THE EFFECT OF COPPER UPON THE DEVELOPMENT OF BACTERIA IN SEA WATER AND THE ISOLATION OF SPECIFIC BACTERIA[1]

By

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INTRODUCTORY AND HISTORICAL

Copper is used extensively in the eradication of various types of disease-producing microorganisms, especially fungi, as well as other organisms which are a nuisance to water supplies, such as algae and certain animal forms. It is also known that traces of copper are essential for the growth of many of the lower forms of life. The extensive literature on the relation of copper to microbial development is largely limited to the above two phenomena. Comparatively little is known, however, of the effect of copper upon a mixed microbiological population consisting of many organisms with different metabolic processes. It is known, for example, that the growth of plants in certain soils, as peats, will respond markedly to the application of small amounts of copper (5). The development of fungi in copper-free media has been suggested as a means of determining the concentration of copper in a given soil; this method is based upon the response of *A. niger* to the presence of small amounts of available copper (3).

Certain microorganisms are known to be capable of tolerating fairly large amounts of copper. However, the organisms investigated so far are found largely among the fungi (1, 6, 7, 8). What role these organisms play in the transformation of copper in nature or even in artificial substrates is still a matter of speculation; high copper concentrations retard the growth of the organisms during the early stages of development. The presence of excessive concentrations of copper brings about the elimination of most microorganisms in a natural substrate with the exception of certain fungi capable of withstanding these high copper concentrations (6). The mycelium of some of the

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copper-resisting fungi was found (1) to contain considerable copper, which is in a fixed state and cannot be readily removed by acids. The absorption of copper during the early stages of growth was found (4) to be followed by the elimination of the copper into the medium in the older cultures.

The relationship of copper to bacteria has been chiefly limited to its disinfectant action, different bacteria varying greatly in their resistance to copper. Some of the bacteria were found (2) to take up enough copper to color them, first green, then brown.

A search for information concerning the relationships of marine bacteria to copper in sea water suggested the desirability of undertaking a study of the effect of copper upon marine bacteria as a whole and of the presence in the sea of bacteria capable of withstanding copper in concentrations greater than mere traces. Particular attention was paid to the influence of copper on the development of marine bacteria in sea water and in culture media. The problems of adjustment of bacteria to varying copper concentrations in sea water and the effect of these bacteria upon the copper have been studied.

EXPERIMENTAL

The effect of copper on marine bacteria. The medium selected for the study of the effect of copper upon marine bacteria was one extensively used in this laboratory for the study of marine bacteria and supplemented with varying amounts of $CuSO_4.5H_2O$. This medium has the following composition:

Agar	15 gm
Glucose	1 gm
Peptone	1 gm
K_2HPO_4	0.05 gm
Sea water	1000 ml

The medium was divided into several portions which received additions of $CuSO_4$ ranging from 0 to 40 mg per liter. These media were tubed, plugged, and sterilized and used for plating out fresh sea water. The numbers of bacteria found in the water taken near the dock of the Oceanographic Institution at Woods Hole showed no appreciable differences in the media receiving the above amounts of copper. Apparently these concentrations were not great enough to have an inhibiting effect upon the growth of marine bacteria. Even in this preliminary experiment, however, the appearance of several red-brown pigmented colonies on the plates containing the largest amount of $CuSO_4$ was observed.

This experiment was repeated using greater concentrations of copper in the medium, as follows:

Medium No.	$CuSO_4.5H_2O$ *mg/L*
1	0
6	50
7	100
8	200

Fresh sea water was plated out in dilutions of 1 and 1 : 10 using the above media. In addition, small amounts of fresh films from plates submerged in the water for several weeks were also plated out in dilutions of 1 : 1,000 and 1 : 10,000. The plates were incubated for two and four days at room temperature (25° C.), and the total colonies developing on the plate were determined. The red-brown colonies, which had a typical appearance of metallic copper, were designated as "copper colonies." The results obtained may be summarized as follows:

NUMBERS OF BACTERIA IN 1 ML. FRESH SEA WATER

Medium No.	*Colonies per plate, dilution* 1 : 10	
	Total colonies	*"Copper" colonies per plate*
1	200	0
6	160	10
7	3	0
8	0	0

NUMBERS OF BACTERIA IN A SMALL AMOUNT OF FILM FROM SUBMERGED SURFACE

Medium No.	*Colonies per plate,* 1 : 1,000 *dilution*	
	Total colonies	*"Copper" colonies*
1	>500	0
6	>500	a few
7	500	many
8	90	0

On continued incubation of the plates, the red color of the "copper" colonies on the media containing $CuSO_4$ disappeared, the colonies losing their red pigment and becoming white again.

There was a slight increase in acidity of the medium with an increase in copper concentration; however, even the highest concentration of copper used in this experiment did not make the medium very acid, changing the pH to 6.9. It is believed that these copper concentrations would not have had a great enough effect upon the growth of the bacteria merely through the pH change.

It was apparent from the results of these experiments that the presence of $CuSO_4$ in the medium was responsible for the pigmented colonies, and the presence of 200 mg of $CuSO_4.5H_2O$ per liter of

medium was too great a concentration for the development of the total bacterial population in the water, as well as of the specific "copper" present. The addition of 100 mg $CuSO_4.5H_2O$ per liter of medium was found to be the optimum for the development of the specific "copper" colonies on the plate. This concentration was used in the subsequent experiments for determining the abundance of the "copper" organisms in sea water. The medium containing this amount of copper will henceforth be referred to as medium 7.

In order to determine whether such "copper" bacteria are also present in soil and in lake water, samples of ordinary soil and pond water were plated out, using nutrient agar media enriched with 100 mg of $CuSO_4.5H_2O$ per liter. The materials were plated out using different dilutions. The numbers of bacteria obtained on this medium were 1,600 per ml of pond water and 60,000 per gm of soil, after 2 days incubation. No typical "copper" colonies appeared on any of the plates. Whether these bacteria are totally absent in soil and in fresh water or whether the salts present in the media used for plating out the sea water had a specific effect upon the development of such bacteria, still remains to be determined.

The effect of additions of various forms of copper to sea water upon the general bacterial population of the water as a whole and upon the development of specific "copper" bacteria was now studied.

The influence of $CuSO_4$ *upon the bacteria and upon oxygen consumption in sea water.* The total numbers of bacteria in the water were measured by the plating method, using the copper-free medium 1; the number of "copper" bacteria were determined by the use of medium No. 7. Oxygen consumption in the water, as influenced by the addition of varying amounts of copper, was also measured by the common Winkler method.

Several preliminary experiments were carried out for the purpose of determining the type of container to be used for incubating the water, the method for treating the water, and the length of the incubation period (Table I).

Only the results of one typical preliminary experiment are reported here. Measured quantities of fresh sea water received varying amounts of $CuSO_4.5H_2O$. They were immediately distributed in oxygen bottles (230 ml portions), which were incubated under water at room temperature (20°–25° C). After different periods of incubation, the bottles were removed and contents used for plating and oxygen determination. One ml portions were removed with sterile pipettes, disturbing the water in the bottles as little as possible. The

TABLE I—Influence of Copper upon the Development of Bacteria and Consumption of Oxygen in Fresh Sea Water Kept in Laboratory

Incubation of water, days	*Copper added as* $CuSO_4.5H_2O$ *mg/l.*	*Oxygen consumed ml/l.*	*Total numbers of bacteria per 1 ml water*	*"Copper" bacteria per 1 ml water*
0	0	0	2.000	.45
1	0	1.204	3.160.000	232.000
1	1	0.777	1.660.000	720.000
1	10	0.018	38.000	6.500
1	50	0.021	73.500	5.000
1	200	0.070	105.000	17.000
2	0	1.708	2.400.000	35.000
2	1	1.544	4.400.000	166.000
2	10	0.679	1.050.000	163.000
2	50	0.665	1.550.000	115.000
2	200	0	24.000	1.500
4	0	2.216	220.000	1.000
4	1	2.590	3.440.000	21.500
4	10	0.952	3.760.000	42.500
4	50	0.892	6.540.000	290.000
4	200	0.574	703.000	682.000
7	0	2.976	200.000	2.000
7	1	4.480	22.000	1.500
7	10	0.952	2.510.000	1.050.000
7	50	0.795	650.000	360.000
7	200	0.795	810.000	31.500

reagents for the oxygen determination were then added to the residual water in the bottles. The bacterial plates were incubated at room temperature for 4 days and counted at the end of the 2d and 4th days. The 4 day counts are reported unless otherwise indicated.

Although the results of this experiment are somewhat erratic, nevertheless they permit certain interesting conclusions. The addition of copper to the water had at first a depressing effect upon bacterial development and oxygen consumption. This effect was soon overcome; the smaller the amount of copper added the shorter was the period of "depression" or "adaptation." Subsequently copper had a stimulating effect, depending on the amounts of copper added; the less the concentration of copper the sooner the stimulating effect was observed. At the highest concentration of copper (200 mg $CuSO_4.5H_2O$), the period of depression was longest and was not completely overcome even after 7 days incubation, the longest period used in this experiment.

In order to overcome the variability in the results which definitely appeared to be due to experimental errors, this experiment was repeated, using a larger number of replicates and extending the incubation period for a somewhat longer time. Fresh sea water, taken some 100 feet away from the pier of the Oceanographic Insti-

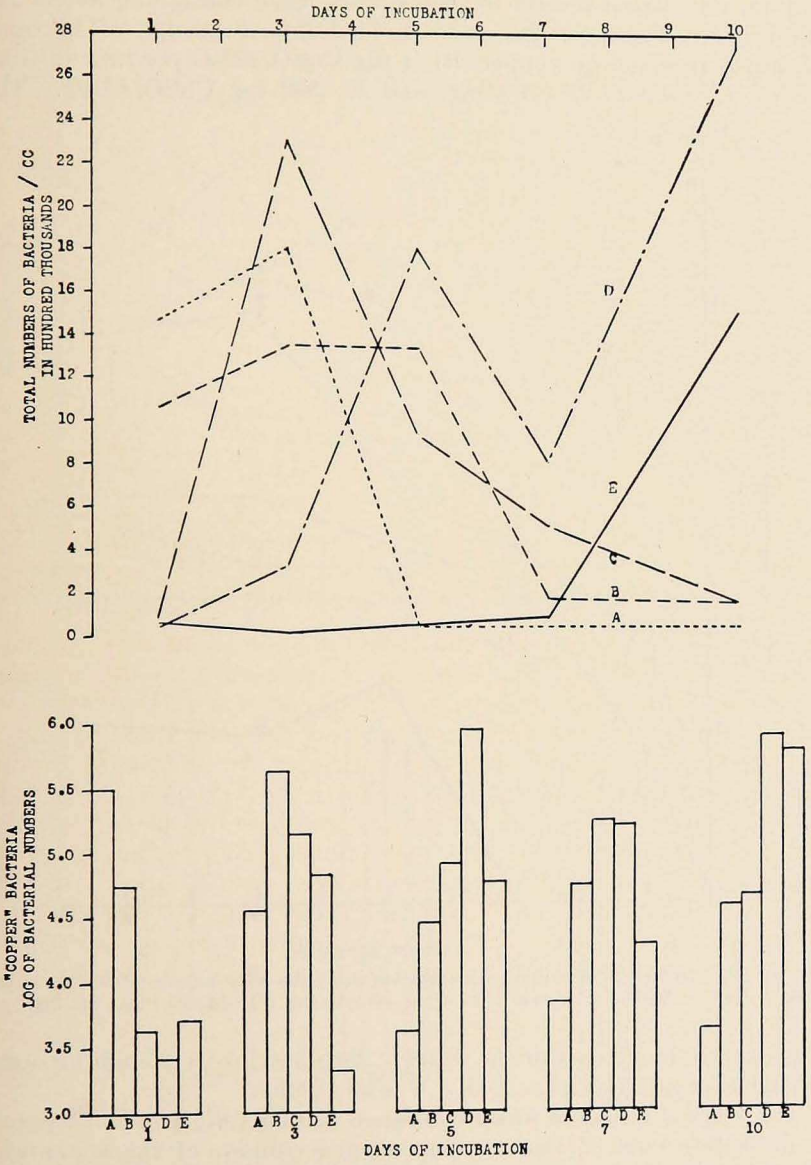

Figure 38. Influence of copper sulfate upon the total bacterial population of sea water and upon the development of "copper" bacteria. $CuSO_4.5H_2O$/L Sea water: (A.) none; (B.) 1 mg; (C.) 10 mg; (D.) 100 mg; (E.) 500 mg.

tution, was placed in fifty oxygen bottles, each containing about 150 ml water. These bottles were divided into 5 groups of 10 each. Group A received no copper, B—1 mg $CuSO_4.5H_2O$ per liter of fresh water, C—10 mg, D—100 mg, and E—500 mg $CuSO_4.5H_2O$. The

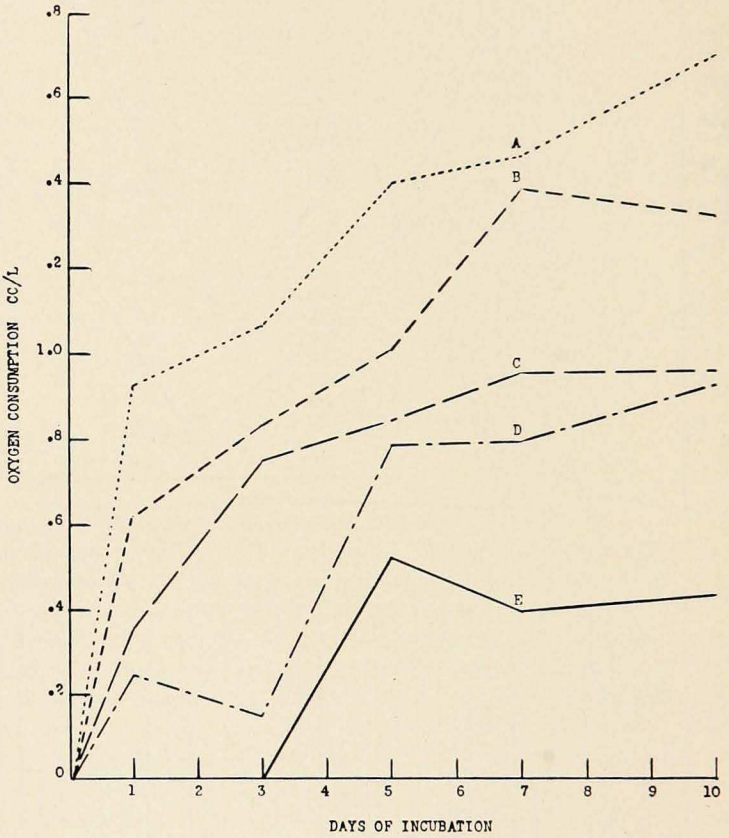

Figure 39. Influence of copper concentration upon the consumption of oxygen in sea water. $CuSO_4.5H_2O$/L: (A.) none; (B.) 1 mg; (C.) 10 mg. (D.) 100 mg; (E.) 500 mg.

bottles were incubated under water. Two bottles from each lot were removed for analysis after 1, 3, 5, 7, and 10 days.

The original fresh sea water contained 670 bacteria and 90 "copper" bacteria per 1 ml of water. The oxygen content of the water was 5.09 cc/liter. Figures 38 and 39 present graphically the results of this experiment. Here again, the addition of copper to the water had a delaying effect upon bacterial development, which was later overcome;

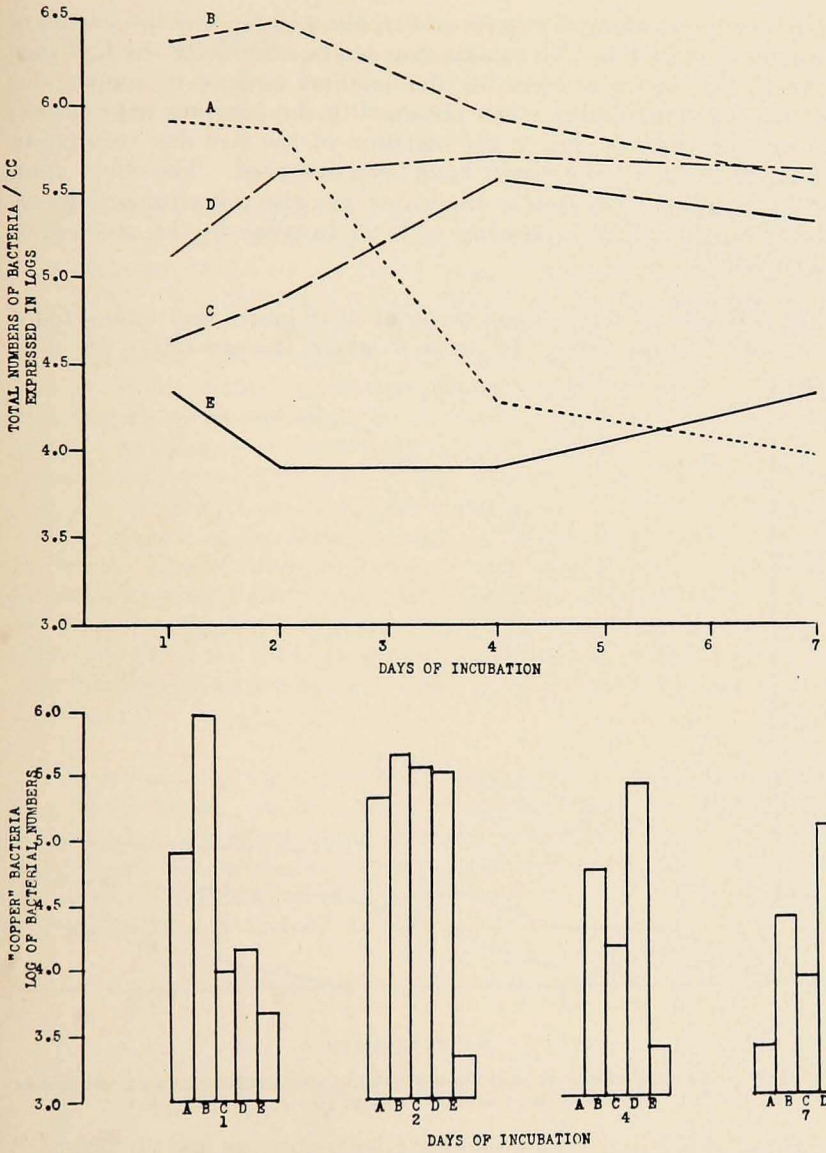

Figure 40. Influence of copper chloride upon the total bacterial population of sea water and upon the development of "copper" bacteria. $CuCl_2$/L; (A.) none; (B.) 1 mg; (C.) 10 mg; (D.) 50 mg; (E.) 100 mg.

the larger the amount of copper added, the greater was the length of the depression period. No explanation can be offered for the fact that curve B, for the water receiving the smallest amount of copper, did not show the stimulating effect obtained in the previous experiment; this may possibly be due to the omission of the two day incubation period, when such a rise might have been observed. The effect upon the consumption of oxygen in the water was also not stimulating but depressive, this effect increasing with an increase in the amount of copper added.

The influence of $CuCl_2$ *upon bacterial development and upon oxygen consumption in sea water.* In order to avoid the secondary effect of

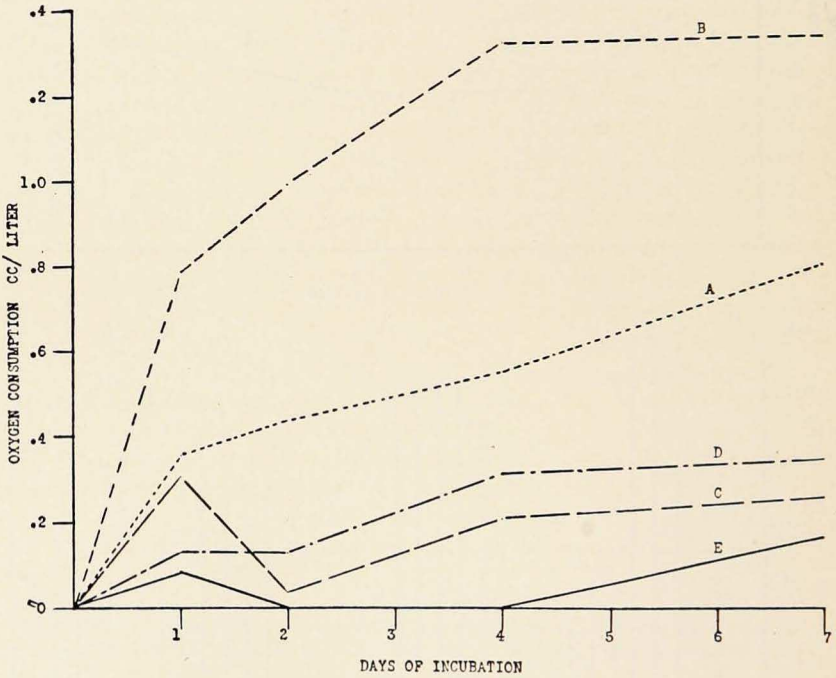

Figure 41. Influence of copper chloride upon the oxygen consumption by bacteria in sea water. $CuCl_2$/L: (A.) none; (B.) 1 mg; (C.) 10 mg; (D.) 50 mg; (E.) 100 mg.

the sulphate ion upon bacterial development, copper chloride was now employed. This experiment was carried out in a manner similar to the previous one. Five series of bottles were used. These received fresh sea water containing the following amounts of $CuCl_2.2H_2O$ per liter of water.

Series	$CuCl_2.2H_2O$ mg/L
A	0
B	1
C	10
D	50
E	200

The bottles were incubated as in the previous experiments and the bacterial numbers and oxygen consumption were determined. The bacteria were plated out on media 1 and 7, in dilutions of 1 : 1,000 and 1 : 10,000. The fresh sea water, plated out at the beginning of the experiment, gave a total of 16,960 bacteria per 1 cc of water using medium 1 and 1,840 "copper" bacteria on medium 7.

The results of this experiment (Figures 40 and 41) are very similar to those obtained with $CuSO_4$. Copper in the form of $CuCl_2$ had, in small quantities, a stimulating effect upon the growth rate of marine bacteria and especially upon oxygen consumption in the water. One mg. of $CuCl_2.2H_2O$ per liter of sea water gave a marked increase in the total numbers of bacteria as well as in the numbers of "copper" bacteria. The addition of 10 mg or more of the copper salt per liter of water showed, however, marked inhibiting effects, which tended to be overcome on further incubation. The largest amount of copper salt added had the greatest depressive effect; the period of depression was practically ended after 7 days' incubation of the water in the laboratory.

The influence of Cu_2O *on bacterial development and oxygen consumption in sea water.* A third form of copper, Cu_2O, was now used for determining the effect upon bacterial activities in sea water. This experiment was carried out in a manner similar to the previous ones. An incubation period of 14 days was used.

The water was treated as follows:

Series	Cu_2O mg/L
A	0
B	1
C	5
D	20
E	100

The fresh sea water plated out at the beginning of the experiment gave 238 bacteria on medium 1 and 10 "copper" bacteria on medium 7 per 1 ml of water. The oxygen content of fresh water was 4.99 cc/liter. This is similar to that found in the other experiments; however, when the water was tested immediately after the Cu_2O was

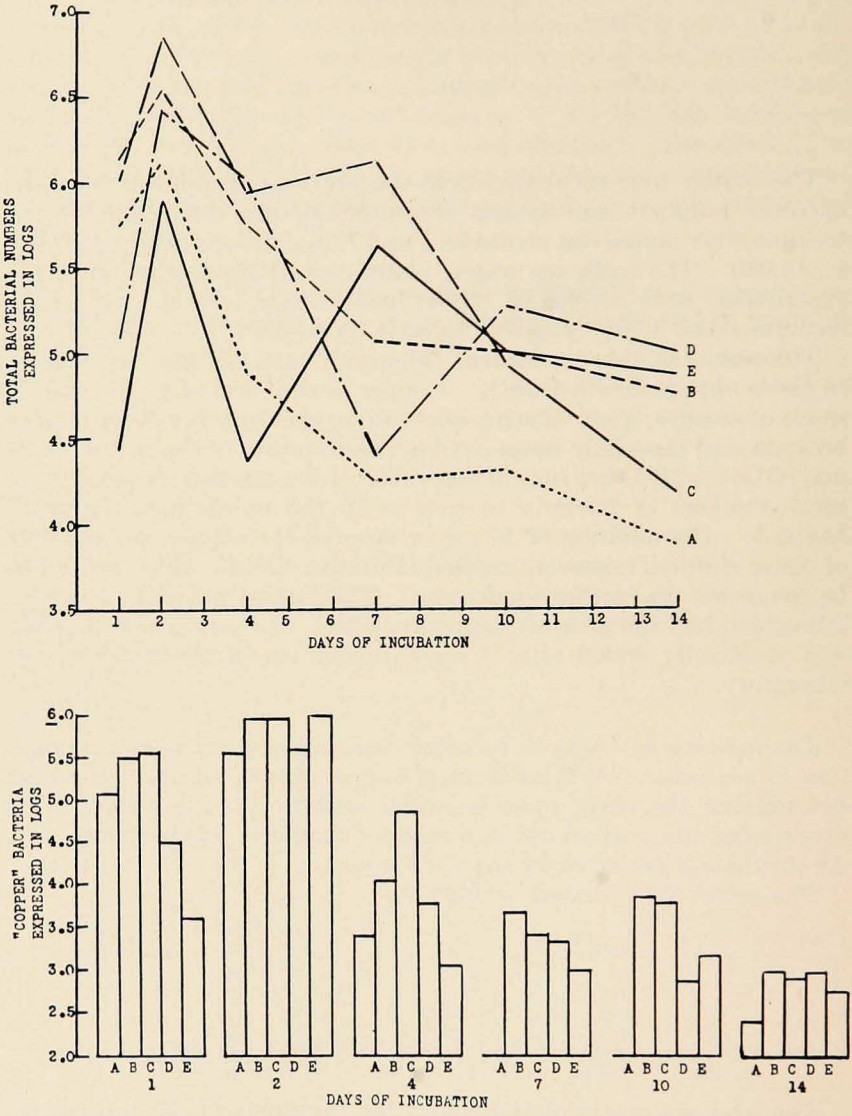

Figure 42. Influence of copper oxide upon the total bacterial population in sea water and the development of "copper" bacteria. Cu_2O/L sea water. (A.) none; (B.) 1 mg· (C.) 5 mg; (D.) 20 mg; (E.) 100 mg.

introduced, there was an immediate marked consumption of the oxygen. This was due to the oxidation of the cuprous oxide to cupric oxide. This reaction consumed approximately 1.5 cc of oxygen per 1 liter of sea water in the E series, to which the highest amount of Cu_2O was added.

This experiment was repeated in order to determine the amount of oxygen taken up chemically and biologically by the addition of Cu_2O to the fresh water. Two lots of bottles were filled with fresh sea water, one lot receiving no copper and the other receiving 100 mg of Cu_2O per liter of water. These bottles were incubated for short period of time and analyzed for oxygen. The results reported in Table II definitely point to the fact that the chemical consumption of the oxygen is immediate and does not increase on further incubation.

The results of the effect of Cu_2O on bacterial development presented in Figure 42 confirm fully the effects of the other two forms of copper, namely, at first a depression followed by a stimulating effect. The greater the amount of copper added, the longer was the depressive action. There were, however, two exceptions; first, no depressive action was observed where the two smallest amounts of copper were added as Cu_2O; second, the depressive effect of the largest amount of copper was attained with the 7 day incubation period.

TABLE II—Chemical and Biological Oxygen Consumption in Water Treated with Cu_2O

Incubation of water, hours	*Water enriched with* Cu_2O	*Unenriched water*	*Biological Consumption*	*Chemical Consumption*
Start	3.41	5.00	0	1.59
2	3.32	4.98	0.02	1.66
4	3.21	4.93	0.07	1.72
6	3.16	4.93	0.07	1.77
8	3.16	4.88	0.12	1.72
10	3.16	4.73	0.27	1.57
24	3.16	4.34	0.66	1.18 (?)
72	2.28	3.82	1.18	1.54

100 mg Cu_2O per liter of water. Data expressed in cc of oxygen per liter of water.

"Copper" bacteria in surface films and copper concentration by "copper" bacteria. A series of films removed from various surfaces which had been submerged near or at the dock of the Oceanographic Institution were examined for the total bacterial content and the percentage of "copper" bacteria. Uniform pieces of film were removed, by means of sterile swabs, suspended in sterile sea water, diluted and plated. The results (Table III) showed no particular uniformity between the total numbers and the numbers of "copper" bacteria. This may have been due to the fact that the films were of

different ages (42–170 days old) and varied considerably in thickness; the amounts of film removed from the different surfaces were also not absolutely uniform. The "copper" nature of some of the bacteria was not always recognized, since the pigmentation of the colonies and the disappearance of the pigment did not take place uniformly.

The results of these experiments point definitely to the fact that copper, in various forms, has some very definite relations to the development of bacteria in sea water. First, there is an apparent adaptation of the bacteria to the copper, whereby the latter may even exert a stimulating effect upon the development and activities of the marine bacteria, within definite limits of concentration and time. Second, there is a marked development, among the marine bacteria, of a specific population, which has been designated as "copper" bacteria; these are present in the water and are capable of developing there in very large numbers.

In order to demonstrate whether the copper is being concentrated in the bacterial pellicle, an attempt was made to produce such pellicles artificially. Sea water was enriched with the nutrients of medium 7 and placed in shallow layers in flasks. Some of these were left in an unsterile condition and others were sterilized and inoculated with some of the pure cultures of the "copper" bacteria. Typically red pellicles were produced in some of the flasks. These pellicles were carefully removed and analyzed for copper. It was found that about 8 per cent of the copper added to the medium was concentrated in the pellicles. Only a few analyses have been made so far, however, and further work is required in order to establish this point. Because of the great variability in the pigmentation of the pellicle and the limited analyses made so far, these results should be taken as merely suggestive. The oxygen tension of the medium appears to play an important role in this connection.

TABLE III—Numbers of Total Bacteria, Developing on Plate, and of "Copper" Bacteria in Comparable Units of Bacterial Films from Different Surfaces

	Medium 1 (no copper)	Medium 7 (copper present)	
No. of submerged plate	*Total numbers of bacteria*[1]	*Total numbers of bacteria*	*"Copper" bacteria*
0	428.000	57.000	8.000
10	1.295.000	361.500	28.500
14	1.680.000	124.500	20.500
20	1.095.000	102.500	7.000
25	1.150.000	114.500	12.000
28	930.000	103.500	21.500
33	400.000	107.000	4.000

[1] Per unit of surface removed by sterile swab.

Nature of the "copper" bacteria. A detailed study of the nature and physiology of the "copper" bacteria will be presented at a later date. It is sufficient to present here only some of their general characteristics in order to emphasize their specific nature.

The development of the "copper" colonies on the agar plate containing a copper salt was rather characteristic. The surface colonies behaved in this respect quite differently from the sub-surface colonies. The former produced round reddish to red-brown and even dark-red colonies; the pigment of the colonies gradually disappeared, either beginning from the center or from the periphery of the colony, until, after 5–6 days, the whole colony lost its red pigment. The sub-surface colonies kept the pigment for a longer period of time. This phenomenon did not appear to be correlated with sunlight. Whether the copper is at first assimilated or absorbed by the cell, then oxidized to give the pigmented form, and finally reduced or excreted remains to be determined. It was observed, however, that when a quantity of bacterial cell material was collected, dried and analyzed, it rapidly lost its red color and became greenish.

In order to study further the physiological characteristics, these bacteria were isolated in pure culture and cultivated on a special copper-containing medium. This medium was comparable to soft agar, thus enabling the organism to grow under partial anaerobic conditions. An extract of *Chondrus crispus* (Carragar) was used as the gel-forming agent. This medium, designated as 6b, had the following composition:

Peptone	1 gram
Glucose	1 gram
K_2HPO_4	0.05 gram
$CuSO_4.5H_2O$	0.1 gram
Carragar	6 grams
Sea water	1,000 ml.

This medium was placed in test tubes and sterilized. When the medium was allowed to set, it separated into two distinct layers; the lower layer was of a gel nature, whereas the top layer was in the form of a supernatant fluid. This enabled the bacteria to grow on the surface of the liquid; when a pellicle of sufficient thickness was produced, it dropped upon the lower or gel layer. The pellicle thus accumulated, without rapidly losing its red color, until several layers of film were formed, one on top of the other.

Colonies were picked from the various plates containing media 1 and 7 and inoculated upon this special medium. Both white and red colonies were picked from the copper-containing medium 7 and white colonies alone were picked from the copper-free medium 1.

These pure cultures were incubated in the dark at room temperature. The red color of the pellicle served as evidence of the "copper" character of the bacteria. Some cultures produced this coloration faster than others. Those colonies which were red on medium 7 gave red growth on medium 6b and those colonies that were white on medium 7, remained white on the 6b medium, whereas the white colonies picked from medium 1 gave either white or red growth on medium 6b. This indicates that the "copper" colonies are masked on medium 1 by the absence of copper in the medium; it tends to prove further that the "copper" bacteria are able to live in the absence of copper in the medium.

The cultures first isolated from colonies were replated, reisolated several times, and retransferred to media 6b and 7. Each reacted true to type. They were separated on the basis of pigmentation characteristics, size and appearance of colony. Thirteen pure cultures of "copper" bacteria were thus selected for further study.

On microscopic examination, various cultures of "copper" bacteria were found to be very small, short rods. Those taken from the pigmented colonies appeared darker and were more readily recognized than the bacteria taken from the white colonies, which had either lost their red-brown pigment or had not acquired it yet. They were found to behave alike toward the gram stains and were gram-negative organisms without any spores.

When plated out, the "copper" bacteria produced colonies both on the surface and below the surface of the agar. The surface colonies pigmented very rapidly, usually within 48 hours, and remained pigmented from two to four days. They were convex, had a very smooth edge and the surface was smooth and shiny. In losing the pigment, the colonies did so in a very characteristic manner. The pigment was gained or lost in the form of concentric zones until finally the colony no longer possessed any pigment at all. This occurred very rapidly in the surface colonies, and often only one concentric ring was noted. In the deep colonies, however, the rate of pigmentation was slower, usually requiring from four to five days to appear; the pigment remained from four to ten days depending on the number of colonies in close proximity to one another. The deeper colonies continued to produce concentric rings of red and white to the extent of seven or eight in number. The areas of white between the rings of red retained a more or less granular appearance. The reason for the occurrence of the rings is not definitely known, but it is thought that the copper nearest the youngest cells, the periphery of the colony, is responsible for the pigmentation, and, as the colony enlarges, this area is no longer adjacent to the fresh supply of copper, and becomes white again.

In the case of streaks of the bacterial cultures, grown on medium No. 7, the pigmentation occurred at the base, the culture continuing to grow along the entire streak. The growth was usually shiny and echinulate in appearance. In time, it became tough and leathery, as did the colonies as well. The culture remained alive but it could be transferred only by removing the whole colony or a mass of growth from the agar. The cultures of the "copper" bacteria died in a relatively short time under aerobic conditions, but not under reduced oxygen pressure. For this reason, the pure cultures are kept under partial anaerobic conditions. This can be accomplished by using deep tubes of soft agar or carragar medium.

CONCLUSIONS

1. The presence of 100 mg $CuSO_4.5H_2O$ in a sea water medium allowed the development of certain red-brown pigmented colonies, from sea water and from bacterial films taken from submerged surfaces. No such colonies were obtained from fresh water or from soil by the use of fresh water media containing the same copper salt.

2. The addition of various forms of copper to sea water had at first a depressing effect upon bacterial development and oxygen consumption. This effect was later overcome, and with time a stimulation resulted. The rapidity of adjustment of the bacteria to the addition of copper depended upon the amounts of copper added to the water. High concentrations often proved toxic to a point where the majority of the bacteria were unable to become adjusted, at least within the short experimental period employed.

3. The consumption of oxygen in sea water by bacteria was parallel to the growth rate of the bacteria, as influenced by the amounts of copper added to the water.

4. Sea water and surface films were found to contain bacteria that have a definite affinity for copper, since they are able to withstand concentrations of 500 mg $CuSO_4.5H_2O$ per liter of medium.

5. When grown upon media containing copper, the "copper" bacteria produce colonies pigmented a red-brown color, whereas no color is noted when the same bacteria are grown in the absence of copper. These "copper" bacteria were also grown in the presence of various forms of copper, including $CuSO_4.5H_2O$, $CuCl_2.H_2O$ and Cu_2O.

6. The pigmentation of the surface "copper" colonies was observed on the plates within 48 hours incubation; it persisted for two to four days. The pigmentation of submerged colonies appeared within four or five days' incubation of the plates and lasted from four to ten days.

7. A number of cultures designated as "copper" bacteria and producing red pigmented growth on media containing copper in the form of $CuSO_4.5H_2O$ were isolated. These bacteria were all small, non-spore forming, gram-negative rods.

8. The red-brown pigment in the sub-surface colonies persisted for a long time; however, in surface colonies it rapidly disappeared, in a characteristic manner, namely through the formation of concentric colorless zones which gradually turned the whole colony colorless.

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