TOXIC EFFECTS OF COPPER ON ATTACHMENT AND GROWTH OF BUGULA NERITINA [1]

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Introduction

This paper describes physiological experiments dealing with the effect of copper on attachment and early development of *Bugula neritina* (L.), a widely distributed marine fouling organism. The studies were designed to gain a clearer understanding of the specific role of copper in prevention of fouling and the mechanism of action of copper paint surfaces. Various experiments using copper solutions and paints were made to gain an integrated concept of copper toxicity.

Copper, in one form or another, has been used since ancient times to prevent fouling and is one of the most effective metallic poisons for marine fouling organisms (Visscher, 1927). The antifouling efficiency of metallic poisons is related to the intrinsic toxicity of their ions and the solubility of their corrosion products in sea water, both relatively high for copper (Parker, 1924). Recently, this concept has been clarified and extended by Ketchum, Ferry, Redfield, and Burns (1945) who show that the prevention of fouling is related to the concentration of toxic dissolved in the water at the surface of the paint, and that the rate of loss of toxic is a measure of the steady-state concentration in a narrow zone at the paint surface. The rate of loss of toxic from a paint surface is known as the leaching rate. The authors show that in the case of copper paints, a leaching rate maintained consistently above a minimum of 10 micrograms of copper per square centimeter per day will prevent serious fouling.

Much discussion and some experimentation has centered around the question of the relative toxicities of different forms of copper. Carritt and Riley (1943), however, were unable to demonstrate any difference in toxicity to *Bugula turrita* between cuprous and cupric ions, while Clarke (1943) found that "all forms of copper have roughly the same toxicity but differ greatly in their solubilities."

The establishment of fouling organisms on a submerged surface depends *a priori* on their ability to affix themselves and to develop after attachment. Effective antifouling surfaces, therefore, must function in one or both of two ways, by repelling (or killing) larvae of fouling organisms, or by inhibiting development of any that attach. Visscher (1927), Neu (1932), and Edmondson and Ingram (1939)

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conclude from indirect evidence obtained mainly in field studies that toxic paints have no effect on growth of fouling organisms once attached, that they function only by repelling or delaying attachment. Without minimizing the repellent action of antifouling paints, evidence is accumulating to show that toxic surfaces function importantly through inhibition of growth of attached larvae. The ingenious experiments of Pomerat and Weiss (1943) showing inhibition of fouling growth on unpainted areas by adjacent toxic paint surfaces strongly support this view. Similar "distance effects" of antifouling paint surfaces on fouling growth on adjacent untreated areas are frequently seen in panel tests (e.g., Edmondson, 1944, Fig. 7a, p. 13).

Toxic effects of copper on various fouling organisms have been described by Bray (1924), Whedon *et al.* (1942 and 1943), Miller and Cupp (1942), Clarke (1943), and Riley (1943) in unpublished reports to the Bureau of Ships, U. S. Navy Department. Prytherch (1934) reports stimulating effects of small amounts of copper on settling and metamorphosis of the oyster, and similar oligodynamic effects on ascidians were observed by Grave and Nicholl (1939).

Jones (1941) reviews theories on the mechanism of toxic action of metals, notably copper. These may be divided into two main groups which maintain that: (1) copper retards vital processes through inactivation of essential enzymes, and (2) copper acts more directly by precipitating cytoplasmic proteins as copper-proteinates. With regard to fouling organisms, Clarke's (1943) studies on barnacles and mussels, and Riley's (1943) investigations and those of the present author on bryozoans favor the inactivation theory. Further studies are needed, however, to elucidate the physiological mechanisms involved.

The results of the present investigation will be presented in four parts: (1) attachment and growth of *Bugula* on copper paint surfaces, (2) growth of *Bugula* in copper solutions, (3) recovery of *Bugula* from copper poisoning, and (4) toxicity gradients of copper paint surfaces.

Materials and Methods

Bugula larvae for these studies were obtained as follows. Mature colonies were collected from docks, piling, etc., in San Diego Bay the day before tests were made, and placed in aquaria of sea water in the laboratory. Early the next morning, these liberated swarms of larvae which were easily collected at the light side of the container since they are positively phototropic and visible to the naked eye (about 0.2 mm. in length and darkly colored). *Bugula* larvae normally attach and begin to grow before mid-afternoon of the day on which they are liberated as described by Grave (1930) and Edmondson (1944). In subtropical localities, as San Diego Bay, larvae may be obtained the year around. From the foregoing facts, it is obvious that this form is remarkably suited for studies on fouling problems.

Primed steel, 1 × 3 inch panels were coated with the various experimental and control paints employed in the first section of this paper. Larger panels (10 × 12 inches) were similarly prepared and exposed in the bay to test their antifouling efficiency under field conditions. In most cases, the small panels were "seasoned" before testing by soaking them for a month or more in large jars of sea water. The seasoning bath was changed at frequent intervals and aerated by means of a stream of air bubbles delivered at the base of the container by means of a tapered glass tube.

For other studies, larvae were allowed to attach to small test panels which were coated with a non-toxic paint composed of equal parts of paraffin and ester gum. So attached, they could conveniently be transferred to the experimental situations or be removed therefrom for observations.

Other procedures will be described as necessary in the various sections of the investigation.

Attachment and growth of Bugula on copper paint surfaces

The first problem was to determine the effect of actual copper paint surfaces on attachment and growth of *Bugula* larvae under simulated natural conditions. The data given are typical of many such tests that have been performed.

TABLE I

Attachment and growth of Bugula larvae on hot and cold plastic paints, each series with graded copper content. (Paints seasoned for two months in sea water bath before tests were made.)

Series	Paint No.	Percentage Cu_2O in dry paint	Leaching rate (2-mo.) μg Cu/cm.²/day		Per cent larval attachment ± S.E.		Growth (3-day) Maximum length [1] (mm.)	
			Filmed	Clean	Filmed	Clean	Filmed	Clean
Hot plastic	1	37.0	28.1	24.1	4±1.6	2±1.1	0.3	—
	2	32.0	19.4	23.7	23±5.3	15±4.4	0.3	0.3
	3	26.0	14.0	14.0	5±3.3	13±6.3	0.2	0.2
	4	19.0	13.5	11.5	79±3.5	72±5.0	0.2	0.2
	5	10.6	6.9	5.8	86±4.4	95±2.6	0.4	0.6
	6	0	—	—	100	100	1.3	1.2
Cold plastic	7	36.0	14.3	14.1	0	3±2.4	—	0.2
	8	30.0	14.1	13.3	5±3.0	1±1.4	0.2	0.2
	9	19.2	14.1	13.0	7±2.4	10±4.5	0.3	0.2
	10	9.3	3.5	3.8	87±3.6	73±6.8	0.6	0.7
	11	0	—	—	78±7.4	98±1.4	1.1	1.1

[1] As attached larvae are approximately 0.2 mm. in length, maximum growth increments may be calculated by subtracting this value from the maximum lengths given above.

Two series of copper paints (one hot plastic and one cold plastic series)[2] were prepared, each having the same matrix composition but graded amounts of cuprous oxide (Table I). Two test panels were coated with each of these paints and seasoned for ten weeks. Prior to testing, one panel in each duplicate set was wiped to remove the slime film that accumulated during seasoning; the other was used without being cleaned.

At the start of the attachment test, the panels were immersed in jars containing 700 cc. fresh sea water and immediately thereafter a known number of *Bugula* larvae (usually 100) were added. Tests were always started in mid-morning when the larvae were active, and counts of the number attached were made late in the after-

[2] A hot plastic paint is one that is applied hot and forms a solid film on cooling; cold plastic paints are one of the types of paints which dry by evaporation of the solvent. The paints used in these experiments were experimental modifications of standard Navy formulations.

noon when all larvae were either attached or dead. The percentage of attachment was calculated by dividing the number of larvae affixed to the test panel by the total number of larvae used.

Data on development, which involves both growth and differentiation, were obtained by measuring the length from base to apex of the young *Bugula* stalks, and by determining the number of polypides developed from time to time. Length measurements were made with an ocular micrometer under low magnification.

The results of the attachment and growth tests on the various copper paint surfaces are given in Table I and in Figures 1 and 2. The data clearly segregate two groups of paints. Those with less than 15 per cent Cu_2O content or leaching rates less than 10 micrograms of copper per square centimeter per day are obviously inferior as over 70 per cent of the larvae were able to attach to them *and* to grow significantly. These paints foul in a more or less short period of time in field tests. The second group, with greater copper content and concomitantly higher leaching rates, were significantly more efficient in repelling attachment and permitted no significant growth. These paints are more effective in the field, the length of their effective periods being related to their copper content.

That leaching rate rather than copper content is responsible for the antifouling performance of a paint can easily be demonstrated by comparing *Bugula* attachment and growth on surfaces with identical amounts of cuprous oxide but different leaching rates. If, for example, ester gum is substituted on an equal weight basis for rosin as the resinous component in our hot plastic series, leaching rates fall far below the adequate level and *Bugula* larvae attach abundantly and develop colonies on such surfaces. Ketchum *et al.* (1945) have shown that substitution of ester gum, esterified Albertol or coumarone-indene for the rosin content of an effective antifouling paint (Navy Department Specification 52-P-161) drastically reduces the leaching rate below the critical level with the result that such paints foul quickly in the field. Thus, copper content is important only insofar as it contributes to an adequate and sustained leaching rate, and may be neglected in the subsequent discussion.

In these tests, the slime film on the various paint surfaces had no consistent effect on larval attachment. Its presence apparently is not necessary for attachment of *Bugula* since larvae affixed themselves to cleaned panels as well as those with a coating of slime film, and since they readily attach themselves to unseasoned surfaces shortly after their initial submergence and certainly before any visible film forms. Whether or not an invisible film is prerequisite to attachment is an academic question. In the tests cited, the larvae had no choice between surfaces with and those without slime film. When such choice is involved, *Bugula* larvae do exhibit certain preferences (Whedon *et al.*, 1943). The role of the slime film on attachment to toxic and non-toxic surfaces under various experimental conditions will be considered in another paper.

Larval attachment to paints with leaching rates above 15 micrograms of copper per sq. cm. per day ranged between zero and 23 per cent with an average of less than 10 per cent. None of these was able to grow, however, and hence to establish a colony. The range of leaching rates between 10 and 15 μg. per sq. cm. per day may be regarded as a "marginal zone," especially as the lower value is approached, since paints with leaching rates in this range often permit considerable attachment of larvae. These may or may not be killed. If not, as will be shown later, some of them might recover and grow should the leaching rate and subsequent surface concentration of toxic fall below the adequate growth inhibiting level. Even if the attached larvae are killed, however, there is still the possibility that their dead bodies might form a less toxic substrate for later larvae than the paint surface

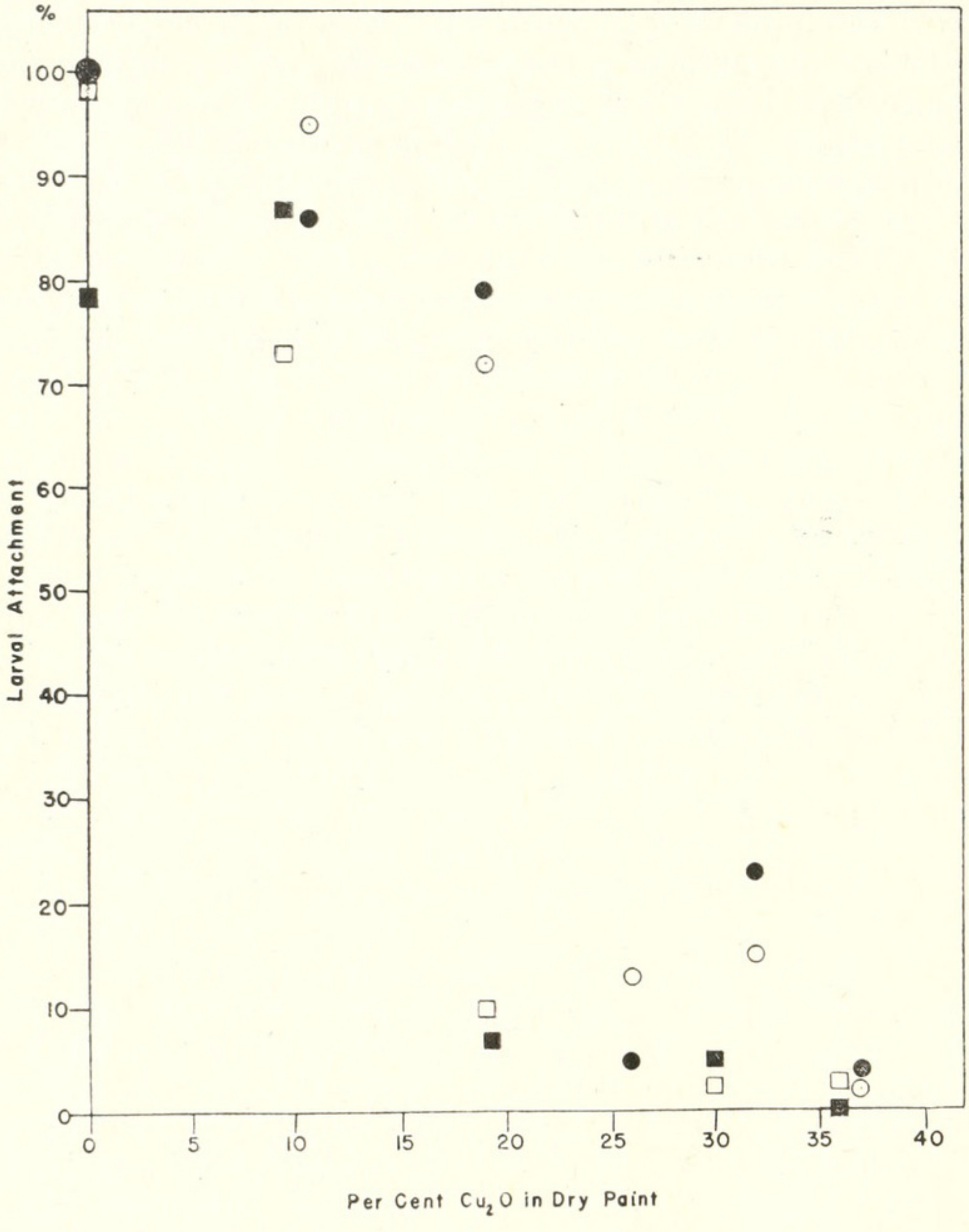

FIGURE 1. Attachment of *Bugula* larvae in relation to copper content of paint. Circles represent hot plastic paints while squares indicate cold plastic paints. Black-filled data points represent paint surfaces coated with slime film while clear data points represent cleaned surfaces (wiped before testing). All paints were seasoned for ten weeks in sea water before testing.

itself. Larvae frequently attach themselves on top of other larvae, often in clusters. Conceivably, those underneath might take up the toxic ions diffusing from the paint or in other ways shield the overlying larvae so that the latter could develop. Certainly, those on top would be further removed from the paint surface and hence would be located in a less concentrated part of the toxic zone which might permit their development. While this sort of attachment is obviously not very secure, firmer attachment may eventuate by means of stolonic outgrowths from developing colonies to the paint surface. Such root-like processes are developed by erect bryozoans and may give rise to secondary colonies.

The fact that at least a small percentage of larvae attach at any one time to quite toxic surfaces indicates that under field conditions the growth inhibiting func-

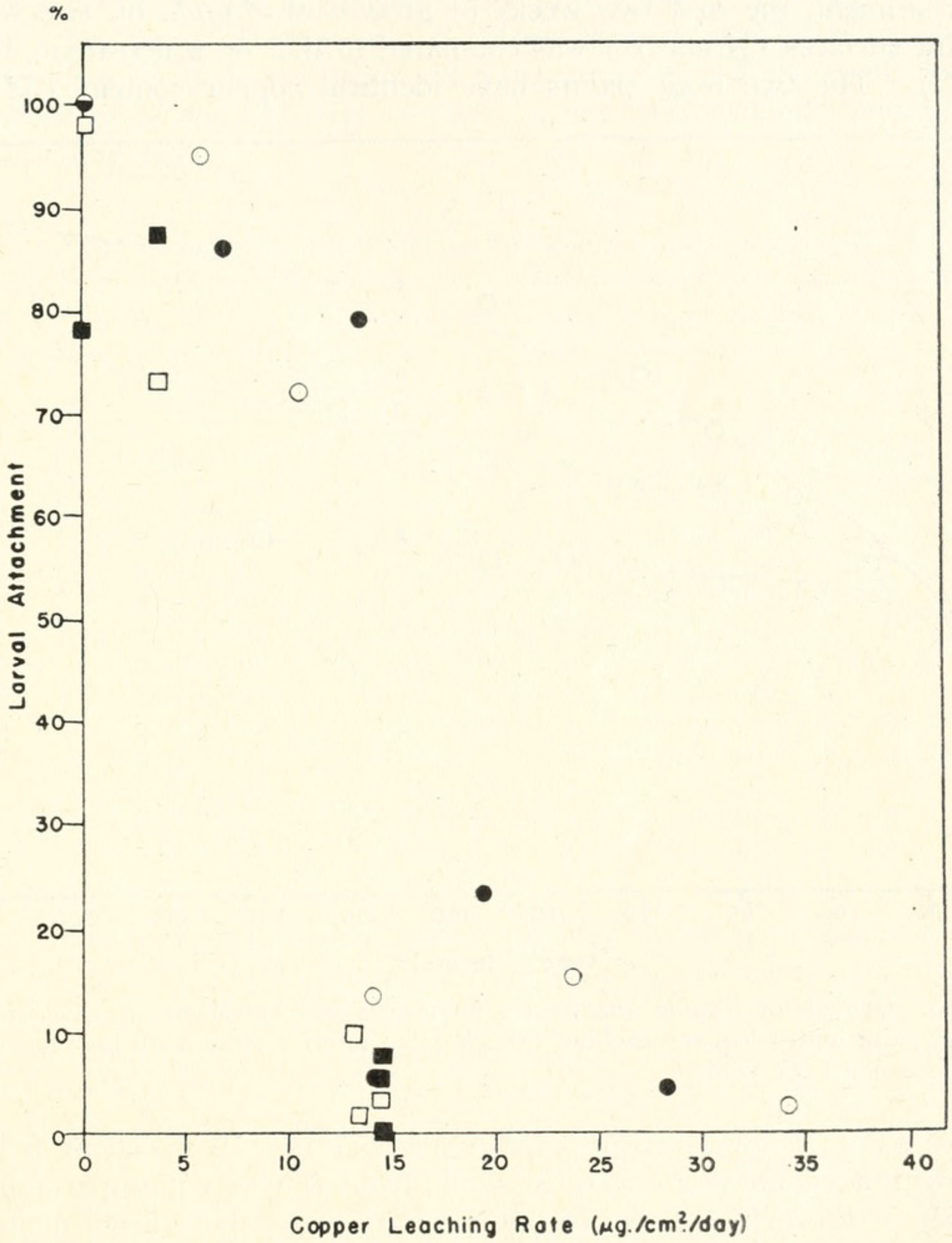

FIGURE 2. Attachment of *Bugula* larvae in relation to copper leaching rate. Symbols as in Figure 1.

tion of toxic surfaces plays an important role in the prevention of fouling. Effective antifouling surfaces must function in part by stopping development of attached larvae. This is contrary to the previously mentioned views of Visscher, Neu, and Edmondson and Ingram. The latter authors report a restricted growth of *Bugula* and serpulid worms on toxic surfaces exposed in Hawaiian waters, but attribute this to "delayed attachment rather than to a slower rate of growth after settling."

Dates of attachment in their tests are not known, and hence rates of growth on treated and untreated surfaces cannot be calculated and compared from their data. In view of the present studies, it would seem that the alternate interpretation of a growth retarding effect is equally plausible, if not more probable.

To illustrate further the growth retarding effect of toxic surfaces, another experiment involving growth of *Bugula* on unseasoned copper paint surfaces may be cited. In this experiment, the first two weeks of growth of *Bugula* on two hot-plastic, copper-paint surfaces (*A* and *B*) was compared to that on a non-toxic, hot-plastic control (*C*). The two toxic paints have identical copper content (32 per cent

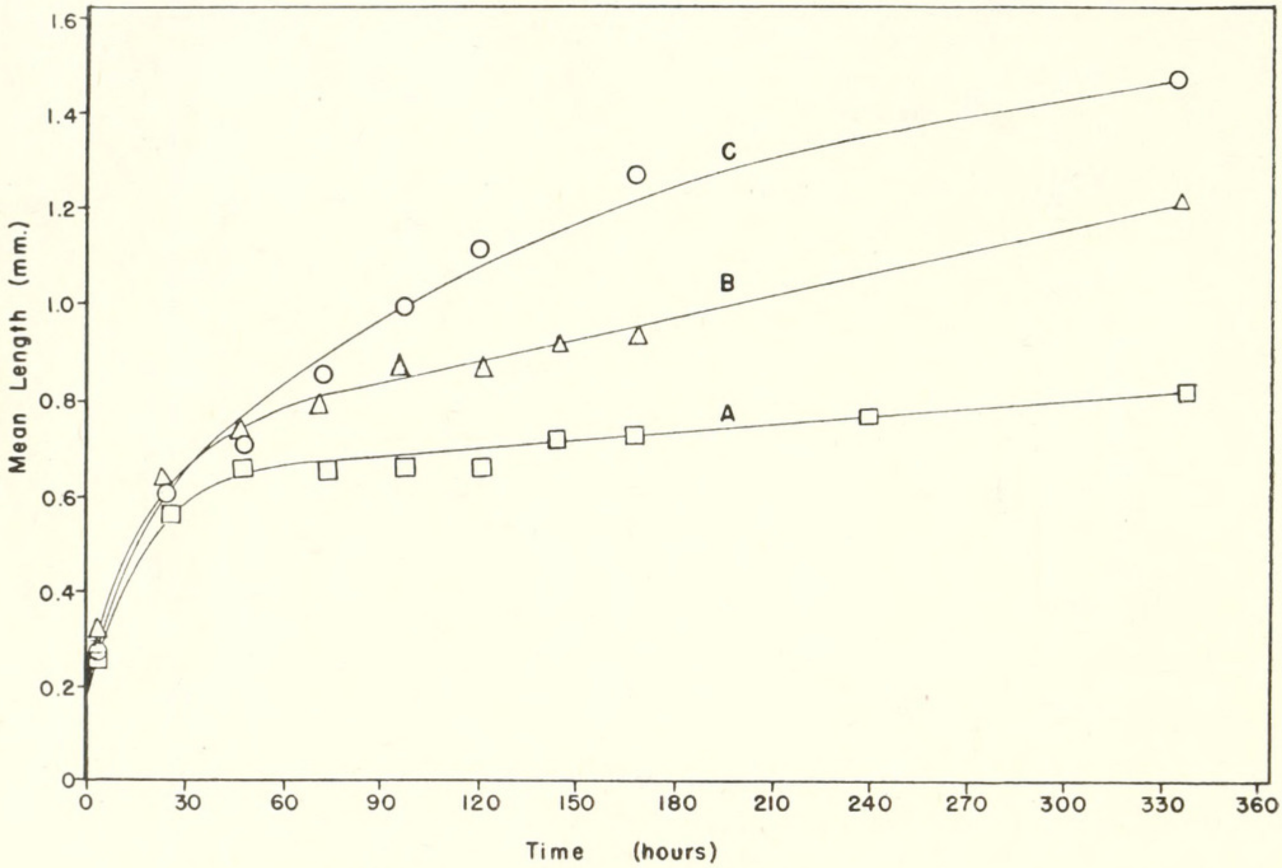

FIGURE 3. Growth of *Bugula* ancestrulae on unseasoned hot-plastic paints. *A* is an excellent copper paint with adequate leaching rate, *B* is an inferior paint with low copper leaching rate, and *C* is a non-toxic control.

Cu_2O), but paint *A* (w.w. rosin-paraffin matrix) has an excellent field record correlated with adequate leaching rates, while paint *B* (ester gum-paraffin matrix) fouls quickly in the field because of low leaching rate. Hot-plastic paints of the type here used have low initial leaching rates and do not develop characteristic leaching rates until they have been submerged for a period of time. Hence, *Bugula* larvae attach abundantly to unseasoned panels coated with these paints and begin to grow normally on them.

As shown in Figure 3, the growth curves in the present experiment do not begin to diverge significantly until after the second day. Growth on paint surface *A* was practically stopped at this time at the first polypide stage. Some individuals developed the first polypide, most did not, and no colonies were established. Colonies were developed on paint surface *B*, but growth and differentiation were significantly less than for controls. The three curves become more and more divergent with time.

The foregoing differences between growth inhibiting properties of paints *A* and *B* must be attributed to differences in their leaching rates as their copper content and growth of *Bugula* on their respective controls are identical. Repeated leaching rate determinations of these paints have shown both to have low initial leaching rates (which accounts for the initial similarities in the *Bugula* growth curves for the two surfaces) but paint *A* eventually develops an adequate leaching rate, while leaching rates of paint *B* do not attain the adequate level. Thus, again the growth retarding property of copper paint surfaces is demonstrated and associated with the copper leaching rate.

Table II

Effect of copper on growth and development of Bugula ancestrulae

Av. copper concentration mg./liter	Growth and differentiation in copper solutions				
	Mean length increments ± S.E.			Polypides per colony	
	1 day	3 days	5 days	3 days	5 days
	mm.	mm.	mm.		
Controls	0.36±.007	0.61±.016	0.77±.021	1–2	1–2
0–.05	0.30±.015	0.55±.013	0.63±.018	1	1–2
0.05–.10	0.26±.009	0.37±.010	0.46±.012	1	1–2
0.10–.15	0.25±.007	0.36±.008	0.48±.013	1	1
0.15–.20	0.14±.008	0.22±.009	0.24±.011	0–1	0–1
0.20–.25	0.08±.007	0.10±.008	0.12±.010	0	0
0.25–.30	0.02±.007	0.03±.008	0.02±.009	0	0
0.30–.35	0.04±.011	0.03±.011	0.03±.009	0	0
0.35–.40	–0.03±.008	0.01±.009	0±.008	0	0
0.40–.45	0.03±.007	0.02±.011	–0.03±.010	0	0
0.45–.50	0.02±.008	0.03±.010	0.03±.010	0	0

The experiments cited in this section are in close accord with results obtained by Ketchum *et al.* (1945) and substantiate the leaching rate theory of action of anti-fouling paints. It is noteworthy that their estimate of minimum adequate copper leaching rate (10 micrograms per sq. cm. per day), which was based on comparisons of numerous leaching rate determinations with field exposure tests, is confirmed by the *Bugula* attachment and growth tests. Data presented in this section show that copper paints with leaching rates less than this value permit larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 10 micrograms per sq.cm. per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms of copper per sq. cm. per day, but these do not develop into colonies.

Growth of Bugula in copper solutions

To determine more precisely the effect of copper ions on growth of *Bugula*, non-toxic panels bearing attached larvae (about 25) were immersed in sea-water solutions of graded copper concentrations. Cuprous oxide was the salt used in making the copper solutions, but the ions were presumably cupric since the oxidation of cuprous ion is supposedly rapid. The young *Bugulae* were measured just before immersion in the experimental solutions and at one, three, and five day intervals thereafter. Polypide development was also observed at these times. Mean length increments after immersion were computed as an index of toxicity of the solutions.

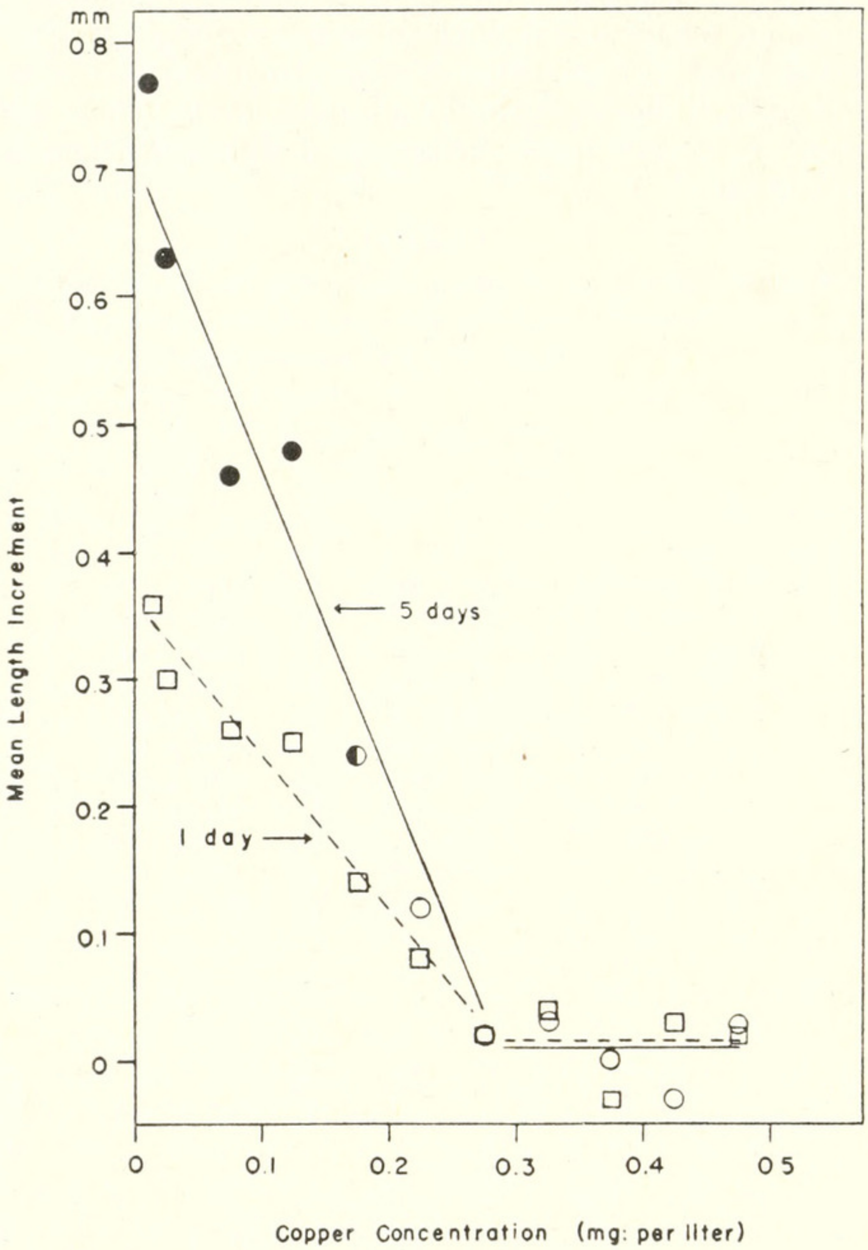

FIGURE 4. Growth of *Bugula* ancestrulae in relation to copper concentration. Curves for one-day (squares) and five-day increments (circles) based on grouped data (class intervals = 0.05 mg. copper per liter). Filled squares indicate one or more polypides per colony, half filled squares show that some stalks did not differentiate polypides, and open data points indicate that no polypides were developed. See also Figure 5.

The copper concentrations of each solution were determined before and after the experiment, and the average values were used.

As clearly shown in Table II and Figures 4 and 5, the degree of early growth and differentiation of *Bugula* is closely related to the copper concentration in the water surrounding the organisms. Up to about 0.3 mg. per liter, increment in length is inversely proportional to copper concentration of the solution. Higher concentrations inhibit growth completely or allow but slight, insignificant increment. The sharp break or inflection in the curves in Figures 4 and 5 at about 0.3 mg. copper per liter apparently marks this as a critical concentration for growth. No stimulating effect of small amounts of copper on growth was noted, since even in the greatest dilutions used growth increments were less (with minor exceptions) than in fresh sea-water controls.

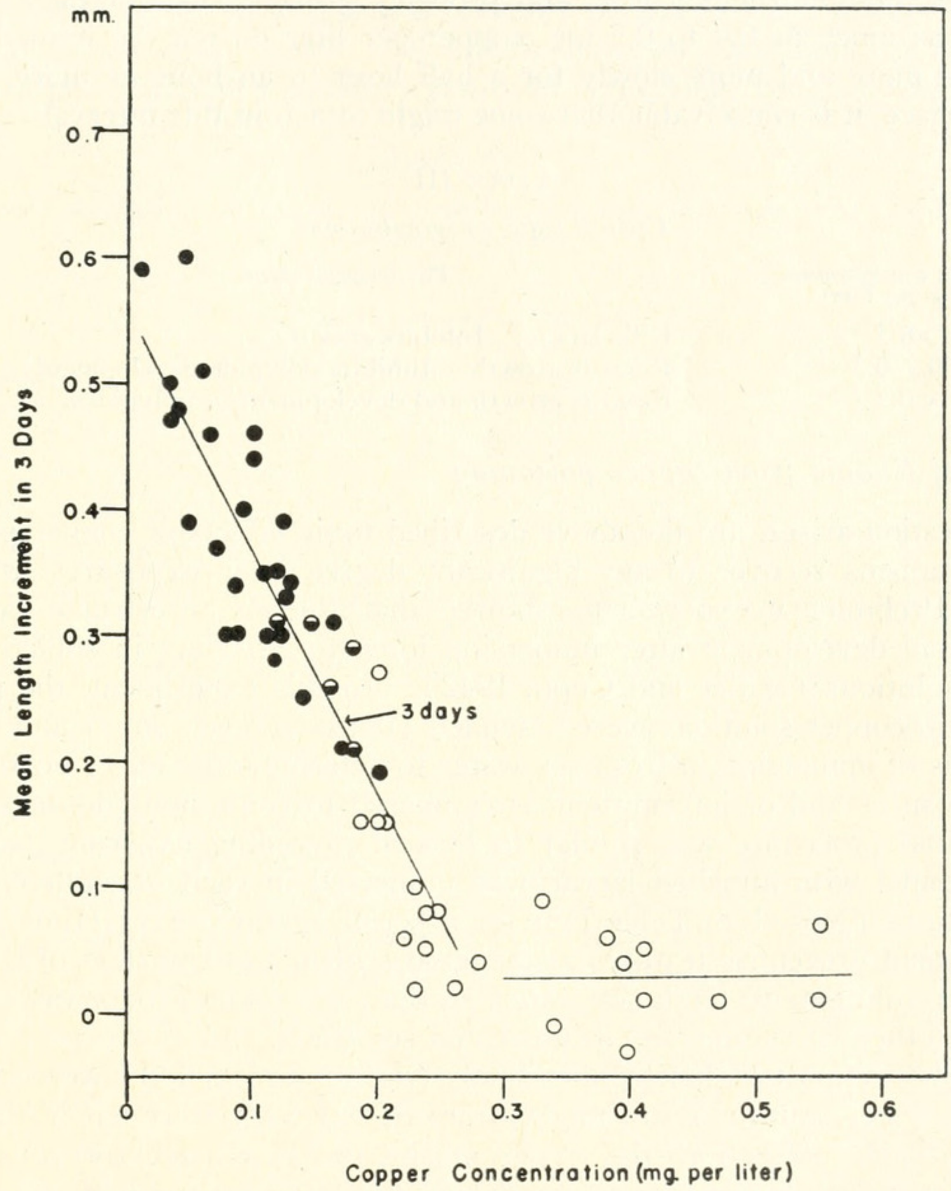

FIGURE 5. Three day growth of *Bugula* ancestrulae in relation to copper concentration. Black circles indicate fully developed polypides, half filled data points represent partly formed polypides, and clear circles indicate no differentiation of polypides. See also Figure 4.

Polypide differentiation is inhibited by copper concentrations greater than 0.2 mg. per liter, the minimal value being less than that required to prevent growth completely. Concentrations less than 0.2 mg. per liter only delay polypide development. This is shown by the fact that only controls attained the second polypide stage at three days (Table II), by the several cases of incomplete or non-functional polypides at three and five days in the range between 0.1 and 0.2 mg.

per liter (Figs. 4 and 5), and by the fact that at five days only the first polypide was developed in concentrations greater than 0.1 mg. per liter.

A series of critical copper concentrations may be postulated from these and other studies (Table III). The minimum lethal dose (MLD) for free swimming larvae is about 0.3 mg. per liter (Miller and Cupp, 1942), the same concentration required to stop growth of attached larvae. No data are available on concentrations necessary to prevent larval attachment. Presumably these would be at least as great as the minimum lethal dose, and probably greater, since larvae in solutions containing as much as 0.4 to 0.5 mg. copper per liter do not die immediately but swim about more and more slowly for a half hour to an hour or more. Given a suitable surface, it is conceivable that some might attach in this interval.

TABLE III

Critical copper concentrations

Copper concentration (mg. per liter)	Physiological effect
>0.3	Kills larvae. Inhibits growth.
0.2–0.3	Retards growth. Inhibits polypide development.
<0.2	Retards growth and development of polypides.

Recovery of Bugula from copper poisoning

The question arises, are the above-described toxic effects of copper permanent, or can organisms recover to any significant degree after exposures to sublethal dosages? Preliminary experiments showed that *Bugula* ancestrulae can recover nearly normal development after immersion for 6 to 24 hours in sublethal copper sea-water solutions (Miller and Cupp, 1942). In this experiment, the periods of immersion in copper solutions were extended (three to seven days) and alternated with periods of immersion in fresh sea water to determine the effect on recovery of longer exposures and of intermittent as compared to continuous dosages.

The initial procedure was similar to that of preceding experiments. A non-toxic test panel with attached larvae was immersed in each of eight copper sea-water solutions (Nos. 1–8, Table IV) for a period of three days (time period I). The subsequent procedure features a staggered schedule of transfers of the animals from copper solutions to fresh sea water, so that, while one group was exposed to copper, the other was immersed in untreated sea water and *vice versa*. After the first three days exposure, half of the panels (group *A*, sets 1–4) were transferred to fresh sea water, and the remainder (group *B*, sets 5–8) were left in the original copper solutions. After four days (period II), group *A* panels were transferred from sea water back to the original copper solutions, while group *B* sets (that had now been in the toxic solutions for a week) were transferred to fresh sea water. After another four days (period III), the process was again reversed—those in sea water were transferred back to copper solutions and *vice versa*. The experiment was terminated on the fifteenth day, the final four days constituting period IV.

Length measurements were made before the original immersion (a few hours after attachment of the larvae), at the time of transfers (on the 3d, 7th, and 11th days), and at the termination of the test on the 15th day. From these data, the rates of growth relative to that of controls for the four time periods in copper solutions and sea water were computed. Concentrations of the copper solutions were estimated from their biological effects (using curves in Figs. 4 and 5) as chemical determinations were not made for this test. Data are given in Table IV, and the salient features are shown in Figure 6.

In all but one case, transfers to fresh sea water were followed by marked and significant increases in growth rate and polypide differentiation, while the reciprocal transfer resulted in sharply decreased, if not completely inhibited, growth (Fig. 6). The exceptional case (set 7) presumably received a lethal dose in the first period as no significant increases in length and certainly no differentiation were observed following transfer to fresh water. All others, however, exhibited an amazingly high degree of plasticity and regulatory ability in recovering from the effects of the

TABLE IV

Growth and development of Bugula ancestrulae during and after immersions in copper sea-water solutions

Symbol No.	Cu. in solns. mg./liter	Mean length increments, time periods I–IV *				Total no. of polypides per colony at end of periods:			
		I (3 days) mm.	II (3–7 days) mm.	III (7–11 days) mm.	IV (11–15 days) mm.	I	II	III	IV
A-1	0.26	0.05	0.34	0	0.14	0	1	1	2
-2	0.27	0.04	0.22	0.01	0.09	0	1	1	2
-3	0.24	0.09	0.27	0.10	0.14	0	1	1	2
-4	0.23	0.11	0.25	0.09	0.06	0	1	1	2
B-5	0.25	0.08	0.01	0.12	−0.02	0	0	1 ab.	1
-6	0.23	0.10	0.01	0.17	−0.02	0	0	1 ab.	1
-7	0.3	0.03	0.03	0.05	0	0	0	0	0
-8	0.20(?)	0.14	0.16	0.23	0.10	1	1	1	2
Control		0.68 (0.68)	0.64 (0.53)	0.11 (0.28)	0.25 (0.19)	1	2	3	4

* Group *A* immersed in copper solutions during periods I and III, and in fresh sea water during periods II and IV. Group *B* immersed in copper solutions during periods, I, II, and IV, and in fresh water during period III.

poison. Even those immersed an entire week in solutions that allowed but slight growth increment and no visible differentiation (e.g., sets 5 and 6) were able to recover significantly, from growth rates of zero to as much as 60 per cent that of controls, when restored in fresh sea water. They also differentiated polypides though these were slightly abnormal in some instances. Set 8 in group *B* was apparently exposed to a weaker solution than any of the others since these individuals not only grew considerably but also developed polypides even while immersed in the copper solution. Nevertheless, significant increases and decreases in relative growth rates for this set were observed in the various periods. Seemingly, one could control rate of growth and differentiation almost at will by appropriate exposures to copper. The ability of young *Bugulae* to recover each time after repeated and long immersions in rather toxic copper solutions is truly remarkable.

In no case was recovery complete as judged either by growth rates or polypide development. Growth rates after transfers to sea water ranged roughly between a third and four-fifths of the normal (see Fig. 6), and the number of polypides developed was less than that of controls.

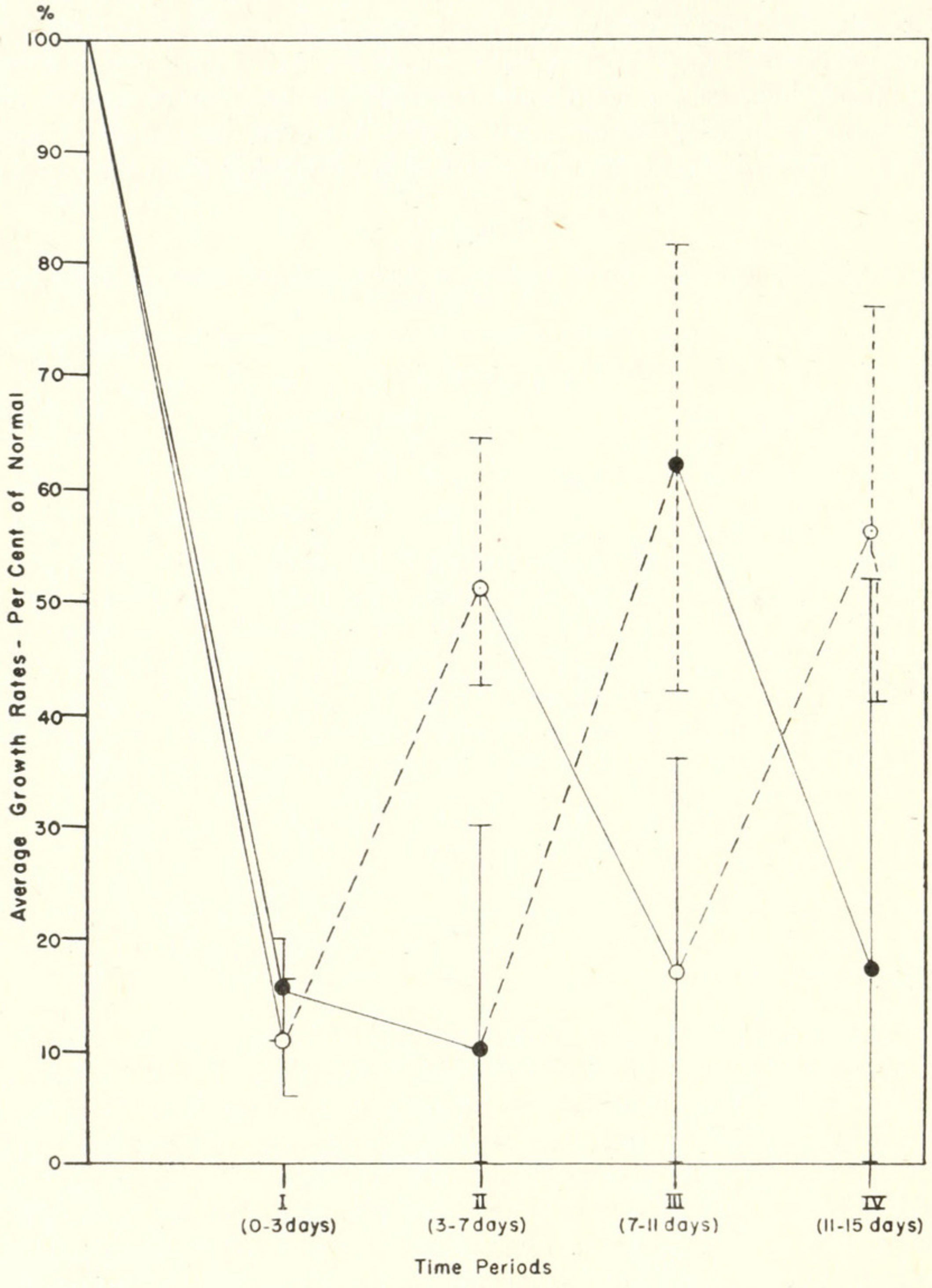

FIGURE 6. Growth rates of *Bugula* ancestrulae (in per cent of normal) during and after exposure to sublethal copper concentrations. Black data points represent averages for group *A* panels (Table IV), and clear circles are averages for group *B* panels (omitting set 7). Solid lines indicate periods of immersion in copper solutions, and dashed lines represent transfer to fresh sea water. Vertical lines through data points show the range between minimum and maximum growth rates for each average.

Length of exposure in sublethal concentrations has relatively little apparent effect on recovery. This is shown by the fact that *Bugulae* exposed to copper for seven days were able to develop after transfer to sea water at a rate comparable to that of specimens which had been exposed for only three days. As previously noted, however, the longer exposures caused abnormal polypide development in some cases.

In contrast to this finding, there is evidence that in the case of barnacles and mussels the length of exposure is as important as the concentration in determining the toxic effects of copper solutions. Clarke (1943) found that low concentrations acting for a long time produced effects equivalent to high concentration acting for a short time. In other words, the toxic effect is proportional to the product of duration and intensity. Further experiments of this general type are needed to determine more precisely immediate and long-term effects of various exposures and concentrations and of continuous versus discontinuous exposures.

These experiments have practical implications since, under natural conditions, the concentration of toxic ions adjacent to a copper paint surface might vary from time to time as a result of fluctuation in leaching rate, formation of surface films, currents flowing past the surface, and other factors. The effect of such changes on attached *Bugula* larvae might be surmised from the foregoing studies showing that growth is an inverse function of copper concentration in the medium and that stunted individuals can resume development when restored to non-toxic situations. Presumably, *Bugula* ancestrulae attached to a surface with fluctuating toxicity might recover to some degree with each significant decrease, if the organisms were still viable. With each increase in length, the ancestrulae greatly improve their chances to establish colonies for reasons which will become apparent in the next section of this paper.

Toxicity gradients of copper paint surfaces

The question of zones or gradients of toxicity adjoining toxic paint surfaces is involved in understanding their antifouling action, and is the last problem to be considered in this paper. Although a toxicity gradient has been assumed as a consequence of diffusion of toxic ions emanating from an antifouling paint, a demonstration of this seemed desirable. Furthermore, information on the effective limits and other characteristics of the toxic zone is of particular interest in connection with the establishment of colonial fouling organisms (e.g., erect bryozoans and most hydroids) that grow, plant-like, more or less perpendicularly away from the surface to which they attach, and develop new individuals at the ends of their branches. It would be useful to know how far from the toxic surface growth inhibiting and growth retarding concentrations are maintained, or how much a colony would have to grow before its terminal polypides were out of danger from poisoning. In the following experiment, the problem was to demonstrate, if possible, the existence of the toxic zone of an antifouling paint and to determine its general characteristics.

The preceding studies, showing rather precise relationships between growth of *Bugula* ancestrulae and copper concentration, suggested a method for attacking the problem of toxic gradients. The essential features of the procedure used and of the results obtained are illustrated in Figure 7. A non-toxic panel bearing attached and growing *Bugulae* was placed perpendicularly against a panel coated with a cold-plastic copper-paint (Table I, No. 7). With this arrangement, the developing *Bugula* stalks maintain practically constant distances between their axes and the toxic surface since they grow parallel to the latter. The non-toxic panel was ruled in millimeter divisions paralleling the toxic surface, and these were used as class intervals of distance in analyzing the data. The ancestrulae in each division were measured at the start of the tests and two days afterwards to determine the effect of diffusing copper ions on their growth at various distances from the paint surface. For control, a non-toxic panel was substituted for the toxic panel. Experimental and control racks were placed in an aquarium containing ten liters of sea water. The water was aerated during the tests by fine streams of air bubbles delivered through pinholes in a piece of rubber tubing stretched along the bottom of the aquarium. This method of aeration caused some circulation of water but no appreciable agitation.

TABLE V

Toxicity gradients of a copper paint demonstrated by growth of Bugula at measured distances from the toxic surface

Distance from paint surface	Test 1 Mean growth increment ± S.E. (2 days)		Test 2 Mean growth increment ± S.E. (2 days)	
	Experimental	Control	Experimental	Control
mm.	mm.	mm.	mm.	mm.
0–1	0.10±.02	0.48±.02	0.01±.01	0.43±.02
1–2	0.27±.03	0.42±.05	0.03±.01	0.46±.02
2–3	0.40±.06	—	0.16±.03	0.44±.03
3–4	0.38±.06	0.50	0.30±.06	0.49±.02
4–5	0.35±.10	0.50	0.40	0.50±.04
5–6	0.45±.08	0.45±.04	0.37±.04	0.50
6–7	0.55±.04	0.45±.04	0.35	—
7–8	0.45±.08	0.40	0.45	0.40
8–9	0.50±.07	0.50	0.35±.04	0.40
9–10	0.50	0.43±.03	0.41±.02	0.50

Two tests were made: the first, using toxic panels that had been seasoned for ten weeks in the laboratory; the second, with the same panels that were seasoned another two weeks. In the first tests, the slime film which had accumulated during seasoning was not removed but for the second tests the slime was wiped off. Data are given in Table V and graphically illustrated in Figures 7 and 8.

As clearly demonstrated by the retarded growth of the colonies near the painted surface, the toxicity arising from the surface decreases rapidly with distance from the surface. The outer effective limit of the toxic zone is variable. Beyond two millimeters from the surface in the first test and four millimeters in the second, no significant difference in growth between experimentals and controls was demonstrated. These values, then, represent the respective outer limits of the toxic gradient in the two tests and indicate the order of magnitude of the width of the toxic zone for a good antifouling paint. Within these zones, growth increments are roughly proportional to perpendicular distance from the toxic surface as might be expected from the diffusion gradient of toxic ions. Ancestrulae immediately

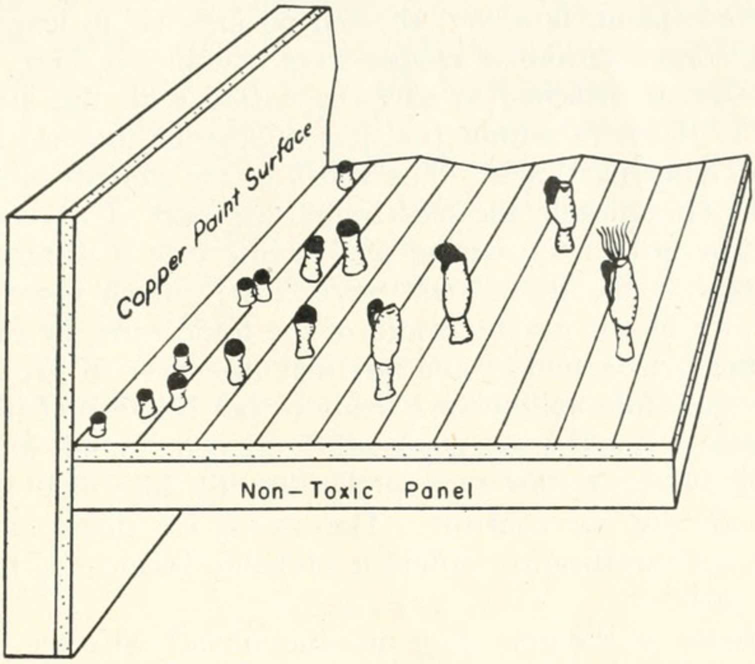

FIGURE 7. The toxic gradient extending from a copper paint surface as shown by growth of *Bugula* ancestrulae at measured distances from the toxic surface. *Bugula* figures are camera lucida drawings made four days after start of test 2 (for two-day growth, see Table V), and are twice enlarged in comparison to the millimeter rulings shown on the non-toxic panel.

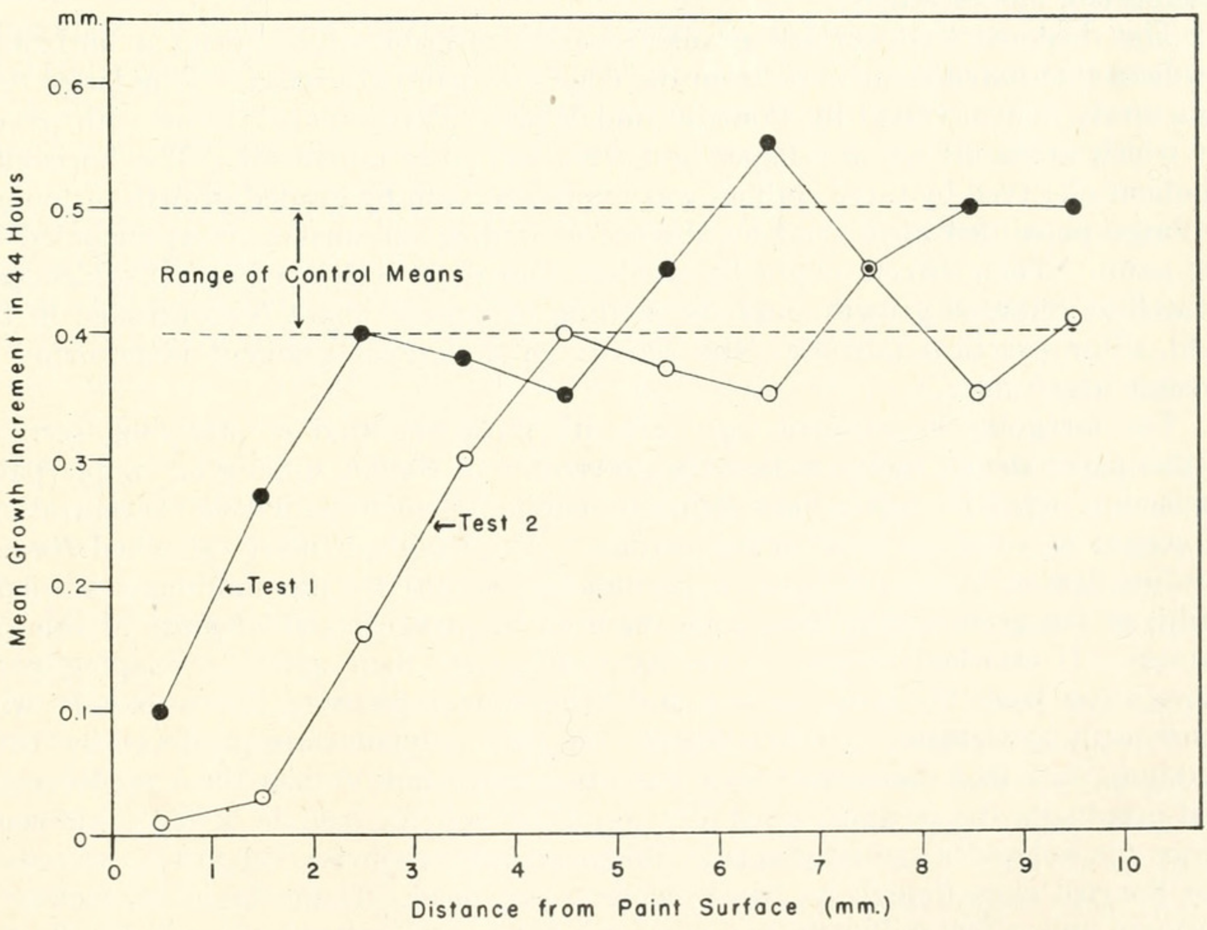

FIGURE 8. Gradients of toxicity of an antifouling paint: test 1—paint seasoned for ten weeks and coated with slime film, and test 2—after twelve weeks seasoning and with slime film removed before testing.

adjacent to the toxic paint, however, showed no increase in length indicating that growth inhibiting concentrations of copper were maintained there. This result was anticipated since larvae attached to surfaces coated with this paint do not grow (Table I, No. 7). Growth within the first millimeter interval is contributed by ancestrulae in the outer part of it. The width of the growth inhibiting portion of the toxic gradient (practically, the most important part of it) could be more precisely determined by further tests using smaller intervals of distance.

The panels used in the second test were clearly much more toxic than those first tested as shown by the greater width of the toxic zone, by the greater growth inhibition near the surface, and by the fact that the curve of growth increments at distances greater than four millimeters tends to fall below that of controls. With respect to the latter point, the mean growth increment for all individuals between four and ten millimeters in test two is significantly less than the corresponding mean in test one or that for controls. The means for single millimeter intervals in this range are not significantly different probably because of the small numbers of individuals in each.

The characteristics of the toxic zone are undoubtedly affected by various factors such as leaching rate and velocity of flow of water across the surface. In the field, currents or movement of the painted surface through the water would probably alter the character of the toxic zone. This and other factors could be simulated in the laboratory and their effects analyzed using the above-illustrated methods with appropriate modifications.

The demonstrated vertical gradient would probably differ from a horizontal gradient that extends outward from the edge of a painted surface. The latter type was nicely demonstrated by Pomerat and Weiss (1943) in field tests with panels on which areas of various shapes and sizes were left unpainted. The horizontal gradient observed by these authors was expressed both by graded growth of fouling on large unpainted areas, and by absence of fouling on smaller areas encircled by the paint. Their effects might be attributed in part to delayed larval attachment as well as retarded growth since the settling of larvae cannot be controlled in the field. For practical purposes, the activity of a vertical gradient is probably of greater importance.

The foregoing experiment together with those reported in preceding sections of this paper clearly indicates that the prevention of *Bugula* fouling on copper paint surfaces is dependent upon their ability to maintain growth inhibiting concentrations of copper in a narrow zone at the surface. The length of newly attached *Bugula neritina* larvae (about 0.2 mm.) presumably represents the minimum adequate width of the growth inhibiting zone required to prevent establishment of this organism. If attached forms are permitted to grow, their apical developing parts move away from the toxic surface and hence into regions of lower toxicity with consequent acceleration of development. As new polypides are produced by distal budding, they find themselves in a less toxic environment than their predecessors and eventually the terminal polypides would lie entirely outside of the toxic zone. Since the polypides are functionally independent (except for support), the colony can flourish even though its basal members are dead. If the toxic zone extends outward only a few millimeters, just a few of the basal polypides would be affected since *Bugula* colonies at the first polypide stage average about 0.9 mm. in length and each successive polypide adds at least a half millimeter to the length.

Larvae that settle on previously attached forms or on any inert particles elevated appreciably above the surface plane of the paint would clearly stand a better chance of survival than those attached directly to the toxic surface. They would occupy less toxic regions of the toxic gradient which might permit their growth, while those attached to the surface itself might be killed or permanently stunted by the higher concentration of the toxic prevailing there. Judging from the steep slopes of the toxicity gradients for an effective paint (Fig. 8), a fraction of a millimeter from the paint surface might make a significant difference in development, especially on surfaces with borderline toxicity.

To summarize: the foregoing preliminary tests clearly demonstrate a zone or gradient of toxicity that extends outward a few millimeters from an effective copper paint surface. Further experiments, using the method illustrated, are indicated to determine more precisely the limits and other characteristics of the toxic zone under various conditions.

Summary

Copper paint surfaces prevent the establishment of *Bugula neritina* (1) by repelling or killing the larvae and (2) by inhibiting growth and metamorphosis of attached larvae.

Copper paints with leaching rates less than 10 micrograms of copper per square centimeter per day permit the larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 15 micrograms per square centimeter per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms per square centimeter per day, but these do not develop colonies.

No consistent effect of slime film on larval attachment was noted. Its presence is not prerequisite to attachment.

Precise relationships between copper concentration and growth of *Bugula* ancestrulae are demonstrated. Growth in sea-water solutions of copper is inversely proportional to the concentration up to 0.3 mg. per liter. Higher concentrations completely inhibit growth.

The critical copper concentrations affecting various stages of the early life cycle of *Bugula* are as follows: (1) Concentrations greater than 0.3 mg. per liter kill larvae and completely inhibit growth of attached forms, (2) concentrations between 0.2 and 0.3 mg. per liter retard growth and prevent polypide formation, and (3) concentrations less than 0.2 mg. per liter retard growth and polypide development.

No stimulation of growth by copper solutions was observed. There was some evidence that small concentrations of copper stimulated attachment of larvae.

Bugula ancestrulae can recover and develop almost normally after seven days exposure to sublethal concentrations of copper. They can recover after repeated immersions in copper solutions that practically prevent growth. Length of exposure has relatively little effect on their ability to recover from copper poisoning.

A gradient of toxicity extending outward a few millimeters from a copper paint surface is demonstrated.

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