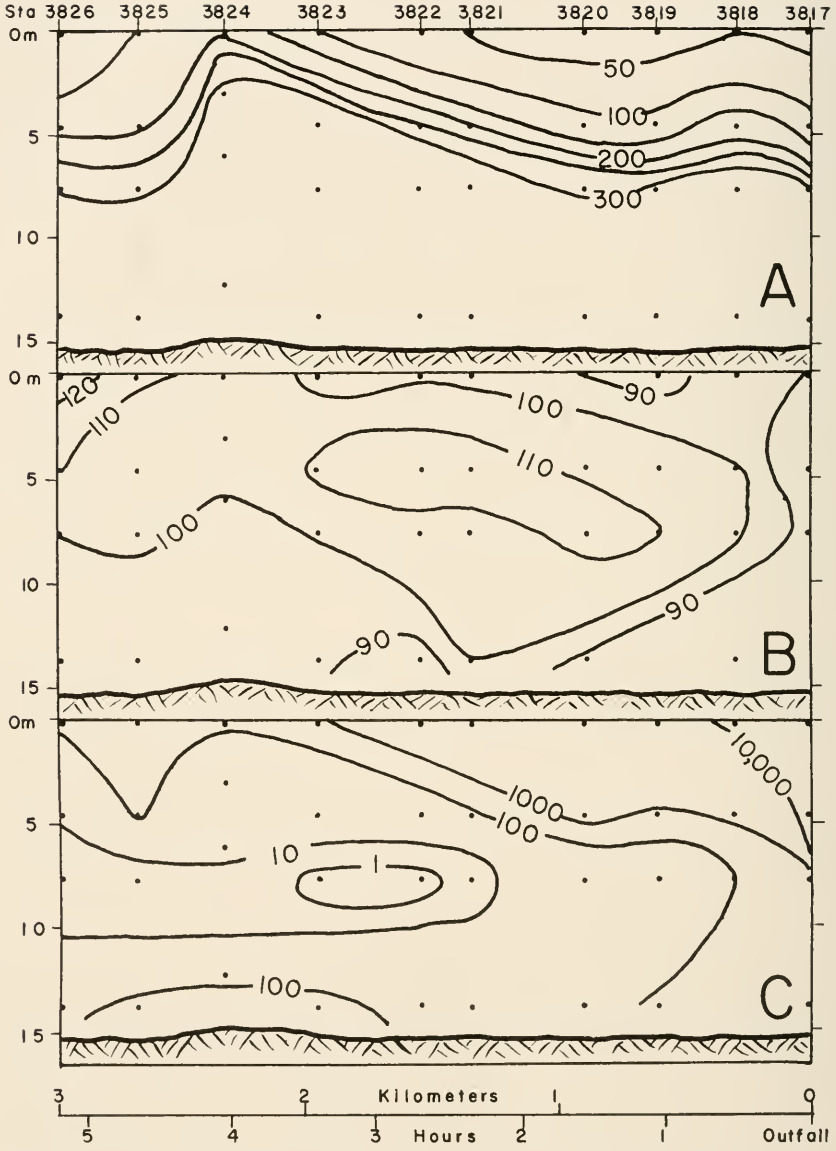

Fig. 1. Vertical distribution of properties in effluent field, Jan. 13, 1956. A—dilution, parts sea water per part effluent; B—oxygen, % saturation; C—coliform bacteria, MPN/ml.

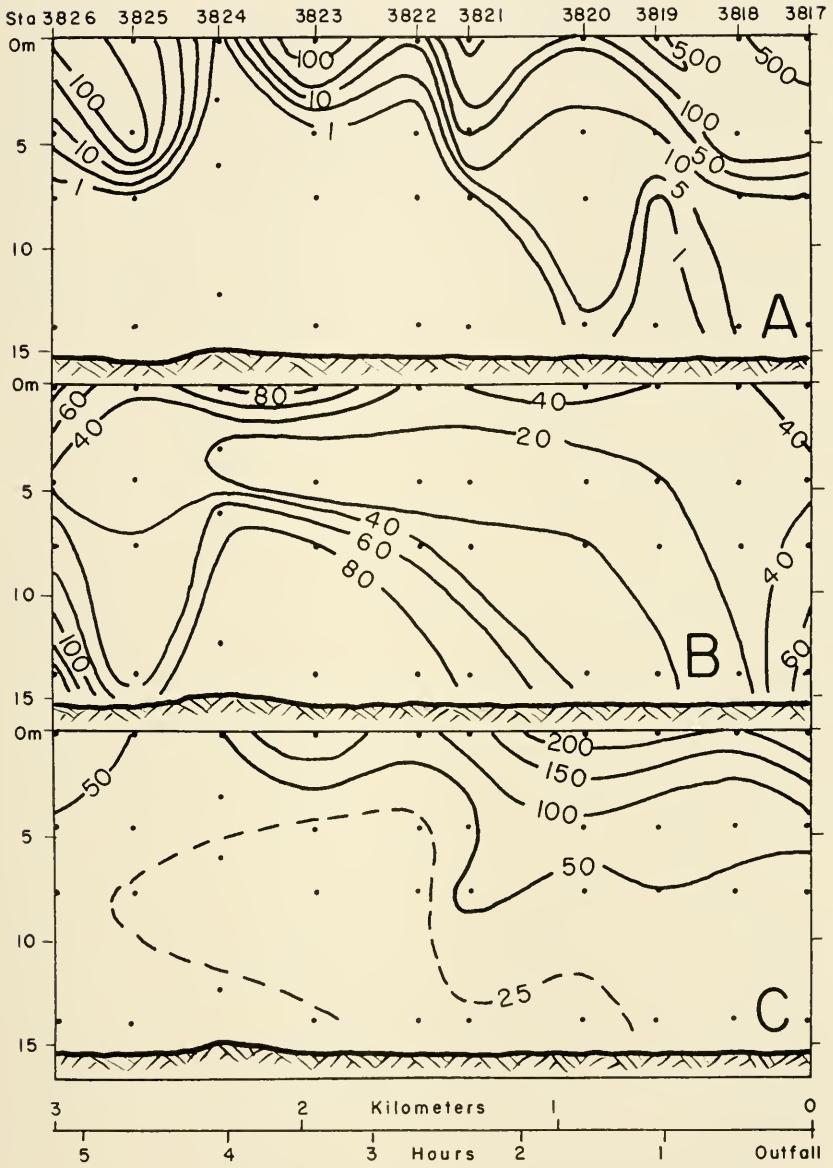

Fig. 2. Vertical distribution of properties in effluent field, Jan. 13, 1956. A—ammonia nitrogen, μg/L; B—nitrate nitrogen, μg/L; C—phosphate phosphorus, μg/L.

that calculated from dilution of the effluent. The reason for this deficiency, which reached a maximum after about one hour, is unknown. After one hour, the observed values approached the calculated values at a decreasing rate; the difference is assumed to have been due to mineralization. A similar relationship existed for PO_4-P. Table 1 summarizes computations of mineralization rates for nitrogen and phosphorus during the first day: the information available for the present study required the use of extrapolated values as indicated. As a first approximation, organic nitrogen and phosphorus are assumed to have been mineralized in the effluent field at rates of about 2 and 0.6 kg/day, respectively.

The NO_3-N and PO_4-P distributions shown in Figure 2 suggest that there are two distinct areas of mineralization, one within the effluent field and one at the bottom; similar distributions have been observed near other Southern California outfalls (12). A comparison of median concentrations of NO_3-N and PO_4-P throughout the water column near Hyperion with background concentrations observed on the same day at a point (AHF Sta 3827) about 20 km south of the outfall indicates that at the 7.5 m depth, the nitrate and phosphate concentrations were essentially normal. In the lower 7.6 m of the water column, NO_3-N and PO_4-P concentrations averaged about 30 and 20 μg/L above normal. If it is assumed that these are steady-state values over an average width of 1 km, and that the net flushing current in this area averages 5 cm/sec, about 1,000 kg nitrogen and 667 kg phosphorus were released daily to the bottom waters from the sediment. The NO_3-N derived from the 20,000 kg of organic nitrogen which settled during the first day. The phosphorus could have come from both the 5,000 kg organic and the 460 kg PO_4-P deposited; accordingly, a lower limit of 200 kg/day from the former is established. An intermediate value of 300 kg/day is adopted for regeneration of phosphorus as being more consistent with the N:P ratio for mineralization in the effluent field.

Nitrification in the effluent field is suggested in Figure 2. An analysis of crude averages of NH_3-N and NO_3-N variations during the first four hours of travel, made with the same provision for

TABLE 1

COMPUTATION OF MINERALIZATION RATES FROM PROFILE DATA

Time Hours	Dilution, Parts Sea Water per Part Effluent	Total Flow, m^3/hr	Inorganic Nitrogen					Phosphate - Phosphorus				
			Calculated from Dilution, µg/l	Average Observed, µg/l	Deficit µg/l	Mineralization		Calculated from Dilution µg/l	Average Observed µg/l	Deficit µg/l	Mineralization	
						Rate µg/l/hr	Total Grams				Rate µg/l/hr	Total Grams
0	20	0.8×10^6	1000	970	30		—	450	280	170		—
						300[a]					90[a]	
1	30	1.2×10^6	660	160	500		360	300	100	200		110
						200					60	
2	46	1.8×10^6	440	135	300		360	200	60	140		110
						125					40	
3	67	2.7×10^6	305	130	175		340	150	50	100		110
						75					30	
4	100	4.0×10^6	200	100	100		300	120	50	70		120
						50					10	
5	125	5.0×10^6	170	120	50		250	110	50	60		50
						½ to 1					~½	
24	300	1.2×10^7	10	20 to 50[b]	-(10 to 40)		110 to 460	26	~14	12		110
							Total 1720 to 2070 g/day					Total 610 g/day

a. Extrapolated from subsequent rates.

b. Estimated from Santa Monica Bay and other data reported by Stevenson and Grady (12).

continuity shown in table 1, indicated a 28 per cent reduction of the NH_3-N by nitrification.

Phosphate concentrations in surface waters of the bay have been observed on more recent cruises made by the Allan Hancock Foundation (AHF Stations 5505; 5536; 5538; 5728-39; and 6985-94). The average surface distribution of PO_4-P indicates that an area of 138 km² was affected by the discharge. Surface temperatures varied generally between 14 and 16 C.

A rate of phosphorus mineralization can be computed from the surface distribution of PO_4-P and a simplified treatment of the time-area-volume relationships within the effluent field. Assume that areas within the roughly bell-shaped surface PO_4-P iso-

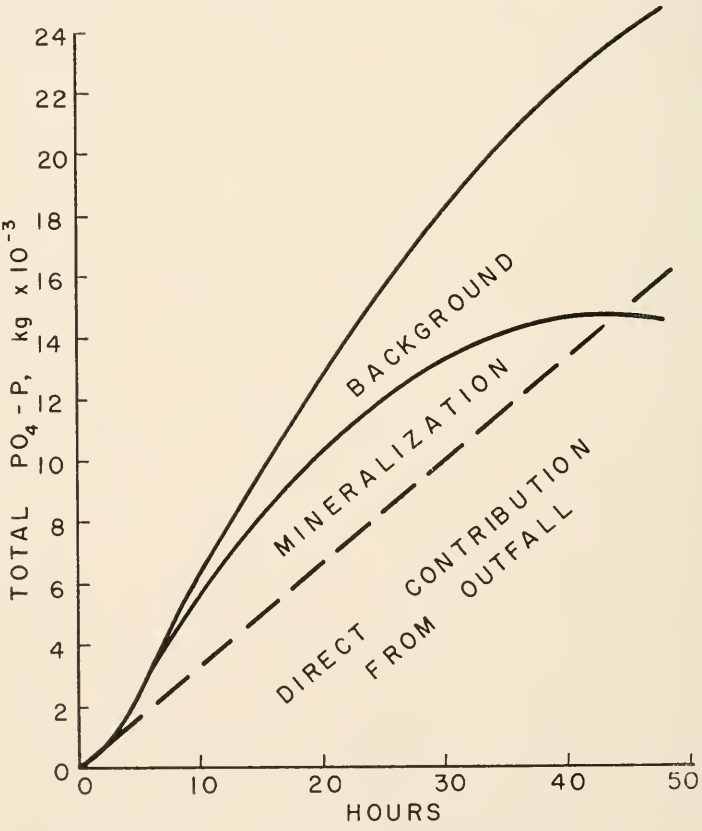

Fig. 3. Total phosphate phosphorus in effluent field.

pleths are equivalent to semicircles, and that they represent horizontal concentrations in one-half of a right circular cone whose apex is located over the outfall at a depth of 10 m. Continuity requirements for dilutions of 20, 100, and 300 parts sea water per part effluent at 0, 4, and 24 hours, respectively, and of extrapolated values to 48 hours, permit computation of the total PO_4-P within the mass for observed concentrations. This calculated value is then corrected by subtracting the background 15-20 μg/L and compared with quantities which represent the PO_4-P discharged with the effluent through various time intervals. Figure 3 shows the results of these computations where the difference between the total excess amount in the water and the increment furnished by the outfall reaches a maximum of 3,500 kg PO_4-P after about one day; this difference may be ascribed to mineralization. The differences indicated after about one and one-half days are of doubtful significance because dilution has brought the concentrations to within the normal variation.

DISCUSSION

Profiles of nutrient concentrations within 3 km of the outfall imply mineralization of organic nitrogen and phosphorus at rates of 1,000 and 300 kg/day, respectively, on the bottom, and 2 and 0.6 kg/day, respectively, in the effluent field. Thus, about 5 per cent of the available material is regenerated in the sediments while about 0.1 per cent is regenerated in the water. Although there are several uncertainties implicit in the analysis, the greater efficiency of mineralization within the sediment is apparent.

Increases in the standing crop of microplankton have been observed (7, 12); these vary from about 20 per cent during most of the year to some 300 per cent during the spring and fall blooms. Dinoflagellates appear to be the most responsive to the nutrients in the discharge. The nitrogen and phosphorus content of the standing crop of microplankton have been computed by means of the conversion factors given by Sverdrup *et al* (14, p. 929); the resulting values were less than normal background variations of the inorganic nutrients. The effects of normal and increased productivity and of the mineralization of plankton detritus have accordingly been neglected.

It is significant that only a portion of the daily contribution of organic material to the bottom is mineralized and returned to the water column. Some of the remainder enters into the food chain of enriched benthic and bottom fish populations. Hartman (6), Gunnerson (3) and Hume *et al.* (7) have discussed the effects of fertilization by discharges from various outfalls. The latter reported populations of capitellid worms of up to 200,000 individuals/m^2, equivalent to a biomass of 6 kg/m^2, near the Hyperion digested sludge outfall which was placed in operation in 1957. Comparable observations were not made near the one-mile outfall discussed in this study. Since operation of the sludge outfall primarily involves a change in the point of disposal rather than a significant difference in the quantity or nature of the settleable solids, the figures are probably indicative of conditions near the one mile outfall in 1956. While there was some increase in the organic carbon content of the sediments (15), there has been no evidence of a progressive buildup of a sludge bank. Accordingly, the unassimilated organics must have been removed periodically by turbulence and wave action, and it follows that essentially aerobic conditions are likely in the sediment.

It is apparent that the phosphorus mineralization rate of 3,500 kg/day computed from the areal distributions does not agree with that derived from the profiles. The possible error introduced by the several simplifying assumptions is not known; however, the larger value could result from the greater extent of the field and from the presence of old effluent in the diluting water. The return of old effluent to the outfall area was demonstrated by a radioactive tracer study of effluent dilution (9). Available data do not permit an adequate evaluation of this phenomenon, but the probability of dilution by older, more mineralized effluent is almost certainly proportional to the age and volume of the field.

The difference may be explained, at least in part, by assuming that the values represent mineralization which takes place over two different areas. The first is the surface area derived from the steady-state volumetric relationships where the equivalent radius for the water mass after one day is 5.8 km; the corresponding area is 1.42×10^7 m^2. The second area is the 1.38 x

10^8 m^2 within which, increased surface phosphate concentrations were observed. If it is further assumed that the lower and higher rates are associated with the lesser and greater areas, it is possible to compare annual rates of nutrient regeneration with those reported for other areas.

The annual rate of nitrogen mineralization may be computed as 1×10^6 g/day x 365 days/yr $\div$ 1.42×10^7 m^2 = 26 g/m^2/yr. Essentially all of this regeneration takes place on the bottom and it exceeds, by two orders of magnitude, the value of 1.57 μg-atoms/cm^2/yr (0.22 g/m^2/yr) reported by Rittenberg *et al.* (10) for the upper 200 cm of sediment in Santa Barbara Basin where the bottom depth is 600 m. A second comparison may be made with the upper 200 m of the ocean by assuming the annual production figure of 150 g carbon/m^2/year adopted by Emery *et al.* (2), multiplying by the factor 0.18g N/g C from Sverdrup *et al.* (14), and accepting Riley's (8) estimate of 90 per cent regeneration within that depth; the resulting rate for nitrogen mineralization is 24 g/m^2/yr.

Phosphorus mineralization may be computed from the profile data as 3×10^5 g/day x 365 days/yr $\div$ 1.42×10^7 m^2 = 7.7 g/m^2/yr and from the areal data as 3.5×10^6 g/day x 365 days/yr $\div$ 1.38×10^8 m^2 = 9.3 g/m^2/yr. Regeneration in the ocean may be computed by using the factor, 0.024 g P/g C, after Sverdrup *et al.* (14) and proceeding as above; this results in 3.2 gP/m^2/yr.

It appears that there is a relative excess of phosphorus regenerated in Santa Monica Bay. This corresponds roughly with the N:P ratio in the dicharge, but it is not known why this imbalance of nutrients should persist through the mineralization processes.

SUMMARY

An investigation has been made of the rates of mineralization of the 22,000 kg and the 5,500 kg organic nitrogen and phosphorus discharged daily with the effluent from the Hyperion Sewage Treatment Plant into Santa Monica Bay in 1956. Regeneration rates of 1,000 kg per day for nitrogen and from 300 to 3,500 kg per day for phosphorus have been computed; on an areal basis, these amount to 26 g N/m^2/yr and 7.7 to 9.3 g P/m^2/yr since different areas were involved. Most of the regeneration took

place on the bottom, in part because of the settling of the larger portion of the organics, but primarily because of the far greater efficiency of mineralization processes on the bottom. It was also found that nitrification commenced immediately in the receiving water, resulting in a 28 per cent decrease in NH_3-N concentrations during the first four hours.

ACKNOWLEDGMENTS

I am indebted to Dr. S. C. Rittenberg, University of Southern California, for suggesting this study, and for his assistance in its pursuit. I am also grateful to Mr. W. F. Garber, City of Los Angeles, for his support during the original investigations of Santa Monica Bay.

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Chapter 61

The Role of Bacteria in the Mineralization of Phosphorus in Lakes

F. R. Hayes

When phosphorus is added to marine or fresh-water ponds, nearly all of it disappears in a few days (6, 7, 9). By analogy with agriculture, the decline has sometimes been attributed to new plant growth. The theory of growth stimulation, however, required reconsideration when it was discovered that tracer phosphorus atoms, measured as radioactivity, diminished in the water in the same way as large masses of fertilizer (4, 8). It now appears that there is a single pool of phosphorus belonging to water and solids, which is distributed between them in a dynamic equilibrium or steady state. Disappearance of phosphorus from water is a consequence of an experimental arrangement in which phosphorus is added to the water phase. Were the opposite technique followed, of taking up phosphorus from the water on, say ion-exchange resins, a continuous replacement from the solids would be expected.

The foregoing interpretation does not deny the general observation that addition of fertilizers stimulates growth. Obviously after equilibration there will be more nutrient in the system than before. The point is that the decline of phosphorus in the water phase is not a measure of increased productivity, since its rate will tend to be independent of the quantity added.

The purpose of this paper is to discuss the effect of microorganisms on the phosphorus exchange reaction between water and the sediments, plants and zooplankton.

For the experiments, a sample of natural water was brought to the laboratory with precautions to prevent undue multiplication of microorganisms. To an 8 oz. wide-mouthed jar of the water, antibiotics (terramycin and later the more powerful tetra-

cycline), plants or zooplankton-like organisms were added as desired. A layer of superficial lake sediment was centrifuged to the bottom of the jar to study exchanges at the mud-water interface. Inorganic $P^{32}O_4$ was added as desired and the course of its decline in the water followed.

The water was kept in motion by bubbles of nitrogen or air which had been passed through a filter to remove bacteria. Checks were made from time to time on the antibiotics to ensure that they were both toxic to bacteria and non-toxic to the experimental plants and invertebrates. For reasons to follow, all samples of P^{32}-PO_4 were autoclaved upon delivery and dilution, and kept sterile until use.

Methods of handling and counting P^{32}, calculations of turnover times, handling of sediment cores, etc., are given by Coffin *et al.* (1), Hayes (3), Hayes *et al.* (4), Harris (2), and Hayes and Phillips (5).

It is well known that most of the phosphorus in natural waters, say four-fifths to nine-tenths in the summer, is in an organic form. There is an inorganic maximum in the winter which quickly declines below the limits of detection when there is enough light to produce a phytoplankton bloom. In an early laboratory observation, it was found that three-fifths of the carrier-free phosphate obtained from an atomic pile in normal HCl will, when diluted with distilled water, go over to the organic form within a few days. This is in the absence of any detectable nutrient source.

The conversion of PO_4 to organic P was further studied on a jar of lake water which was allowed to settle for twenty-four hours, filtered and kept in the dark. The upper curve of Figure 1 shows that after six days, 17 per cent of the P^{32} had disappeared from the water, presumably to sessile bacteria growing on the walls. The center curve shows how inorganic phosphorus declines, mostly within the first day, to approach an equilibrium in which it is about equal to the organic fraction. The lowest curve is organic P^{32} in the water. To find the total organic phosphorus production, the decline as shown in the top curve should be added to take in the sessile forms. The dotted line gives the revised values. The turnover time for organic P was calculated at

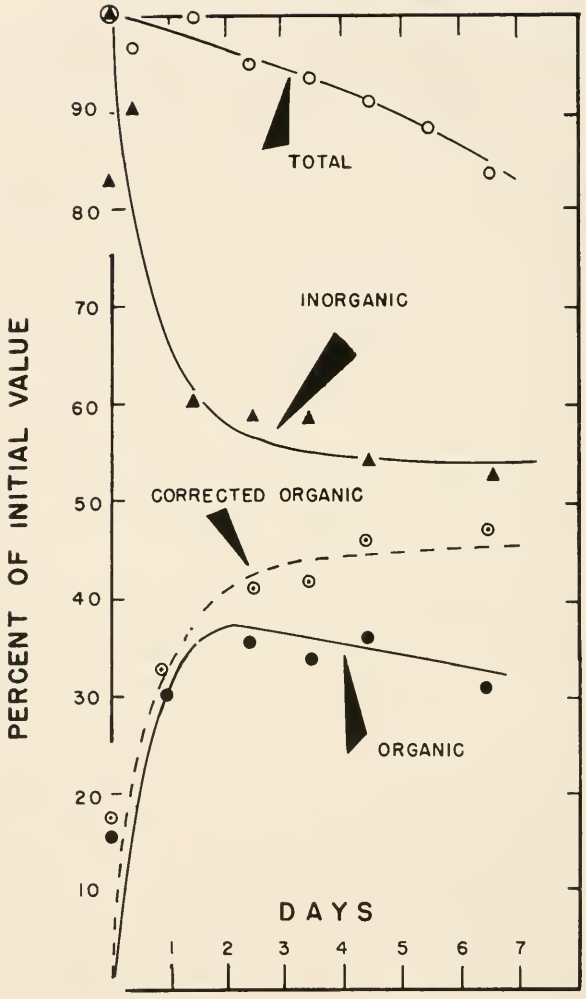

Fig. 1. Synthesis of organic phosphorus in water from Chocolate Lake, Nova Scotia. Water was kept in the dark throughout, and prior to the test was stored twenty-four hours and filtered. The decline in total P is attributed to uptake by sessile organisms. Solid "organic" line, direct observations; dotted "organic" line incorporates correction for loss to sessile forms (i.e., solid line points plus 100 minus values in top line).

nineteen hours, which means that in this interval as much organic phosphorus as was present would be expected to cycle through the inorganic state.

Additional information was obtained from an experiment in which bacteria and higher plants were placed in competition for added radiophosphorus. If filtered lake water is set up in the light, control value will be similar to the top line of Figure 1. To replicates under test are added a plant sprig with or without antibiotic as well. The loss of P^{32} from the water is shown in Figure 2. In the absence of bacteria, *Eriocaulon* very rapidly takes up

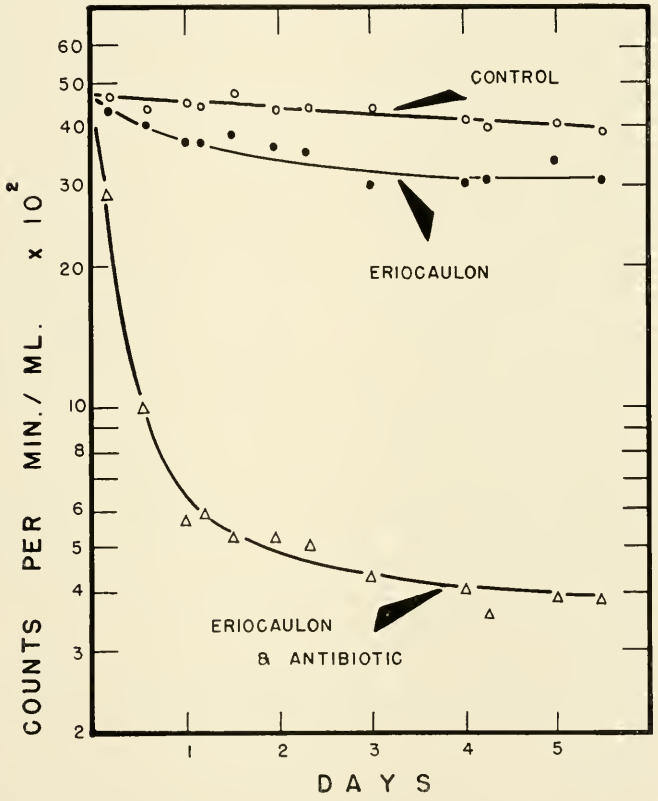

Fig. 2. Radiophosphorus remaining in aerated water from Grand Lake, N. S., containing plants, with and without antibiotic. One gm. of sprigs of the flowering aquatic, *Eriocaulon* (pipewort) were added to 150 ml. of filtered lake water. Similar results were obtained with the plants *Utricularia* and *Sphagnum*.

radiophosphate and reaches an equilibrium at which 10 per cent of the P^{32} is in the water (bottom curve, Fig. 2). The phosphorus turnover time for *Eriocaulon,* in the absence of bacteria, was calculated to be 3.0 days. The effect of bacteria (middle curve, Fig. 2) is to hold phosphorus in the water and prevent the large loss to the bottom plants. This is because bacteria hold large quantities of phosphorus in their cells and produce soluble organic radiophosphorus, which the plant presumably cannot assimilate. The experiment was repeated with the plants *Sphagnum* and *Utricularia* with similar results.

An experiment was done with a layer of sediment at the bottom of the bottle and with added plants and bacteria, thus placing all three components in competition. As Figure 3 shows, the addition of *Eriocaulon* to the control resulted in a loss of 70 per cent of the radiophosphate from the water after six days. The ability of the plant to remove so great a quantity of radio-

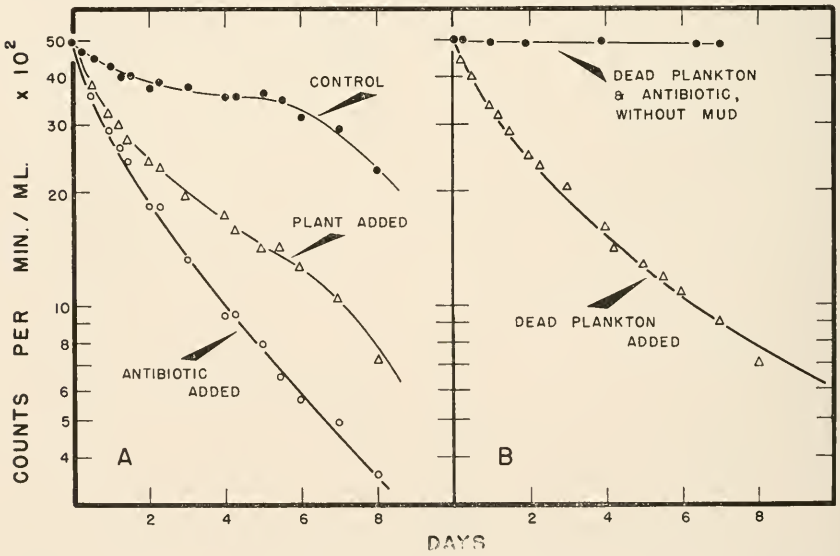

Fig. 3. A. Amount of P^{32} remaining in an aerated Grand Lake mud-water system to which 2 g of *Eriocaulon* sprigs or 30 mg of tetracycline was added. *B.* Effect of adding dead plankton in the presence and absence of bacteria. Comparing the lowest curve of A with the top one of B it is shown that mud is necessary for the inorganic exchange of P^{32} and that sterile plankton will not serve as a substitute for mud.

phosphate from the water when the mud control was unable to do so in the presence of bacteria, indicates the affinity of *Eriocaulon* for phosphate. The control curve in Figure 3A is an extreme illustration of another phenomenon which was common to most experiments. While a control mud-water system seemed to reach equilibrium after three to eight days, a gradual loss of radiophosphorus in water continued over a period of weeks until practically none remained in the water. This decline is attributed to a fall-out of radiophosphorus in the bodies of bacteria.

In summary, when bacteria and higher plants compete for inorganic phosphorus the bacteria get there first and change a part of it to organic forms which are apparently unavailable to plant use. In the absence of bacteria the aquatic plants tested take up enormous quantities of P^{32} within 12 hrs, indicating a rapid exchange and the establishment of an equilibrium with most of the phosphorus in the plant body.

A large fallout of plankton following a bloom might be expected to affect bacterial activity at the mud surface of a lake and thus alter the exchange of phosphorus. Plankton was netted from Grand Lake, autoclaved, and approximately 0.3 g was pipetted into duplicate mud-water systems from the same lake. The plankton sedimented immediately to the mud surface. Equal quantities of dead plankton were placed in two antibiotic treated glass blanks, omitting mud. Results are shown in Figure 3B. Obviously sterile dead plankton, of itself, is unable to absorb significant quantities of phosphate by a colloidal mechanism or by other means. The addition of dead plankton to a mud-water system resulted in an enormous and rapid loss of P^{32} from the water. This greater uptake at the mud surface, as compared with the control in Figure 3A, was evidently caused by increased activity there, due to the large amount of readily decomposable organic matter. An uptake of phosphate by bacteria attached to falling organic matter in lakes is probably of importance in the natural cycle.

The first experiment described showed that bacteria in the water convert a large part of inorganic P^{32} to the organic form in a few hours. The question arises: do bacteria serve as necessary intermediates in uptake by invertebrates? To test this, experi-

ments with the beach flea, *Gammarus*, were set up with and without pretreatment by terramycin. The results, as seen in Figure 4, show that lack of bacteria inhibits radiophosphorus absorption. Evidently direct absorption of inorganic P does not occur appreciably either through the body wall, intestine or gills. This result is in general agreement with various recorded experiments which show that higher invertebrates obtain their food by

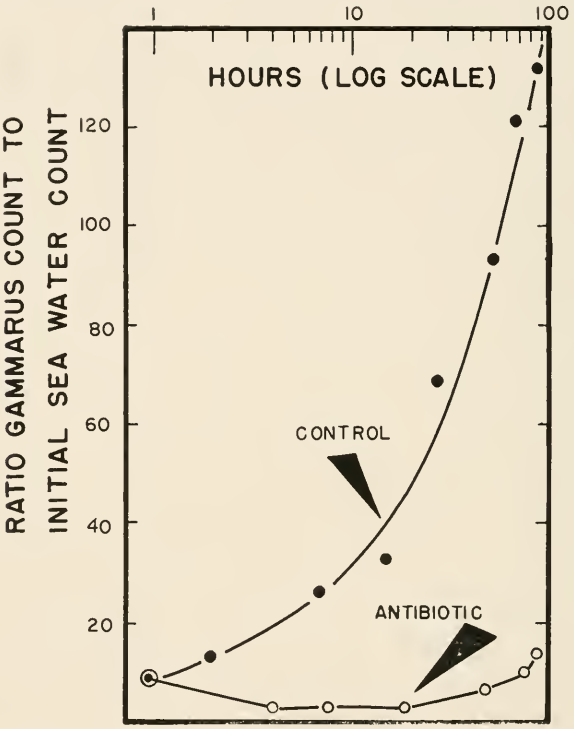

Fig. 4. Uptake of P^{32} by marine *Gammarus* after antibiotic treatment. Specimens were washed in sterile sea water, then treated for one hour on each of two successive days with terramycin in a concentration of 100 mg per 100 ml water. After treatment, the animals were transferred to flasks of sterile sea water for the experiment. Checks were made for bacterial growth in each flask as animals were removed from it. No flask was completely free, but bacteria could be reduced to as few as five colonies per ml. In the present test, the flask with antibiotic treated *Gammarus* began with about 200 colonies per ml and had 5000 by the end, as compared with 100,000 or more in the control. Evidently the intermediary activity of bacteria is necessary to permit inorganic phosphorus to be used.

digestion of particulate matter rather than by direct absorption of inorganic or organic solutions.

There are now sufficient data, from experiments here described and the literature, to warrant a preliminary assembly of time relations of the several phosphorus reactions as they might occur in a lake or bay. References are given by Hayes and Phillips (5). In Figure 5 the numbers are all days except for one five minute entry. Four kinds of line are used as qualitative indicators of time. It is seen that upon addition of phosphate to water the immediate reaction, within minutes, is a transfer through the bodies of unicellular floating forms of life (heavy lines to right). Next, as shown by the light solid lines to the left, there occurs within a matter of hours, i.e., two orders of magnitude slower than the above, an exchange in which the floating cells and the higher aquatics compete on approximately equal terms for the PO_4. This they make into their own body structures, throwing some of it back to the water as soluble organic phosphorus. We have cancelled out the virtually instantaneous passage through floating cells and set the process down as an equilibrium between inorganic and organic phosphorus in solution, for which the turnover time is 0.3 days.

At top left is indicated a doubt as to whether higher plants can utilize organic phosphorus, and at top right the feeding of zooplankton is given a turnover time, and their inability to utilize inorganic P is noted.

The lower part of Figure 5 brings in the sediment surface. At right, bacteria are noted to fall out at a few per cent per day. This is in bottles and not to be read as net fallout in natural waters, which is subject to wide variation. The fallout at right probably describes the same phenomenon as the line leading down at left, i.e., the settling of organic matter to be reduced again by bottom microorganisms for regeneration to the water. The turnover time for leaving the water and for return is here three days, an order of magnitude slower than for exchanges with floating life.

The inorganic mechanism, which can be observed when bacteria are suppressed, is shown at lower center. The observed time relations are not affected by the redox state. In taking out

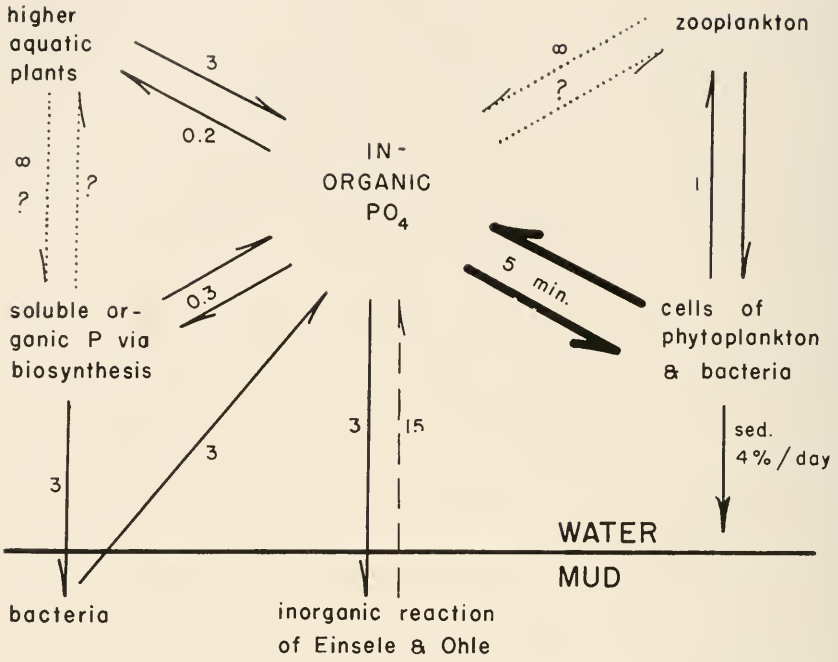

Fig. 5. Transformations of phosphorus in a lake with turnover times for different equilibria. Very heavy lines indicate the first action with floating cells, for which the time is given in minutes. Other times in days. Lighter solid lines are reactions at intermediate speeds—two or three orders of magnitude slower than the initial one. Dashed line is the return from mud by inorganic release, a still slower turnover. Dotted lines at top indicate reactions too slow to measure, called infinitely slow by comparison with the rest.

inorganic P the mechanism has the same turnover as the bacterial action just described, namely three days. The return to water however is much slower, at fifteen days.

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Chapter 62

Release and Capillary Movement of Phosphorus in Exposed Tidal Sediments*

Carl H. Oppenheimer and R. A. Ward

INTRODUCTION

The shallow marine bays of the central Texas Gulf Coast contain relatively large areas of intertidal mud flats (Figs. 1 and 2A) which are alternately exposed and inundated by wind driven tides. The surface of the gradually sloping mud flats is covered by a growth of blue-green algae accompanied by diatoms, flagellates, protozoa and bacteria. Water does not stand and evaporate when the tide goes down, but either runs off or sinks into the sediment. Thus, the water table in the sediment rises and falls with the tides. The sediments consist of varying size sands with some broken shell and fine layers of clay which are trapped by the blue-green algal mat.

The winds normally blow from the south east during the summer at average velocities of 5 to 35 mph. During the winter, northers occur at biweekly intervals causing north west winds with velocities up to 70 mph. The alternation of the wind direction moves the water back and forth over the mud flats. This motion is accompanied by the normal lunar tide of approximately six inches. When the flats are exposed for some days or weeks, the wind draws water to the surface of the sediment by capillary action. Salts dissolved in the capillary water are deposited and encrusted on the mud surfaces as the water evaporates (Fig. 2B). When the flats are again covered by water or during rain fall, the salts are dissolved.

The water within the bays is highly turbid with living or-

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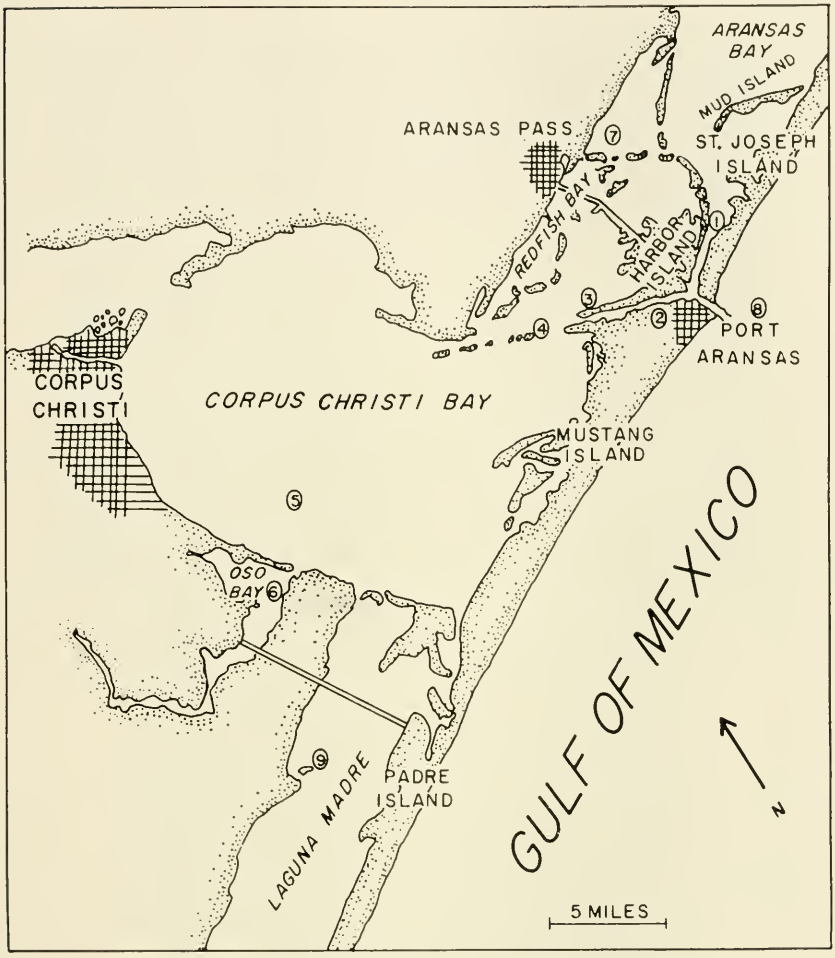

Fig. 1. General description of the shallow marine bays near the Institute of Marine Science. Numerals indicate various sampling areas.

ganisms, detritus and clays (8) and visability usually extends only a few cm into the water. When the wind blows the water over the exposed flats, the organic materials, clays and other solids, settle out or are entrapped by the blue-green algal mat and become a part of the sediments. In this way phosphate is trapped, in the remains of dead organisms, in the detritus adsorbed to iron hydroxide or clays, and as insoluble phosphate minerals. Redfish

A

B

Bay sediments may contain up to 5 per cent organic matter. The surface organic matter (0-0.5 cm depth) may fluctuate during a year cycle from 4.6 per cent in the summer to 0.8 per cent in the winter (16). Information on the sorption of phosphate to ferric hydroxide as a possible mechanism in sediment building is summarized by Hayes, Reed, and Cameron (5). Stephenson (13) indicates that agitation of sediment affects the release of phosphate due to the mechanical disruption of organisms and the liberation of bacterially produced phosphate.

This paper reports measurements of phosphate and chloride contents of the salts, sediments below the salts, and water in adjacent ponds or bays in vicinities where salts are present on the sediment surface. The experiments were prompted by the idea that soluble phosphates are transported by capillary action to the sediment surface as part of a cycle between sediment and water. This release of bound phosphorus may in part explain the fairly high productivity of these shallow bays reported by Odum and Hoskin (6). Emphasis will be placed on the possible explanation of bacterial activities which effectively release bound phosphorus from the sediment.

PROCEDURE

Samples were collected from areas indicated by numerals in Figure 1. Each area was representative of an extensive mud flat which was alternately dry or covered by water. The salts precipitated on the surface and underlying sediments were carefully removed with a spatula and placed in small glass containers. Water from adjacent bay or pond was collected in glass bottles. The water samples were immediately analyzed for chloride and total and inorganic phosphorus. The sediment samples were returned to the laboratory and refrigerated or dried immediately at 110 C. The dried samples were weighed into flasks and the salts dissolved in double distilled water. In aliquots for chloride analysis, the solids were allowed to settle out. Chloride was determined by the standard silver nitrate method for sea water. Inorganic phos-

←

Fig. 2. (A) Aerial photograph of Lydia Ann Channel showing intricate pattern of mud flats. (B) Mud flat with salt precipitated on surface.

phate was determined by the method of Wooster (17). For organic phosphorus determinations, the samples were digested by the method of Strickland (14) and the resulting phosphorus was measured by the method of Wooster (17). Before the colorimetric phosphorus determinations were made, solids were removed by passing the sample through a Millipore Filter. Residual P was washed from the filter and apparatus. The phosphorus adsorbed to the glass receptacles was removed by acid rinsing. All phosphate data were corrected for salt error.

RESULTS

Data for total and inorganic phosphorus and chloride for water and salt samples collected during July to September, 1959 are given in Tables 1 and 2. Data for sediment at depths immediately below the salt layers to 3 cm are not provided in table form. Total phosphorus in the sediments below the salts, ranged from 0.25 to 8.9 μgA/100g with an average of 1.7. The inorganic phosphorus ranged from 0.11 to 0.65 with an average of 0.38. Ten samples were analyzed from the several areas.

TABLE 1

Total and Inorganic Phosphorus in Salts Collected From Sediment Surfaces at Locations Shown in Figure 1

Area	*Inorganic P* μgA/100gm-Cl‰	*Ratio*	*Total P* μgA/100gm-Cl‰	*Ratio*
Lydia Ann Channel (1)				
	3.7/12.2	.30	24.2/12.2	1.99
	0.8/ 6.9	.11	1.2/ 6.9	.18
	*0.3/ 5.7	.04	0.3/ 5.7	.04
	2.5/ 5.7	.44		
Oppenheimer Slough (2)	0.8/17.7	.04	1.4/17.7	.08
	2.6/16.5	.16	3.5/16.5	.21
	*0.2/ 2.0	.11		
Redfish Bay (3)	2.2/20.6	.11	11.4/20.6	.55
	7.1/ 6.8	1.05	10.0/ 6.8	1.47
Redfish Bay (7)	3.9/ 6.9	.57	22.8/ 6.9	3.27
	1.9/ 5.6	.33	6.6/ 5.6	1.18
Average		.30		1.00

* Samples taken immediately after a heavy rain. Little salt remained on the surface sediment.

TABLE 2

TOTAL AND INORGANIC PHOSPHORUS IN BAY WATERS NEAR THE INSTITUTE OF MARINE SCIENCE AT LOCATIONS SHOWN IN FIGURE 1

Area	*Inorganic P*		*Total P*	
	μgA/L-Cl‰	*Ratio*	*μgA/L-Cl‰*	*Ratio*
Lydia Ann Channel (1)	0.83/19.22	.064	290/19.22	.151
	0.54/18.20	.029	3.23/18.20	.177
	0.94/19.74	.048	1.45/19.74	.073
Oppenheimer Slough (2)	1.00/25.64	.039	2.95/25.64	.115
	1.01/21.16	.048	2.26/21.16	.107
	1.27/16.47	.077	2.05/16.47	.124
Redfish Bay (3)	0.60/25.49	.023	2.26/25.49	.089
	0.87/18.01	.048	4.02/18.01	.223
Corpus Christi Channel (4)			1.90	
			1.62	
Corpus Christi Bay (5)			0.80	
			0.97	
Oso Bay (6)			0.80	
			1.40	
Redfish Bay (7)			0.95	
			0.62	
			0.80	
			1.38	
			1.05	
			1.38	
			1.27	
			1.08	
Gulf of Mexico (8)			0.91	
			0.92	
			1.08	
			1.27	
Laguna Madre (9)			2.25	
Average		.047		.132

DISCUSSION

The data in Tables 1 and 2 indicate that phosphates are concentrated at sediment surfaces where water which is sucked from the sediments by capillary activity evaporates. A similar correlation is indicated by the relatively higher phosphorus content

of the bay and pond waters adjacent to the flats as compared with the open bay and Gulf of Mexico waters shown in the data in Figure 2, areas five to eight. It is difficult to calculate the relative surface of intertidal mud flats because of the type of terrain. The phosphorus values in the sediments below the salts are large enough to account for the phosphates found on the surface in the precipitated salt. The phosphate values for the bay waters and Gulf of Mexico are within the range 0.60 to 4.02 μgA-total P/L. Surface phosphorus values for other bays and coastal water are also within this range. Strickland and Austin (15) give values between approximately 1 and 3 μgA-total P/L at 5 meters depth in Departure Bay and Pacific Oceanic values from approximately 1 to 5 μgA-total P increasing with depth. Redfield (9) lists average sea water as containing 2.3 μgA-total P/L, with the North Atlantic 1.25 and the North Pacific 3.0.

The data suggest that the productivity of the bay waters is probably not due to an abnormal amount of phosphorus but perhaps to the rate at which the phosphorus is cycled within the waters. Bruce and Hood (2) have indicated a rapid turnover rate as shown by diurnal inorganic phosphate curves which vary from 2.4 μgA/L at 6 a.m. to 1.4 at 6 p.m. The relatively large variation of inorganic phosphorus certainly suggests caution in establishing routine collecting procedures for such measurements in water systems.

The release of phosphates from the sediments is apparently part of the cycle of this and perhaps other nutrients. Sedimentation rates are relatively rapid: up to 19 cm per 100 years (11) and ¼ to ⅓ inch per year variation in the Laguna Madre (4). The high rate of sedimentation and continued movement of sediments from month to month during rather gusty wind conditions entraps organic and inorganic phosphorus.

There are several mechanisms by which the entrapped phosphorus may be solublized in the sediments. Baas Becking and MacKay (1), Carrett and Goodgal (3), and Sperber (12) discuss the release of soluble phosphate from sediments which accompanies an increase in pH from 6 to 8 due to bacterial metabolism or a decrease in Eh to the anaerobic state and accompanying action of sulfide produced by sulfate-reducing bacteria (7).

When the Eh decreases to a -200 mv, ferric hydroxide is dissolved and any adsorbed phosphates are released. The action of hydrogen sulfide also will release phosphate from iron phosphate by the coupling of the iron with sulfide. In addition to the above mechanisms, bacterial activity will release phosphates during the decomposition of organic matter which may be entrapped within the sediments. Rice (10) has shown by tracer experiments that phosphorus is exchanged between the medium and the living cells of phytoplankton. Carrett and Goodgal (3) discuss the mechanisms by which adsorbed phosphorus may be released when colloidal materials are transported from one environment to another. They discuss the relative sorption of materials from the dissolved state and point out that any colloidal sorption system is very complex in the natural environment, and before it can be understood one should know the relative sorption-desorption pathways. These are complex in our turbid bays where suspended matter is abundant. Hayes discusses the sorption of phosphate in lake sediments in a previous chapter in this publication.

Most of the mud flats have an annual redox potential cycle whereby the oxygen transition zone may be at the surface of the sediment during summer with high bacterial activity and oxygen consumption, and deeper in the sediment (a few cm) during winter when the bacterial activities decline. The pH, in contrast, goes through a diurnal change from approximately 6.5 to 8.0 during respiration and photosynthesis respectively. The diurnal change takes place only to the depth of light penetration, which is about 0.5 cm in algal mats, clays, or silt sediments and to 1.5 cm in sandy sediments.

SUMMARY

Salts are found precipitated on exposed mud flats of the bays studied. The ratio of soluble phosphorus to chloride in the salts was 0.3, whereas for adjacent water it was 0.047. Up to 7.1 μgA of inorganic phosphorus per 100 g of salt was found on the flats. The data indicate that phosphorus was being liberated in the sediments presumably through the action of bacteria and chemical equilibria and concentrated in the salts evaporated from sediment capillary water. Total phosphorus content of the bays,

which ranged from 0.6 to 4.2 μgA/L, is not appreciably greater than for other bays of near shore waters. Thus, it suggests that the higher productivity of the bays is the result of a rapid turnover of P. The total phosphorus in sediments varied from 0.25 to 8.9 μgA/100 g.

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Chapter 63

Experiments Regarding the Sulfide Formation in Sediments of the Texas Gulf Coast

W. Gunkel and Carl H. Oppenheimer

The formation of hydrogen sulfide by bacteria is a widespread phenomenon in nature produced by sulfate reduction and by the decomposition of protein. It is generally assumed that sulfate is more responsible than organic sulfur transformation for the production of sulfide, however, there are very few quantitative investigations in this regard available. Kriss (3) for example, points out that in the Black Sea there are no data which show the prevalence of one or the other reaction. He says (3) that the statement of Danelcenko and Cigirin (1926), that the sulfate reduction plays nearly an exclusive role, is not only lacking in proof but also that their statements have the character of an argument which is in contrast to their actual data. Reviews about sulfate reducing bacteria are in Bunker (2), Rubenchik (10) and Postgate (7).

Experiments were conducted in shallow marine bays at the Texas Gulf Coast, described by Oppenheimer (5), and at the German Coast of the North Sea. Our intention was to obtain quantitative data in model experiments about the formation of sulfides in marine sediments. We were particularly interested in: 1) The amount of sulfide formed with time, 2) The sources of sulfides, and 3) The numbers of bacteria of different physiological groups present.

MATERIALS AND METHODS

Two different experiments were used:

Natural Environment Studies

Opaque plastic tubes, diameter of 6 cm and a length of 25 cm were inserted into the soft sediment in a shallow bay of the Gulf of Mexico near Port Aransas. They contained, after being

closed with neoprene-stoppers, nearly no water above the sediment layer. The sediment consisted of a sandy clay and had a high content of organic substances. Only a very thin top-most layer was aerobic; all the other sediment was anaerobic and black, due to metallic sulfides. It was very homogeneous and gases were formed. Sample tubes were removed at intervals (for 11 weeks) from the sediment for analysis.

Laboratory Studies

The superficial layer of the bottom of a very productive shallow water pool near Port Aransas was collected, homogenized by a mixer and filled into a number of glass containers as shown in Figure 1. The sandy-clay sediments contained a large heterogeneous living population of blue green algae, flagellates, protozoans, diatoms, bacteria and nematodes. In the experiments conducted at the German coast, sediments consisted mainly of sandy clay and decomposing benthic algae like Laminaria, Fucus and red algae. The containers were stored in Texas at 30 C and in Germany at 18 C. The containers were closed by neoprene-

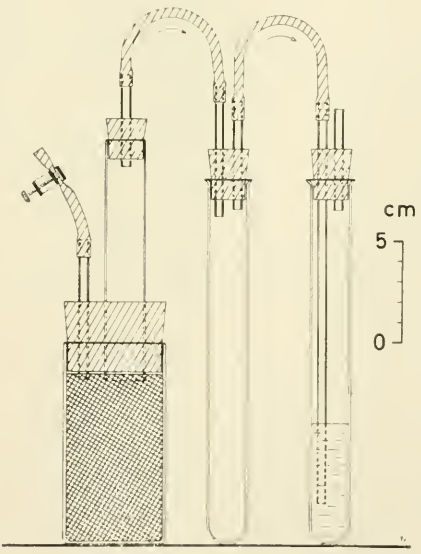

Fig. 1. Container used for the storage of homogenized sediments. Explanation see text.

stoppers with two glass tubes on top. One was used as an inlet for nitrogen to replace all oxygen present. The second one with the greater diameter permitted an increase of the volume of the mud which occurred during formation of gas during the incubation of the containers. An effluent tube was connected, which passed into an empty test tube and then into a test tube filled with a solution of zinc acetate. So as the mud volume expanded, gases could escape and hydrogen sulfide present in the gases was precipitated as zinc sulfide and could be determined. Completely closed containers either broke or the contents emerged.

Chemical Determinations

The determination of sulfate was conducted by the usual gravimetric method after precipitation with $BaCl_2$. The determination of the sulfides followed the titrimetric method, recommended in the "Standard Methods for the Examination of Water, Sewage and Industrial Wastes, 1955" (1). The total-sulfur was determined as sulfate after oxydation to sulfate with nitric acid, bromine and perchloric acid. This wet-ashing technique followed the procedure recommended by Patterson (6). The iron was determined colorimetrically wtih 1,10-phenanthroline (11). The organic bound carbon was determined as CO_2 using a wet-combustion technique after removing the carbonate carbon (12).

Determination of Bacterial Numbers

The numbers of bacteria were determined using the MPN-dilution technique with ½ oz. prescription bottles. The following media were used:

1. For the determination of the aerobes:

5.0	g	caseinhydrolysate
0.05	g	K_2HPO_4
trace		$FePO_4$
750	ml	aged sea water
250	ml	aqua dest.

   The bottles were filled with 9 ml of medium.

2. For the determination of the sulfate-reducing bacteria we used a modified Starkey's medium:

3.5	g	sodium lactate
1.0	g	NH_4Cl
0.5	g	K_2HPO_4

2.0	g	$MgSO_4$
0.1	g	ascorbic acid
0.05	g	asparagine
trace		Mohr's salt
750	ml	aged sea water
250	ml	aqua dest.

3. For the enumeration of the bacteria producing hydrogen sulfide from organic sources:

1.0	g	yeast extract
5.0	g	neopeptone
0.1	g	cystine
0.5	g	K_2HPO_4
1.0	g	NH_4Cl
trace		$FeCl_3$
750	ml	SO_4-free artificial sea water
250	ml	aqua dest.

4. For the enumeration of the anaerobes we used a modified Zo-Bell's 2216 medium:

5.0	g	Bacto-peptone
1.0	g	Bacto-yeast extract
5.0	g	glucose
0.1	g	ascorbic acid
0.01	g	$FePO_4$
750	ml	aged sea water
250	ml	aqua dest.

The last three media were used in completely filled prescription bottles. They were incubated in Texas at 28 C and in Helgoland at 18 C. The pH was in all cases 7.6.

RESULTS

Figure 2 shows the results of the first series of experiments in the natural environment. The sulfides increased within eleven weeks, 0.284 mg/g wet sediment, while the sulfates decreased, 0.577 mg/g wet sediment. A part of the sulfate must have been transformed into something else than sulfide. It would be conceivable that a certain amount of hydrogen sulfide formed from organic sources during the decomposition was transformed into such another form. The Eh became unexpectedly less reducing

during the length of the experiment. The organic bound carbon decreased significantly due to the activity of the bacteria.

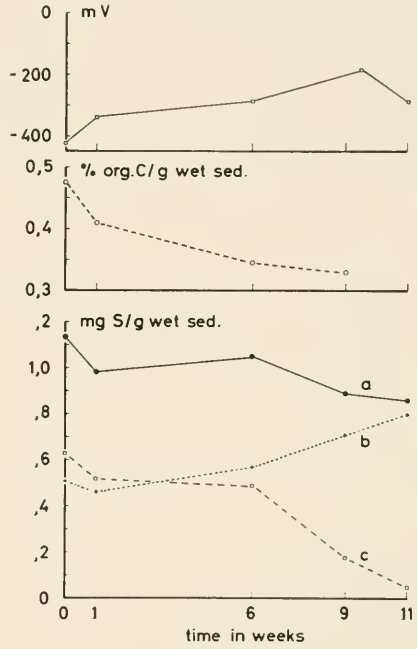

Fig. 2. First experimental series. Sulfate-sulfur (c), sulfide-sulfur (b), product of both (a), organic bound carbon and Eh after different times. Sediment trapped in plastic tubes and left in the natural environment.

Figure 3 shows the numbers of aerobic, anaerobic, bacteria which transform organic sulfur to sulfide and sulfate reducing bacteria in a semi-logarithmic plot. About 100 times more bacteria were found which had the potency to form hydrogen sulfide from organic sources than sulfate reducers. Most bacterial numbers decreased during the length of the experiment, only the sulfate reducing bacteria increased.

Figure 4 shows another series of experiments. A suspension of an anaerobic mud was added to a medium made of aged sea water and containing among other ingredients Difco neopeptone, yeast extract and sodium lactate. The redox potential was reduced to minus 112 mV by bubbling hydrogen through it before the

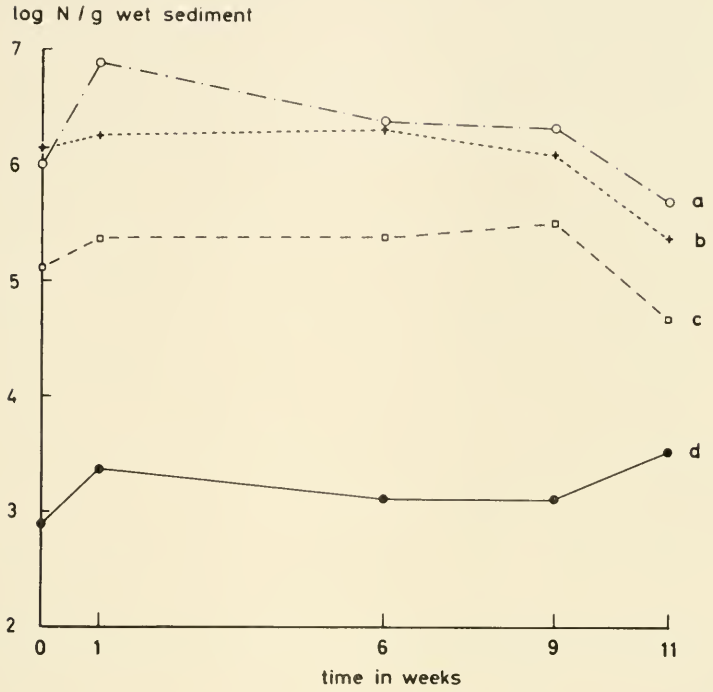

Fig. 3. Bacterial numbers corresponding to the chemical data of Figure 2. Aerobic- (a), anaerobic- (b), H_2S from organic sources producing- (c) sulfate reducing bacteria (d).

inoculation. After different times the sulfide and sulfate of the containers were determined. It is evident that in this series of experiments the increase of sulfide was less than the decrease of sulfate and a part of the sulfur must have been transformed into another form than sulfide.

Figure 5a shows the results of experiments in which the superficial layers of sediments were collected, filled into glass containers and stored for different lengths of time. In addition to sulfate and sulfide, we determined that part of iron which was not soluble in hot diluted hydrochloric acid but only in hot nitric acid. Based on the investigations of Sugawara, Koyama and Kozawa (9) we assumed that this iron is bound in the form of pyrite. In both experiments a considerable increase of sulfate-sulfur, sulfide-sulfur and pyrite-sulfur took place. The increase

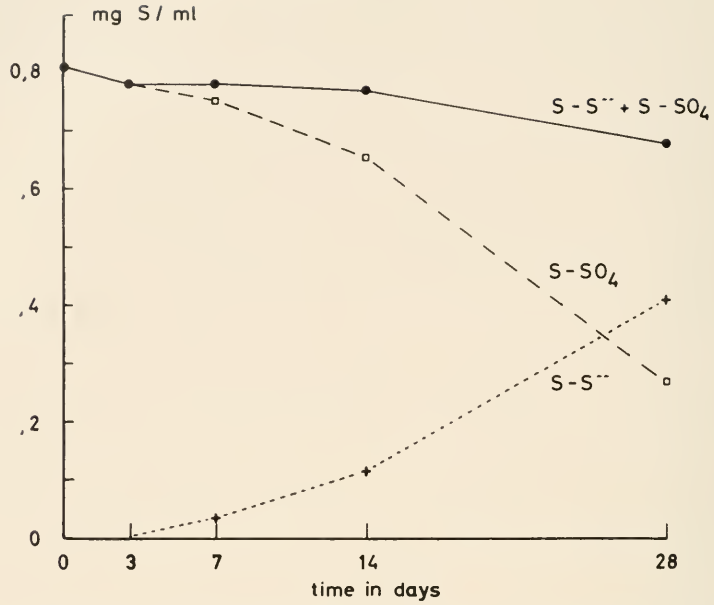

Fig. 4. Decrease of sulfate and increase of sulfide in a medium inoculated with a suspension of anaerobic mud.

amounts in the first experiment to 0.174 mg/g wet sediment and in the second to 0.372 mg/g wet sediment after eight and four days. The sulfur must have derived from another source than from the sulfate: e.g. the originally organic bound sulfur. The increase of the sulfides of the first experiment was 0.387 mg/g wet sediment after eight days and 0.870 mg/g wet sediment in the second experiment after four days. The share of the originally non-sulfate bound sulfur amounts in experiment (1) to 45.36 per cent and in the experiment (2) to 42.77 per cent. As expected, all bacterial numbers were higher than in the first series of experiments. Again the aerobic bacteria showed the highest numbers, followed by the anaerobes, the organic-H_2S-producers and the sulfate-reducers. The sulfate-reducers were about 1000 times less than the organic H_2S-producers, but the absolute number was even more than in the first setup. The increase or decrease of the bacterial numbers are shown in the figure.

Figure 5b shows experiments which were conducted in Hel-

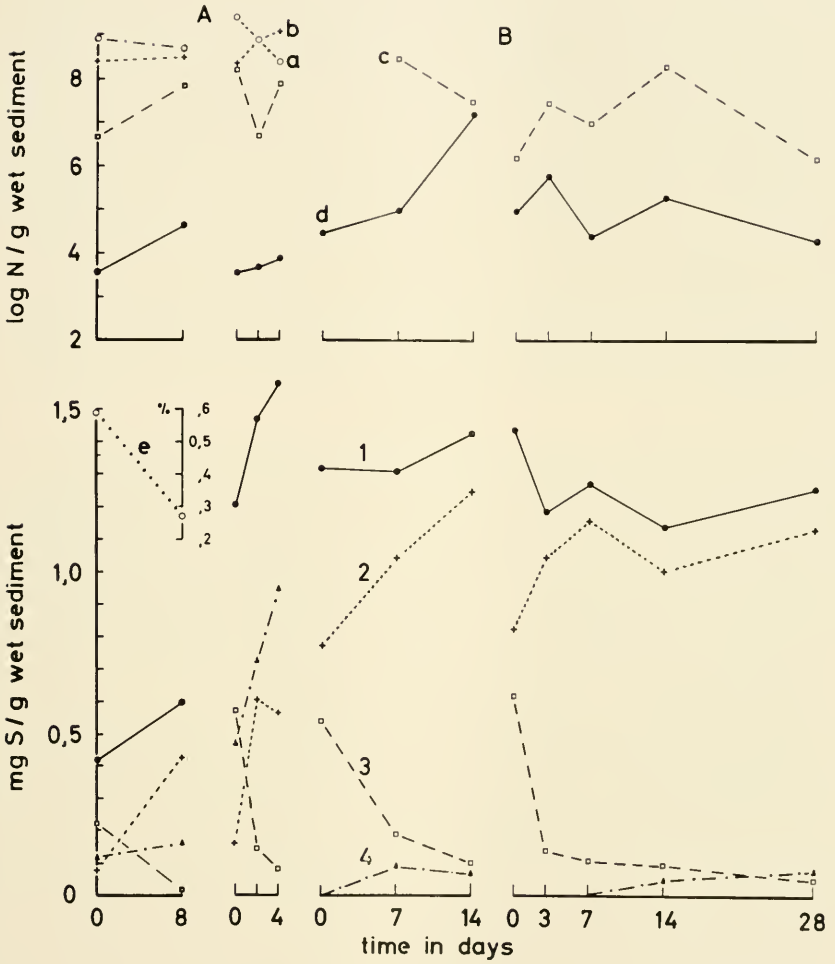

Fig. 5. Chemical alterations and bacterial numbers in homogenized sediments, stored in containers. A—Experiments conducted in Texas and stored at 30 C. B — Experiments conducted in Helgoland, Germany and stored at 18 C. a = aerobic, b = anaerobic, c = bacteria producing H_2S from organic sources, d = sulfate-reducing bacteria, e = organic carbon. 1 = product of sulfide, sulfate and pyrite-sulfur. 2 = sulfide-sulfur, 3 = sulfate-sulfur, 4 = pyrite-sulfur.

goland, Germany.* Also here we have a strong increase of the sulfides and decrease of the sulfates. The incerase after fourteen days was 0.540 mg/g wet sediment and in a second experiment 0.218 mg/g wet sediment after 28 days. The results are somewhat different than those in 5a. In one experiment 19.6 per cent of the formed hydrogen sulfide came from other sources than sulfate (compared with about 50% in the Texas experiments). In the second experiment the decrease of the sulfate was greater than the increase of sulfides, a part of this sulfur must have gone into another form. The numbers of the SO_4-reducers were ten times higher than in the Texas experiment. In one experiment we had a very strong increase of their numbers, in the other one no increase. That the organic bound sulfur could contribute a considerable amount of sulfur is shown by experiments we conducted in Texas. Here we analysed algae and higher plants for their con-

TABLE 1

SULFUR CONTENT OF PLANTS PER GRAM DRY WEIGHT IN MG

	Sulfate-Sulphur	*Total Sulfur Minus Sulfate Sulfur*
	mg/gm	mg/gm
Higher plants		
Thalassia testudinum (König)	1.16	4.65
Diplanthera wrighti (Asherson)	1.52	7.05
Digenea simplex (Wulfen)	0.52	7.94
Algae		
Ulva spec.	6.50	35.52
Gracillaria-type red algae	3.35	23.05
Gracillaria blodgettii (Harvey)	6.39	29.49
Red algae (unknown species)	4.30	29.28

* The sediment in Helgoland consisted partly of weathered red sandstone. The aged iron oxides of the sandstone were also not soluble in HCl and behaved in the same way as pyrite. Therefore the data of the starting point was subtracted from all further data of the pyrite.

tent in organic bound sulfur. We determined the total-sulfur and the sulfate-sulfur. The results are shown in Table 1.

DISCUSSION

Our experiments show that different amounts of sulfide are formed in different sediments. In no experiment was the decrease of sulfate the same as the increase of sulfides. In some experiments different amounts of non-$SO^{=}_4$ sulfur are transformed into sulfide, in other experiments a part of the sulfate is transformed into other forms than sulfide. With our methods we found, in all experiments, much higher bacterial numbers of organic H_2S-producers than sulfate-reducers.

Further experiments, in addition to the used techniques, are planned, including determinations of elementary sulfur, determinations of the different organic sources and tracer experiments.

SUMMARY

Model experiments regarding the sulfide production in sediments rich in organic matter were conducted at the Texas Gulf Coast and in Helgoland, Germany. Chemical determinations of different sulfur compounds, organic bound carbon, Eh, and the bacterial numbers of aerobic-, anerobic -, sulfate-reducing - and organic-H_2S producing bacteria were accomplished.

ACKNOWLEDGMENTS

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Chapter 64

Nutritional Patterns in Marine Bacterial Populations

T. M. Skerman

INTRODUCTION

It has been stated that among the most important activities of bacteria in the sea is the mineralization or modification of organic matter (22). Heterotrophic bacteria, of which the majority are probably saprophytes, appear to outnumber autotrophic varieties in the sea, their abundance closely parallels the distribution of organic matter and they seem capable of utilizing virtually all types of organic compounds (26). Yet accompanying the degradation of such a variety of organic molecules are many intricate biochemical relationships including such phenomena as symbiosis, antagonism and syntrophism, which impart a complex history to the mineralization process. Fundamental to studies of marine bacteria concerned in these activities is an understanding of the molecular relationships between each organism and its environment: experimental observations designed to elucidate some of these relationships of marine heterotrophic bacteria have been initiated by the author.

The Indigenous Heterotrophic Flora

Up to the present, valuable data have accrued from numerous studies of known biochemical processes such as ammonification, nitrification and those concerned with the transformation of sulphur and other elements. Some detailed investigations have been made of the types of marine bacteria taking part in such processes as well as of those engaged in the decomposition of residues such as cellulose, agar, chitin and lignin. Comparatively less attention has been devoted to heterotrophs whose functions are less well understood, but which doubtless comprise a large

proportion of the microflora indigenous to oceans and sediments. Investigations of the latter groups have been hampered by technical and theoretical difficulties in their isolation and the lack of appropriate criteria for their classification.

The majority of forms cultivated from marine sources have been described as Gram-negative asporogenous short rods, many of them motile and proteolytic, possessing a relatively low degree of biochemical activity as judged by standard laboratory tests (8, 26). Yet direct microscopic observations of planktonic bacteria indicate the existence of a variety of morphological types which cannot be reproduced in the laboratory by traditional cultural methods (12). Using similar direct techniques, Jannasch (9) discussed evidence for the existence of a component of the microflora of natural waters consisting of "dwarf" forms that escape detection until supplied with concentrated nutrients. He thus extended for aquatic systems the considerations of Winogradsky (24), who viewed the bacterial flora of soils in two categories, autochthenous and zymogenous. The autochthenous (indigenous) organisms, whose numbers remain fairly constant, were regarded to be physiologically unreactive, and characteristic of soils poorly supplied with fermentable substances and possibly taking part in the slow combustion of humic constituents of soils. Zymogenous organisms, which included the spore-formers, were recognised as being scarce in normal soils but becoming very active upon addition of readily fermentable substances. More information in regard to the marine microorganisms is desirable, yet the observation of Jannasch stresses the important point that different types of organisms may become selected by different methods of examination and isolation.

General Approach to Present Study

To gain a further appreciation of heterotrophic bacterial activity in the sea, more objective qualitative studies on the marine microflora and the types predominating are required. Since the nutritional factor is of basic significance in determining microbial interrelationships, a reconnaissance of the nutritional propensities of the various constituents of bacterial populations of different marine habitats is particularly desirable. Although

antagonistic reactions may complicate the relationship, the incidence of any group of organisms at any one time will depend largely upon the availability of appropriate nutrients, and information on the spectrum of nutritional requirements within a given population may promote understanding of the nature of such rerelationships as those between heterotrophs and their organic milieu.

A unique approach to the study of bacterial activity in soils by Lochhead and co-workers (13) culminated in a series of valuable articles on nutritional relationships of the soil microflora. By developing a method of characterizing bacteria according to their nutritional needs, they utilized fundamental nutritional differences as a basis for comparing both qualitatively and quantitatively the bacterial microfloras representative of different soils and soil habitats. Changes in the relative incidence of different nutritional groups or organisms were related to effects on the soil populations of seasons and soil treatment (10), of the growing plant (14), and other studies yielded data on the utilization and biosynthesis of amino acids and growth factors by certain groups of soil bacteria.

The present study was undertaken to explore the possibilities of applying similar nutritional criteria in the examination of bacterial activity and relationships in marine environments. For these purposes it was planned to compare qualitative characteristics of representative marine bacteria from populations of a selected number of widely differing habitats, *viz.* neritic and oceanic surface waters, deep ocean waters, sublittoral and deep sea sediments.

Cultivation of Marine Bacteria

Many cultural studies of marine bacteria have been primarily directed towards devising complex media to provide maximum plate counts (25) and it has commonly been stated that so diverse are the nutritional requirements of marine bacteria that no one medium can suffice for the growth of all (2). The problem of obtaining random samples of isolates representative of indigenous soil populations was approached by Lochhead by choosing an isolation medium of soil extract agar with no added energy ma-

terial (21). This was regarded as being less selective than conventional rich laboratory media likely to encourage disproportionate growth of zymogenous organisms. Preliminary trials by the author in developing a medium of similar properties suitable for marine materials indicated that sea water extracts of marine muds when enriched with phosphate and iron would support good growth of many marine bacteria. Extracts have been prepared* from freshly collected mud obtained locally in Wellington Harbour by dredging surface sediments. In contrast to the rapid effusive growth frequently encountered when plating marine samples on yeast extract peptone sea water agar, bacterial colonies developing on mud extract agar are small, discrete, and show little tendency for surface spreading. Antagonistic effects on primary isolation plates, possibly leading to the suppression of some forms, are thereby minimised using the latter medium, which moreover permits a relatively long incubation period (14-20 days at 18-20 C) during which time many pin-point colonies arise that could otherwise be difficult to isolate.

MATERIALS AND METHODS

Sampling

Two oceanographic stations were worked on 24/25 June, 1960, one, B 294, (41° 34′S, 175°02′E) 3 miles offshore in 50 fms, and the second, B 293 (41°55′S, 174°57′E) 30 miles offshore in 1200 fms. Using ZoBell samplers, water samples were taken from 25 m at both stations and from 1500 m at the second. Sediments were sampled using gravity corers.

On ship, 100 ml - 200 ml volumes of the water samples, depending upon the expected abundance of bacteria present, were concentrated by membrane filtration (pore size 0.45μ) in a sterile apparatus until 1-2 ml water remained in the well of a rubber gasket above the filter. To reduce carryover of any factors in the raw samples inhibitory to bacterial growth, this concentrate was diluted twice in succession by adding aseptically 50 ml filter-

* Current procedure is to autoclave for one hour at 115 C 1 kg wet mud with 1100 ml aged sea water diluted to a salinity of Ca. 6‰. After filtering, the volume of the extract is adjusted to 1 litre with distilled water, 1 mg Fe (as $FeCl_3.6H_2O$) and 15 g agar are added, and after sterilization, 0.1 g K_2HPO_4 is added.

sterilized aged sea water and refiltered as before. The washed concentrated sample (together with the filter and well) was then transferred aseptically to a jar and made up to 100 ml with sterile aged sea water containing 10 p.p.m. of surface-active agent "Tween 80." After shaking the jar to resuspend the microorganisms and as far as possible disperse cell aggregates, dilutions were prepared and plates poured with mud extract agar in quadruplicate from each dilution.

From the sediment cores, 1 g quantities of surface mud were aseptically transferred to jars similarly containing sterile aged sea water and surface-active agent, and plates were poured from dilutions as above.

The isolation medium used in all cases was prepared from the same batch of marine mud extract.

Isolation of Cultures

Plates were incubated for fourteen days at 18-20 C and counted. To avoid haphazard selection of organisms, *all* colonies were systematically picked off selected plates of known dilutions carrying well spaced colonies. These isolates were successively streaked twice on mud extract agar and finally inoculated as stab cultures in mud extract semisolid agar containing 0.1 per cent yeast extract to serve as stock cultures which were stored at 4 C. Approximately 80 isolates were obtained from each of the original samples.

Nutritional Requirements

The nutritional requirements of the isolates were evaluated by observing their growth response in a series of five media of varying complexities. Organisms were thus differentiated according to their requirements for maximum growth into the following groups:

1. *Bacteria with Simple Requirements.* These organisms grow well in a basal medium (Medium B*) of synthetic sea water enriched solely with phosphate, glucose and inorganic (am-

* Basal medium B. As a synthetic sea water substitute, the ASP2 medium of Provasoli *et al.* (19) was used. From the formula there given, silicate and vitamin B12 were omitted, phosphate was increased from 0.5 mg to 2.0 mg K_2HPO_4/100 ml medium, nitrate was replaced by 0.1 g. $(NH_4)_2SO_4$, and glucose was added at 0.1 per cent. The complete trace metal solution was retained at 3 ml/100 ml. pH = 7.4

monium) nitrogen. Growth is not substantially better in any of the more complex media.

2. *Bacteria Requiring Amino Acids.* These produce maximum growth in the basal medium supplemented with 0.4 per cent vitamin-free casamino acids (Medium BA†).

3. *Bacteria Requiring Growth Factors.* These organisms grow in medium BA if a mixture of vitamins* is added (Medium BAG).

4. *Bacteria Requiring Unidentified Substances in Yeast Extract.* These are capable of maximum growth in Medium BY, the basal medium + 0.1 per cent yeast extract, and show little or submaximal growth in previous media.

5. *Bacteria Requiring Unidentified Substances in Both Yeast Extract and Mud Extract.* Abundant growth is produced only in Medium BYM, the basal medium supplemented with 0.1 per cent yeast extract + 25% v/v mud extract.

Media were dispensed in 5 ml amounts in 125 x 16 mm tubes which were capped and sterilized at 115 C for ten minutes. Duplicate tubes of each medium were inoculated by loop transfer from a five day culture of each organism grown in sea water-mud extract + 0.1 per cent yeast extract. After five days' incubation at 20 C growth responses of the organisms in the test media were compared as optical densities read in a "Spectronic 20" (Bausch and Lomb) photometer set at 600 mμ. Uninoculated tubes served as blanks. Isolates were then assigned to one or other of the five above groups according to which medium supported clear cut maximum growth. Small differences in growth response were not regarded as significant.

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† Added from a sterile 10 per cent stock solution of Difco "Vitamin-free" Casamino Acids. This solution had been extracted twice with charcoal and reconstituted according to the method of Ford *et al.* (6).

* Vitamin mixture /100 ml medium: Riboflavin 50 μg, Ca pantothenate 50 μg, biotin 0.2 μg, thiamin HCl 50 μg, pyridoxine HCl 50 μg, pyridoxal HCl 50 μg, nicotinic acid 50 μg, p-aminobenzoic acid 50 μg, choline chloride 2 mg, folic acid 10 μg, inositol 5 mg, cyanocobalamin 0.2 μg.

RESULTS

Results showing the relative incidence of the different nutritional groups of organisms within populations derived from the various sources are summarized in Table 1.

DISCUSSION

Results indicate that organisms isolated on sea water mud extract show considerable diversity in their nutritional requirements for maximum growth. Further, there is some correspondence in the relative distribution of "nutritional groups" of organisms between the two surface water populations and similarly between those originating from sediments. On the other hand outstanding differences between surface water and sediment bacteria are apparent in the comparatively higher proportion of sea water organisms that showed a requirement for amino acids, and the incidence among bacteria indigenous to sediments of a group dependent, under the experimental conditions, upon nutrients provided by sea water mud extract. In contrast to surface water forms, bacteria from deep waters showed little or no growth on the simpler media (B and BA) and demanded complex addenda of vitamins, yeast extract or mud extract for maximum growth. While estimated absolute numbers (per unit volume) of bacteria of any one nutritional group are highest in the sediments, nevertheless outstanding qualitative differences (expressed by changes in the relative proportion of different groups) are discernible among the sampled populations.

Previous studies have shown amino acids are a class of compounds which satisfy the growth requirements of many bacteria of marine origin (16). Nearly all of fifteen "representative" marine bacteria studied by Ostroff and Henry (17) utilized amino acids which also supported growth of many of the strains isolated from sea water and fish by MacLeod *et al.* (15) either with or without non-amino sources of carbon and energy. There is thus some agreement between earlier observations and the present finding that isolates from surface waters include a high percentage of amino acid-requiring bacteria. If this high percentage of this group of organisms is also characteristic of indigenous bacterial populations *in situ*, it raises speculation as to the relationship of

TABLE 1

Estimated Numbers, and Percentage Incidence of Different Groups of Bacteria in Relation to their Nutritional Requirements, Among Populations Endemic to Certain Selected Marine Environment

Source	*Waters*						*Sediments*			
	Neritic 25 m		*Oceanic 25 m*		*Oceanic 1500 m*		*Sublittoral 100 m*		*Bathyal 2400 m*	
*Total Plate Count**	61,000/100 ml		8,200/100 ml		1,500/100 ml		121 x 10^3 /g		41 x 10^3 /g	
Nutritional Requirements for Maximum Growth	%	Est. no. x10^2/100 ml	%	Est. no. x10^2/100 ml	%	Est. no. x10^2/100 ml	%	Est. no. x10^3/g	%	Est. no. x10^3/g
1. Growth in basal medium-salts, glucose, inorg-N	10.5	65	11.5	9.4	0	—	20.0	24.1	25.0	10.3
2. Amino acids	62.7	382	40.6	33.3	0	—	6.3	7.7	13.7	5.6
3. Amino acids + vitamins	4.7	29	25.0	20.5	34.7	5.2	23.7	28.6	22.5	9.2
4. Yeast extract	19.8	120	20.8	17.1	53.8	8.1	27.5	33.3	21.3	8.7
5. Yeast + mud extracts	2.3	14	2.1	1.7	11.5	1.7	22.5	27.3	17.5	7.2

* Mean of 4 replicates of known dilution.

this group to the sources of free amino acids in surface waters. Decomposing organisms and residues, dissolved organic matter and the excretions of other organisms all contribute to meeting the organic nitrogen needs of planktonic bacteria. However, Waksman *et al.* (23) noted that much of the nitrogenous material of phytoplankton origin undergoes rapid decomposition in the sea and it is likely that compounds such as amino acids will be more readily available in upper levels of the ocean where there may be a continuing supply of them. In view of the fact that the majority of planktonic bacteria are adsorbed upon particulate material, including the cells of marine unicellular algae, the ability of certain components of the phytoplankton to excrete soluble organic products of metabolism is of further interest. Such evidence as the production of extracellular peptide and amide - N by algae (5) and of polysaccharide and organic acids by chlamydomonads (1) supports a likelihood that relations between phytoplankton cells and epiphytic bacterial populations may not be incidental, and that biosynthetic capabilities of certain algae may qualitatively influence the local bacterial flora in a selective, though not necessarily in a direct, manner.

Studies of the influences of the growing plant upon the soil microflora have repeatedly demonstrated that one of the most characteristic effects is the preferential stimulation in the rhizosphere of a group of bacteria dependent on amino acids (14). This was ascribed on experimental evidence (7, 11) primarily to the liberation of amino acids from the plant roots. Further, the presence, also in the rhizosphere, of high numbers of organisms for which growth factors were essential, was attributed to the stimulatory effect of the associated amino acid-requiring forms - a group with a pronounced capacity for vitamin synthesis (4). Little is yet known of specific reciprocal relationships of an analogous nature that may integrate the activities of certain groups of bacteria and other components of marine communities. It has been found that, when added to vitamin-deficient media, the culture filtrates of many of those amino acid-requiring bacteria that were dominant in surface waters will support growth of various vitamin-requiring strains. This finding may be of significance to the ecological status of the amino acid-requiring group

of organisms. Although there is much circumstantial evidence that certain auxotrophic algae and flagellates are ultimately dependent in the sea upon products of bacterial metabolism (cf., 18), a great deal more information is required to evaluate relations between bacteria and marine algae. A notable observation was Collier's (3) record of a persistent association in culture between the dinoflagellate, *Gymnodinium breve*, a vitamin B12 auxotroph, and a dominant type of bacterium found to be a B12 producer. It was suggested that the alga may have supplied a substrate utilized, perhaps preferentially, by the bacterium. These considerations indicate that further studies of the nutritional requirements and biosynthetic potentialities of marine bacteria may be usefully supplemented with appropriately selected syntrophic experiments with unicellular algae and protozoa.

Sediment bacteria which showed maximum growth only in the medium supplemented with marine mud extract have not yet been investigated as a group. As slight or submaximal growth in most cases occurred in other media, it is unlikely that the marine mud extracts supplied any unknown growth factors, neither can these isolates be regarded as necessarily having complex growth requirements, for the extracts could well have supplied simple non-amino carbon or energy sources other than glucose that were unavailable in other media. It is noteworthy that all agar-digesting forms, which comprised some 2 per cent of strains isolated from surface inshore waters, developed preferentially in the yeast mud extract medium. Although extracts may contain humic constituents that serve as metal chelators, their effects in this respect cannot yet be assessed. Some of the marine mud extracts that have been prepared have been found to be inhibitory to certain isolates - a variable that may militate against their value in isolation media.

For important proportions of the strains isolated from sediments, oceanic surface and deep waters, vitamins were essential for growth. Preliminary studies indicate that the specific vitamins most frequently required by these isolates are, in order, thiamin, biotin and cyanocobalamin; these, coincidentally, are listed as the three most important vitamins in the ecology of auxotrophic marine algae and flagellates (18). Folic acid, nicotinic acid,

riboflavin, pantothenic acid and p-aminobenzoic acid are required by a smaller number of strains, but for none are pyridoxine, pyridoxal, choline or inositol necessary. Few isolates demand single vitamins for growth, the majority requiring combinations of two and some of three. There is as yet no evidence of any differences between bacteria from waters and from sediments in respect to their specific growth factor requirements.

No obvious relations are apparent between the morphology of the various isolates and their nutritional grouping. There is some evidence of a trend in which Gram-negative non-sporing short motile rods are characteristic of organisms with simple requirements while those developing in the more complex media include a higher proportion of pleomorphic forms and branching types possibly related to *Mycoplana*. Pigmentation (on mud extract agar) was noted less frequently among sediment isolates (12-14%) than among surface and deep water forms (20-29%). Organisms with inorganic requirements satisfied by sea water and incapable of growth in nutrient broth prepared with demineralised water comprised 95 per cent of the isolates from inshore waters, 72 per cent from ocean waters, 66 per cent from sublittoral sediments and 85 per cent from deep sediments.

As the investigations considered here are based on material obtained in one series of samplings, it is likely that repetitive similar examinations of the indigenous microflora of these environments may reveal fluctuations in the relative incidence of the different nutritional groups of organisms. Such apparent instability in the balance between constituents of microbial populations would not necessarily invalidate the Lochhead method of characterizing them, for as has been pointed out elsewhere (10), in a given environment, components of the microbial populations are in dynamic equilibrium with one another. Variation in this state of balance may therefore be a reflection of, and indeed may indicate the nature of, biologically influential environmental changes ranging from seasonal factors to the physiological activities of other organisms. A more problematical question is whether the nutritional idiosyncrasies of organisms determined in cultivation bear a real relationship to their activities pursued as members of native populations. "The abundance of the various groups

of microorganisms does not necessarily reflect a proportional activity in the transformations implied by their designations, nor does it exclude this." (20) Furthermore there would be little useful application of laboratory data on the nutritional requirements of bacteria from deep sea sources if incubation temperatures and atmospheric pressures inactivate constitutive enzymes to induce certain nutritional deficiencies. It was of interest therefore to observe the similarity in the distributions of nutritional groups in sediment bacteria from 100 m and in those from 2400 m. Sediments from both localities were fine grey muds.

The relevance of serious sampling problems in the primary isolation of organisms is self-evident. Discrepancies between plate and direct counts would be of small consequence in the present method of analysing bacterial populations if all components were proportionately represented among isolates. Although an isolation medium enriched with materials imitating the natural environment has many theoretical attractions, any "non-selective" capacity can by no means be assured. It is also clear that the method is of little value in the classification or taxonomic sense for there is unlikely to be an unequivocal differentiation between organisms merely according to their growth response in the standard media.

The technique developed by Lochhead does provide however a means of comparing some of the potential functional relations of marine bacteria in a variety of ecological circumstances. Despite limitations of the present data, there are indications that outstanding distinctions between marine bacterial populations in regard to their nutritional activity may be related to differences between their particular environmental associations, and it is felt that these preliminary studies justify extension. Further investigations may be helpful in resolving some of the less well known implications of heterotrophic bacterial activity, in particular, processes involving the utilization, production and exchange of critical nutrients within marine ecosystems.

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Chapter 65

On the Quantitative Significance of Microorganisms in Nutrition of Aquatic Invertebrates

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A rather large number of works have been dedicated to the study of the question under consideration. It was repeatedly observed that zooplankton are concentrated where microorganisms are abundant, the number of the latter being reduced on account of consumption (11, 16, 20, 28). The absence of microorganisms in the environment affects the normal development of animals (2, 17). Numerous experiments carried out by Gayevskaya (4) and Rodina (23) proved convincingly that microorganisms form a necessary component of the nutriment of aquatic invertebrate animals. Vegetable remains, devoid of bacterial cells, lose their nutrient value. On the other hand, pure bacterial cultures can serve as the sole source of nutriment of aquatic animals (1, 7, 18, 25, 26, 27, 31).

However, a number of authors suggest that the nutrient value of microbial cells cannot be important because of the small size of microorganisms and their low concentration in natural conditions (3, 12). But there are no good reasons for that suggestion.

Microorganisms occur in the seas and oceans in great quantity and despite the minute volume of the cells, their biological mass is considerable. As a rule, the number of bacteria (by direct microscopic counting) per ml of sea water amounts to tens and hundreds of thousands, and the biological mass up to some mg per cubic meter (Table 1). In the surface strata of sediment deposits the number of microbial cells is still greater, their biological mass reaching, in individual cases, up to some tens of grams per square meter. Under most favourable conditions the fission

of microbial cells takes place every twenty minutes. Under natural conditions the division of cells takes a less rapid course. The ratio of accretion of the biological mass in a certain period of time (P) to its initial size (B) is a coefficient, which biologists consider as a measure of the rate of multiplication. It has been determined that the twenty-four hours' coefficient P/B for the microorganisms inhabiting the Sea of Azov is 1.2-6.2. In the northern part of the Caspian Sea, the microorganisms are able to increase five to six times their biological mass in twenty-four hours, the coefficient P/B being 3.01-6.5. Such a high rate of multiplication is indicative of an active consumption of bacteria by higher organisms since their quantity preserves a rather constant level.

TABLE 1

Quantity and Biological Mass of Microorganisms in the Water of Various Oceans and Seas

Oceans or Sea	*Bacteria per ml*	*Biomass of bacteria in mg/m³*	*Reference*
Arctic Ocean (region of North Pole)	35-39,000	0.03-1.8	Kriss, (13)
Pacific Ocean (north-western part)	33-180,000	0.008-25	Kriss and Biryuzova, (14)
Atlantic Ocean (north-western seaboard of Africa)	24,000-54,000	1.7-61.7	Zhukova, 1959
Black Sea	3,000-500,000	7.7-43.1	Kriss, Lebedeva and Rukina, (15)
Sea of Azov	40,000-140,000	16-76	Zhukova, (32)
Caspian Sea (northern part)	500-500,000	30-750	Osnitskaya, (21)

The work of the predominant number of various physiological groups of bacteria is directed at the destruction of the organic matter. The half-decayed remains of animals and plants, united by the concept of "detritus," contain masses of microbial cells. One gram of damp detritus contains up to 5 billion cells. The microbial cells create detritus and at the same time they are a component part of it; together with detritus they get into the intestines of invertebrates. The analysis of the contents of intestines of aquatic invertebrates shows, as a rule, that it consists, in the main, of an amorphous mass with a small percentage of preserved

plant cells therein. It is known for *Calanus finmarchicus* that detritus fills up, in individual cases, nearly 80 per cent of the volume of its intestine. In the stomach of animals living on the bottom, the percentage of detritus is still greater.

Bacteria ingested as nutriment by invertebrates may either be digested with other components of food, or pass through the intestine unchanged. In the intestine of most of the invertebrates enzymes are present, which stimulate the digestion of proteins, fats and carbohydrates. Taking into consideration the composition of bacterial cells, we may assume that the microorganisms getting into the intestine of invertebrates with other food will be digested by the same enzymes that are in the alimentary canals of the animals.

Quantitative data of the nutrient value of bacterial cells were absent until very recently. Only exact quantitative research in processes of nutrition can enable us to establish the relationship between the separate links of the food chain in the reservoir. With the purpose of meeting this lack, we undertook an investigation of the nutrition of some sediment invertebrates. The animals investigated were polychaeta *(Nereis diversicolor)*, molluscs *(Monodacna edentula)* and amphypoda *(Pontogammarus maeoticus)* taken from the northern part of the Caspian Sea.

It was necessary to determine: 1) Whether the bacterial cells are digested in the intestine of animals existing under natural conditions. 2) Whether they are assimilated, if digested. 3) What is the proportion of microorganisms in the nutrient of the animals.

Animals were fixed with formalin, immediately after they had been caught, it having been proved that their intestine contained food ingested in the places they usually inhabit. Contents of each of the three divisions of the intestine were weighed and preparations of each of the three parts were made according to Winogradsky *for direct microscopic counting* of the number of bacteria. The investigation of *Nereis diversicolor* proved that the number of microbial cells sharply decreases as the food passes from the oral aperture to the mid-gut, whereafter, in the hind-gut it rises slightly (Fig. 1A). In worms the digestive ferments are secreted in the upper one-third of the mid-gut, the end of this division of the alimentary canal secreting no ferments and being

solely sucking. Hence, it is quite understandable, that the number of microbial cells sharply diminishes as the food passes from the fore-to the mid-gut. It is obvious that the number of digested microorganisms represents the difference between the respective numbers of cells in the fore- and mid-gut. The hind-gut contains no ferments, and here the multiplication of undigested microbial cells takes place. As proof thereof, the results of observations by Winogradsky's technique, of the micro colonies consisting of a few bacterial cells, showed that the number of bacterial cells increases from the mid- to the hind-gut in *Nereis diversicolor.*

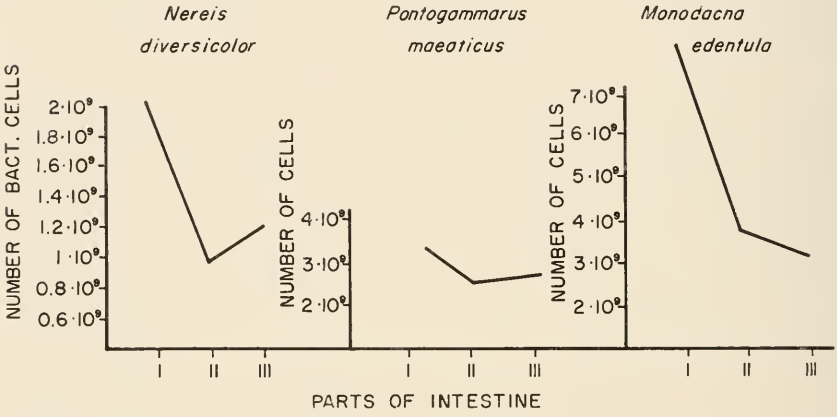

Fig. 1. The change in number of microorganisms in 1 gm. of intestine content.

The character of digestion of bacterial cells in the intestine of *Pontogammarus maeoticus* is analogous to that which was found in *Nereis,* that is, the total number of cells diminishes in passing from the fore- to the mid-gut and increases in the hind-gut (Fig. 1B). This phenomenon coordinates well with the facts of digestion in *Crustacaea.* Digestive ferments in the representatives of this group are secreted on the border of the fore- and mid-gut (29). Uptake can take place only in the mid-gut, the fore- and hind-gut being chitinized.

In the molluscs *(Monodacna edentula),* unlike the representatives of *Articulata,* we observed the uninterrupted diminishing of the number of microbial cells all along the digestive tract

(Fig. 1C), the rate of this diminishing noticeably slackening as food enters into the hind-gut. The same regularity was established in another representative of the family *Cardidae, Adacna plicata.* ZoBell and Feltham (31) extracted from the intestinal tissue of the molluscs a ferment which provokes lysis of 28 strains of sea water bacteria. Yöng (29) mentions that small particles of food, including bacteria, can be digested intercellularly by means of phagocytes, which exist in the fore- as well as in the mid-part of the intestine of molluses. Digestive ferments are secreted on the border of the oesophagus and the stomach, where the uptake of digested food takes place by the special organs-diverticules. Hence, microorganisms are digested in the intestine of molluses as well as in the intestine of *Articulata* identically, that is, when the food passed the fore-part of the intestine and entered its mid-part. No further multiplication of survived bacteria takes place because of the acid reaction of the medium, which reaches pH 3 in the intestine of molluscs (29, 30).

The fact that bacterial cells are digested cannot be regarded as proof that they are assimilated. Digestive ferments could have effected the lysis of bacterial cells. The ability of the newly hatched young of *Nereis diversicolor* for assimilation of bacterial cells was tested experimentally. Worms were offered food of different composition (table 2). It was found that the young of

TABLE 2

Results of Growing Young Nereis diversicolor Fed on Various Food-materials

Food Materials	*Initial Weight of Worm in mg*	*Total Increase in Weight in Course of the Experiment*	*24 Hours Increase in Weight*	*Food Coefficient*
		In %% from the initial weight		
Vegetable	49.0	22.5	1.5	27.3
Yeast	45.7	91.2	6.0	4.9
Bacteria	74.8	12.3	0.9	15.5
Control (animals were not fed)	60.0	–18.4	–1.3	—
Natural sediment (Jablonskaya and Belyaev)	—	—	4.7	142.1

Nereis diversicolor, being fed for fourteen days exclusively on bacteria, did not perish; moreover, they increased in weight by 12.3 per cent of the original weight.

Having made certain that microorganisms had been assimilated in the intestine of analysed animals, we attempted to calculate the twenty-four hours' consumption of bacteria from quantitative data on the diminution of the number of bacterial cells when the food mass passes from the fore-part of the intestine to the mid-part. We calculated mass of digested cells from the data of the cells' volume. Specific weight of microorganisms was taken as equal to 1 (Table 3). It has been found that under conditions of the northern part of the Caspian Sea and in the places where the samples had been taken, the microorganisms supply from 1 to 10 per cent of the twenty-four hours' nitrogen requirement of the sediment invertebrates.

TABLE 3

THE TWENTY-FOUR HOURS' FOOD RATION OF INVERTEBRATES (IN DAMP WEIGHT)

Animals	*Food Ration of an Individual Animal (in mg)**	*Assimilation of Nitrogen by an Individual Animal (in mg)*	*Assimilation of Bacterial Cells by an Individual Animal (in mg)*	*Nitrogen Contained in Assimilated Cells (in mg)*	*Rate of Nitrogen Assimilated from Bacteria in Per Cent of the Total Amount of Assimilated Nitrogen*
Nereis diversicolor	330	0.4	0.11	0.01	9.1%
Pontogammarus maeoticus	14.7	0.015	0.035	0.0004	1.2%
Monodacna edentula	0.608	0.0015	0.0004	0.00004	10%

*According to Karpevitch and Jablonskaya (9).

It is more difficult to determine the quantitative role of microbial cells in the nutrition of planktonic forms. Although *Daphnia magna* was used in most of works where qualitative methods were made use of, its small size greatly complicated the task of obtaining quantitative data for this plankter. The method of

quantitative and weight analysis employed in our experiments with the benthic forms was inapplicable in this case, for it was impossible to produce sufficiently fine preparations and weights. The chosen method was based upon the principle of observing the changes of concentration of cells used as food, in vessels containing small planktonic crustaceans *(Copepoda)* with oar-like swimming feet, *Calanipeda aqua dulcis*, taken in the Sea of Azov. The mixture of cells of algae and bacteria developed in flasks containing phytoplankton, which is a nutriment similar to that under natural conditions, was offered as food. In each variant of the experiment, the numbers of both the bacterial and phytoplanktonic cells were counted simultaneously. This enabled us to compare the consumption of the two kinds of food without previously placing the animals under conditions of forced feeding solely on bacterial cells. The duration of the experiment was shortened as much as possible, so that the numerical values for ingested cells should not be influenced by the reproduction of bacteria. At the same time, the possibility of polluting the medium by undigested microbial cells was minimized. In the course of time that the small crustaceans were in the experimental vessels, a decrease of concentration of the cells of the two food components was, as a rule, observed. It became possible to determine, on these grounds, the rate of filtration according to the formula proposed by Gauld (6). Having both the rate of absorption of food cells from a definite volume of water by one animal in a unit of time and the volume of water filtered in twenty-four hours, it is easy to calculate the twenty-four hours consumption of food by one small crustacean.

The problem arose as to the definition of the optimum rate of filtration. The rate of filtration is not a constant; it depends upon a number of factors, of which the most important is the initial concentration of food cells (Fig. 2). Calculations were made using food-cell concentration proper to the habitats of calanipeda (10,000-20,000 cells per 1 ml). The size of that rate of filtration fluctuated from 0.11 to 0.25 ml per hour.

In order to compare the two kinds of food, i.e., algae and bacteria, we calculated the rate of consumption of cells by one small crustacean in an hour. Those rates were respectively for phyto-

plankton and bacteria, 1.44 per cent and 1.04 per cent of the total number of food cells. This means that the cells of both food components are ingested simultaneously, but at unequal amounts.

Taking into account the natural concentration of cells of bacteria and planktonic algae in the habitat of calanipeds in the Sea of Azov as well as the composition of the cells, their size and species and the rate of consumption of food cells, the figures show

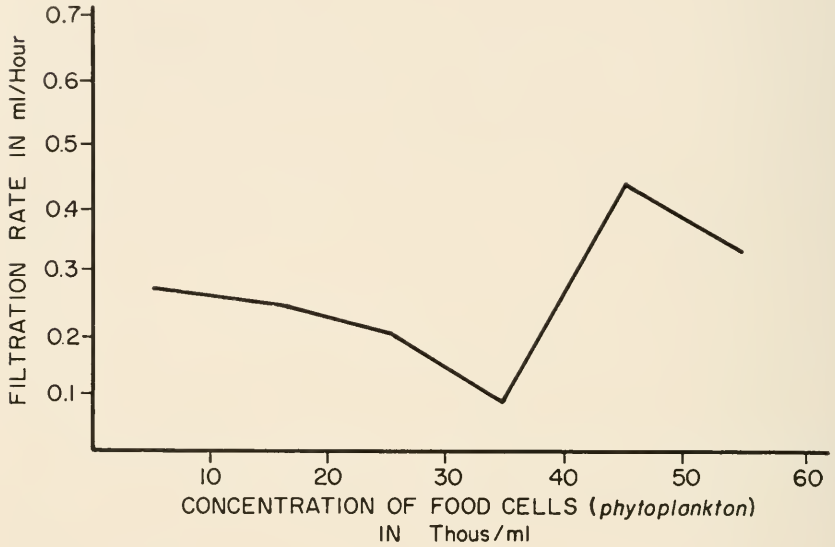

Fig. 2. Dependence of the filtration rate on food concentration.

that the weight of bacteria consumed is 1/5 that of the weight of phytoplankton (Table 4).

Using the radioactive tracer, C^{14}, it has been proved that bacteria are not only ingested by the representatives of plankton, but are also assimilated. The experiment was designed to obtain bacterial cells bearing the isotope tracer which could serve as food for plankters, and after the bacteria were ingested, to find the tracer in the body of the filtrator.

It is comparatively easy to obtain bacteria which bear the tracer by adding a radioactive element to a compound in an artificial nutritive medium in which microorganisms are cultivated. However, bacteria cultivated in artificial nutritive media

TABLE 4

IMPORTANCE OF VARIOUS FOOD COMPONENTS IN NUTRITION OF *Calanipeda Aqua- Dulcis* (IN DAMP WEIGHT)

Number of Crustaceans (Copepods) per Cubic Meter (Sea of Azov) July, 1955	*Volume of Water Filtered in 24 Hours*	*Components of Food*	*Number of Food Cells Ingested in 24 Hours Per Cubic Meter*	*Biological Mass. of Ingested Cells (in mg)*
300	1200 ml	bacteria	87,264,000	0.042
		phytoplankton	2,073,600	0.218

differ strongly from those living under natural conditions and their nutrient value is doubtful in a number of cases. Therefore we used a different way to obtain bacterial food, which would in its composition resemble as nearly as possible the natural food. Microorganisms, intended to serve as the food for small crustaceans, received the radioactive tracer from the cells of phytoplankton in process of its decomposition. The bacteria were separated from phytoplankton by the aid of membrane filters. The plankters were placed into experimental vessels and lived there 48 hours during which time they were fed exclusively on bacteria. In course of that period their bodies acquired the tracer element which they firmly retained after the experiment had been carried out (Table 5). The data presented do not elucidate the balance characteristic of the process, but they establish the presence of the carbon tracer in the body of the filtrator, acquired from the microorganisms, and prove the assimilation of the latter.

It should be supposed that the results of the experiment can be extended, and applied to other representatives of plankton. There is no fundamental difference in composition of the food of

TABLE 5

CHANGES IN RADIOACTIVITY OF THE BODIES OF PLANKTONIC CRUSTACAEA FED BY BACTERIA CONTAINING TRACER ELEMENTS (IN AMP/MIN)

Expt.	*Radioactivity of Small Crustaceans (Copepods)*		*Radioactivity of Bacteria*	
	Initial	*Final*	*Initial*	*Final*
I	0	408	600	327
II	0	553	394	117

Copepoda in various seas and oceans and the rate of filtration in *Calanipeda* is nearly that which has been found in *Calanus finmarchicus* (3, 5, 8).

The wealth of microflora in various seas and oceans has been shown at the beginning of this report. The marine plankton is greatly accumulated in zones where the phytoplankton "in full bloom" is fading away, that is, just where the quantity of bacteria begins to increase sharply. The works dedicated to the study of the extremely complicated question of interrelations of phytoplankton and bacteria testify that the cells of planktonic algae in state of vital activity either are entirely sterile, or suppress, in considerable measure, the multiplication of microorganisms. Only the dying, atrophying cells of vegetable plankton stimulate the quick development of bacteria. Probably owing to that very fact, zooplankton accumulates along the borders of the "blooming" phytoplankton, where the decomposition of the latter begins and the nutriment is concentrated in an immense quantity of microbial cells, which apart from their being of a considerable nutrient value, supply a great number of vitamins.

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Chapter 66

Significance of Microorganisms of Upper Sediment Layer of Shallow Water Basin in Transformation of Organic Matter

A. I. Zhukova and M. V. Fedosov

The chemical conditions of shallow water basins are determined to a considerable degree by the biochemical processes which take place in the upper sediment layer.

Our southern basins, such as the Azov and North Caspian Seas, are characterized by a rapid turnover of organic substance, i.e., its production in the form of phytoplankton during "blooming," and then dying off and rapid decomposition under high temperature conditions in summer.

The intensity of the process of photosynthesis in these basins is very great. According to previously obtained data (3) phytoplankton in the Azov Sea in spring, summer and autumn periods produces on the average from 280 to 340 mg of organic substance per twenty-four hours.

As is known from the work of Gorshkova (6), the organic substance produced by phytoplankton is very unstable and rapidly decomposes. The experimental investigation of the decomposition rate of blue green algae showed that one day after death of the vegetable cells, the water gets about 20 per cent of their phosphorus and 30 per cent of their nitrogen, the decomposition of the phytoplankton being completed on the whole within five days. Similar data were previously obtained by Skopintsev (10) in the course of an experiment with fresh water plankton. Dead planktonic organisms sinking slowly to the bottom begin to decompose in the water column.

According to the calculations made by Skopintsev (10) the nannoplankton, with an average diameter of 50 μ (μ = micron), sink on to the bottom at the rate of 1.9m per day at t° = 22 C (S

= 35‰). Taking into account the fact that the density of water in the Azov and North Caspian Seas is much less than that for which the above calculations were made, it is easily understood that plankton particles will sink much more quickly. At the shallower depths which are observed in the North Caspian and Azov Seas (maximum 15 m, average 5-6 m) plankton particles reach the bottom with three to four days under ideal conditions of calm.

Despite the fact that a certain part of the dead organic substance decomposes, as has already been said, in the water column, not only hard-to-decompose compounds are found on the sediment surface, but also labile organic molecules, the latter mainly from benthic organisms.

Table 1 shows the initial decomposition of the components of plankton.

TABLE 1

Composition of Organic Matter of Plankton and the Surface Sediments of Beloye Lake (From the Data of Speranskaya [14])

	Plankton Per Cent Dry Weight	*Upper Silt Layer*		*Quantity of Decomposed Substance of Plankton in Per Cent of the Initial Quantity*	*Quantity of Stable Substance which Passed into Sediments in Per Cent*
		Total Per Cent	*Calculated as Plankton Ash*		
Ash	6.4	46.4	6.4	—	100
Wax and bitumen	14.15	6.5	0.89	93.6	6.4
Hemicellulose	29.7	7.1	0.98	96.7	3.3
Cellulose	10.2	5.1	0.84	91.7	8.3
Lignin	31.4	30.1	4.27	86.8	13.2
Total nitrogen	8.1	2.5	0.35	95.7	4.3

Hemicellulose decomposes most rapidly; lignin-humus complex-most slowly.

According to the data of Gorshkova (6) on the total amount of carbon in sediments of the Azov Sea and Taganrog Bay, the carbon of sugars and hemicellulose constitutes up to 5-10 per cent; the carbon of cellulose: 4.6-14 per cent; the carbon of

soluble compounds (without sugars and cellulose) from 18 to 37.7 per cent and the lignin - humus complex, from 47 to 63 per cent. The composition or organic substance of the North Caspian sediments is very close to that of the Azov Sea and Taganrog Bay.

The composition of organic substance of sediments is such that its decomposition may take place mostly with the help of bacteria. Therefore we inevitably deal with bacterial processes when considering the process of regeneration of bio-microelements from sediments.

The uppermost silt layer situated at the sediment water interface is most active with respect to the processes in question. As far as the rate of regeneration of bio-microelements in the silt layer is concerned, it should be noted that the upper layer consumes from 1.5 to three times more oxygen than those situated somewhat lower. Such an upper layer does not exceed, as a rule, 1-1.5 cm and is formed in the Azov Sea in fifteen to thirty years.

The sediment layer bordering on water is most active also with respect to bacterial processes. The number of bacteria in silt distinctly decreases with depth but at a depth of 1 m it becomes stable and does not exceed 100-200 million per g of wet silt.

The most distinct reduction in number of bacterial cells is observed at a depth of 25-30 cm from the sediment surface. The number of cells amounts to 1×10^9 per g on the interface with water, whereas it does not exceed 4×10^8 at a depth of 30 cm. The accumulation of bacteria on the sediment surface is often so great that it forms a dense bacterial film. In 1938, Butkevich, noted a dense film of bacterial origin in the North Caspian Sea. The film was of protective value for the water column; Butkevich thought that it absorbed the gases H_2S, CO_2, N_2, CH_4, H_2 which were formed in the silt. The recent investigation carried out in the North Caspian Sea in 1951-1952 (9) showed that despite the absence of dense bacterial film, the upper sediment layer is very rich in bacteria and contains representatives of such physiological groups as those that oxidize methane and hydrogen, as well as those of putrefactive and sulphurating type. The latter two are of considerable importance for biogen regeneration; putrefactive

bacteria, by destroying protein combinations, contribute to the release of various forms of nitrogen while sulphur bacteria are able to transfer various reduced sulphur combinations into oxidized ones containing ions of $So^{=}_{4}$.

A similar type of the upper sediment layer is found in the Azov Sea. In 1923, Isachenko, and then in 1937 a group of microbiologists under Butkevich, found denitrating, desulphurating and thiobacteria in the Azov Sea upper sediment layer.

Almost all of the enumerated physiological groups of microorganisms that were found in the upper sediment layer of the North Caspian and Azov Seas belong to the group of autotrophic microorganisms which do not need organic substance for their development. Autotrophic microorganisms, however, constitute only a small percentage of the total number of bacteria in water basin sediments (8, 13). Therefore while considering the question of transformation of organic substance, one should not confine oneself to consideration of only those groups that "complete" the processes of decomposition (by transforming CH_4, H_2, H_2S, CO_2).

To get an idea of the intensity of bacterial biochemical activities on the surface of sediments one should be aware not only of the qualitative composition of separate groups but also of the total number of microorganisms inhabiting sediments and their weight, i.e., biomass.

The number of bacteria in sediments is great: in the Azov Sea in July it fluctuated from 6.3×10^9 to 17.2×10^9 cells per g of natural silt; the biomass varied from 17 to 57 g/m^2/cm layer (16).

In July of 1951 and 1952, high concentrations of microorganisms in silts were also observed in the North Caspian Sea. In the area of the approaches to the Volga river delta the number of microorganisms was found to have reached 12×10^9 per gm of natural silts soon after the highflood. The biomass changed at this time from 2-6 g/m^2 to 50-52 g/m^2/cm layer (15).

According to the data found in literature, a bacterial cell expends for the formation of its body, an amount of organic substance exceeding its own weight by three fold. Consequently, three times as much organic substance was consumed in the for-

mation of the bacterial biomass observed in the upper sediment of the Azov and Caspian Seas.

The biomass of microorganisms in sediments is not constant. The presence of organic substance changing in different seasons of the year is the principal factor for determining the total number of bacteria (15).

But the mere data of bacterial biomass does not lead to an understanding of the process. The cells in the state of tranquility are passive with respect to the transformation of organic substance. In the course of the observations we were able to ascertain the abundance of cells which were in the state of anabiosis.

Besides, the process of decomposition of the bodies of defunct bacterial cells takes a certain period of time.

In order to comprehend the actual extent of bacterial activities, the value of diurnal production of microorganisms in the near bottom water layer was determined both for the North Caspian and Azov Seas.

Studying the attachment of bacteria to glass plates, it was found that the daily coefficient changed from 1.2 to 6.2. That means that biomass of bacteria can increase by 1.2 to 6.2 times.

As far as seasonal changes in bacterial propagation are concerned, the following has been noted for the Azov Sea: the value of the daily coefficient P/B (production/biomass) kept approximately at the same level both in spring and summer, whereas the percentage of dividing cells increased from 4.7 per cent in spring to 8 per cent in summer. The amount of organic substance in the surface film of the Azov Sea sediments was estimated to nearly meet the requirements of microorganisms under the observed rate of summer propagation.

In order to reveal the connection between microorganisms and the dynamics of biogen elements we have conducted some experiments on the regeneration of bio-microelements from sediments. Simultaneously, observations were made on the dynamics of microbe cells.

The previous works on the North Caspian and Azov Sea sediments showed that the rate of regeneration of bio-microelements in these seas is proportionate to the quantity of organic substance in sediments and to the amount of biochemical oxygen

demand, the latter being determined experimentally (2). The quantity of microbe cells in the North Caspian silts was also shown to be proportionate to the amount of organic substance.

This fact proves once more that bacteria participate in the process of regeneration of bio-microelements. The transition of biogen elements from sediments into water in the Azov Sea is quicker than in the North Caspian Sea.

The quantity of labile organic substance in the Azov Sea sediments (on the average) is 2.4 times greater than in the sediments of the North Caspian Sea. The five days' biological oxygen demand is also higher: 27.2 mg O_2 per g of natural sediment in the Azov Sea against 6.25 mg O_2 per g of natural sediments in the North Caspian Sea. The number of bacteria in the Azov Sea sediments is correspondingly higher.

Theoretical calculations show that the regeneration rate of biogens is such that it can ensure the development of 340 mg/m^3 phytoplankton per day in the summer period at a depth of 7 m (a 5 m deep photosynthetic layer: this actually takes place in the summer period).

Anaerobic reactions are of particular importance for the process of decomposition of organic substance in sediments. Anaerobic processes precede the transition of biogens from silt into water.

Sediments being kept without an access of oxygen prior to the beginning of the observations, were found to transmit into water 1.5 to three times as much biogen elements as sediments being kept aerobically.

In the Azov Sea, under high temperature and restricted conditions (less than 3 ml O_2/L) in summer, the content of ammonium nitrogen salts and phosphates in the near-bottom water increases by two to three times. Such greatly inhibited oxygen conditions near the Azov Sea bottom were found to have formed during stable periods of calm weather favouring the warming up of the upper water layers only. At the same time the difference in the density of different water layers results in their stabilization, which impedes the mixing still further.

If, in addition, there is a difference in the salinity of water layers (as a result of the inflow of water from the Black Sea or

rivers) the stratification is still more stable; this causes continuous oxygen impoverishment in nearbottom layers. On the basis of these observations an experiment was developed on the regeneration of biogen microelements under inhibited oxygen conditions near the silt surface.

The Taganrog Bay argilaceous silt was taken as a sample. The salinity of the initial water samples was 6.5‰. The volume of samples was about 5 liters, the silt layer—5-10 cm. The experiment lasted 10 days under the conditions of artificial depletion of oxygen. The samples were analysed for the content of O_2, NO^{-}_{2}, NH_4^{+}, NO_3^{-}, PO^{-}_{4} and pH. The total number of bacteria and the number of putrefactive bacteria on the mud-water interface were determined. Some samples were kept without silt to reveal its significance in providing the nearbottom water with bio-microelements. The results of the experiment showed that bottom silt brings about a more even distribution of microorganisms in time (in the course of 10 days experiment); silt contributes to a "buffering ability" of the bacterial medium. The presence of silt leads to the increase of the content of phosphates in water because of the regeneration of phosphates from the compounds contained in bottom silt. The pH, in the silt - containing samples, was 0.2-0.5 lower than in those without silt owing to an extra elimination of CO_2. Saprophytic bacteria did not differ in number either in silt- devoid or silt- containing samples. The increase in the content of NH_4^{+} is in parallel with the increase in the total number of bacteria. This fact and the one cited above on the number of saprophytic bacteria not increasing in silt- containing samples, give some reason to think that along with the processes of nitrification an active process of dentrification takes place resulting in the elimination of NH_3, the later being bound by anions.

On the third and sixth days of the experiment the number of bacteria and NH_4^{+} showed a marked increase. After that the content of NH_4^{+} begins to fall, simultaneously accompanied by a reduction of the total number of bacterial cells and an increase in amount of oxygen.

At this time, the oxidation of the organic substance is generally terminated and replaced by the beginning of the nitrification of ammonium. This regularity is clearly seen in Figure 1.

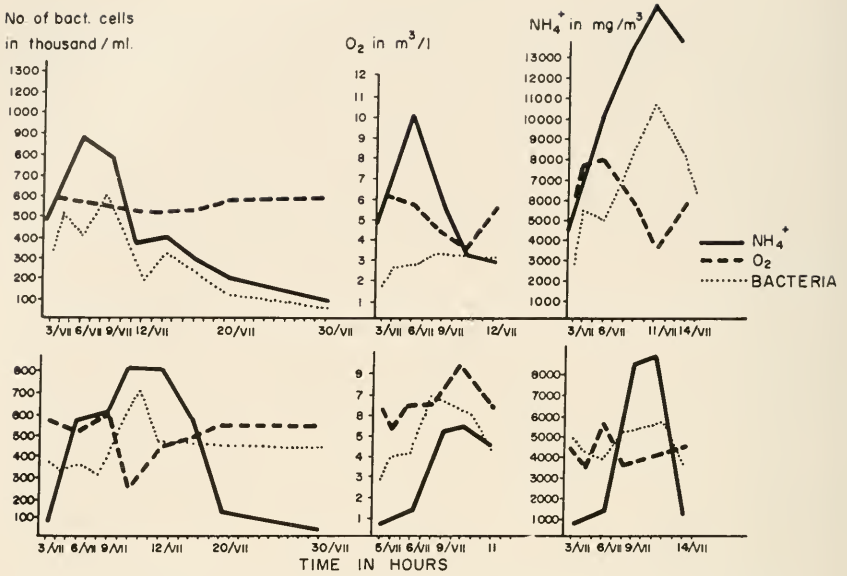

Fig. 1. Role of bacteria in regeneration of biogens from sediments.

Thus, the gas conditions of a basin and the number of biogens in water were experimentally demonstrated to be governed by microflora activities on the silt-water interface.

The above stated reasons prove convincingly that microorganisms participate most actively in the processes of decomposition and regeneration of organic substance in marine shallow water basins. Microbiological investigations conducted by means of a direct count method help to gain more profound and detailed knowledge of the processes of transformation of organic substance in water basins and to assess their quantitative value.

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