A BACTERIAL BASIS FOR THE GROWTH OF ANTIBIOTIC-TREATED BIVALVE LARVAE

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ABSTRACT

Routine addition of the proprietary antibiotic formulation "Combistrep" (dihydrostreptomycin-streptomycin sulfates) to larval cultures of clams, Mercenaria mercenaria, or oysters, Crassostrea virginica, usually results in a significant increase in growth rate of larvae. It had been assumed that this increase was effected by the elimination or suppression of bacterial flora, but plate counts show that the total number of marine bacteria increases in almost direct proportion to the added Combistrep up to 2,000 parts per million. Bacteria-free clam larvae showed no growth when cultured in autoclaved sea water to which Combistrep had been added. In Combistrep-treated cultures inoculated with a mixed flora of marine bacteria, the larvae showed significant growth, while cultures that received the bacterial inoculum but no Combistrep showed little or no growth. These results suggest that the antibiotic-induced bacterial flora in the Combistrep-treated cultures may be utilized by larvae as a food source.

INTRODUCTION

Since the development of methods of rearing larvae of clams, Mercenaria mercenaria, and oysters, Crassostrea virginica, the role of bacteria and dissolved substances in their nutrition has been a matter of conjecture. It has been shown that supplemental live algal foods are primarily responsible for growth and development of these larvae (Davis and Guillard, 1958). On occasion, however, other factors have been observed or suspected to play an important part in nutrition of larvae. Davis and Chanley (1956) reported an increase in the growth rate of clam and oyster larvae on the addition of several vitamins and antibiotics but offered no suggestion on how the increased growth was achieved. Carriker (1956) reared clam larvae to metamorphosis on an extract of cereal, Pablum, and concluded that the good growth of larvae was the result of increased microbial populations stimulated by the addition of the Pablum filtrate. Loosanoff, Davis, and Chanley (1955) stated that clam larvae seem able to utilize sulfur bacteria. Coe (1947) and Rodinca (1948) believed that bacteria played a part in the diet of adult mollusks. On the other hand, Davis (1953) fed pure cultures of nine species of marine bacteria to larval oysters with no success. Loosanoff, Davis, and Chanley (1955) stated that

lack of success in growing oyster larvae on several species of marine bacteria contradicts the generally accepted view that marine bacteria constitute an important part of the oyster diet.

The present paper reports experiments which show the growth-producing effect on clam and oyster larvae of a commercial antibiotic preparation Combistrep[1] (dihydrostreptomycin and streptomycin sulfates). It is furthermore demonstrated that the increase in growth of larvae is associated with a stimulation of marine bacterial populations by the Combistrep. This antibiotic preparation was originally used at the Milford laboratory in an attempt to prevent mortalities of larvae resulting from bacterial diseases, and its effect in increasing growth rates of bivalve larvae was first noted by Chanley (personal communication).

MATERIALS AND METHODS

Methods of conditioning and spawning adult bivalves and rearing larvae in the laboratory have been described in detail (Loosanoff and Davis, 1950). To determine effects of Combistrep on clam and oyster larvae, fertilized eggs were cultured 48 hours at concentrations of approximately 30 per ml in filtered ultraviolet-light-treated sea water. Forty-eight-hour veliger larvae were then collected on stainless steel screens and diluted to a known volume. After the number of larvae per unit volume was determined, appropriate volumes were used to set up experimental cultures with about 10 larvae per ml.

The culture medium, including test materials, was renewed every second day by collecting the larvae on a stainless steel screen and transferring them to new media. Temperatures were held to 24 $\pm$ 1C throughout. After 10 days' exposure to the experimental conditions clam veligers were sampled quantitatively. Oyster veligers were similarly sampled after 12 days. Effect of experimental treatment on growth of larvae was determined by measuring the long axis of 50 clam or 100 oyster larvae. The generally uniform size of clam larvae permits good accuracy with the lesser sample size.

Methods used in determining the effects of Combistrep on marine bacteria and the effects of the stimulated bacterial populations on bivalve larvae are included briefly within the respective result sections.

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[1] Reg. U. S. Pat. Off. Chas. Pfizer & Co., Inc.

Combistrep is a proprietary compound of Chas. Pfizer & Co., Inc., and has the following composition:

dihydrostreptomycin base (as sulfate)	125 mg/cc
streptomycin base (as sulfate)	125 mg/cc
phenol	0.25%
sodium citrate	1.3%
sodium bisulfate	0.2%
water	77.4%

RESULTS

A compilation of data from experiments over the past several years demonstrates that both clam and oyster larvae receiving Combistrep have consistently shown more rapid growth than larvae in comparable cultures that did not receive Combistrep (Figs. 1 and 2). At concentration ranges of 100 to 300 parts per million (ppm), Combistrep generally increased the rate of growth of clam larvae by more than 100% in cultures not receiving a supplemental feeding of algae (Fig. 1). Growth of these untreated, unfed larvae varied considerably, i.e., the mean length at 10 days varied from about 115 to about 148μ. Such differences in growth are certainly due to variations in the amount of food present in the filtered ultraviolet-light-treated sea water. In all cases, however, above average growth in the untreated, unfed cultures was accompanied by a correspondingly more rapid growth of larvae in the Combistrep-treated unfed cultures.

Clam larvae receiving 200 to 400 ppm of Combistrep and no supplemental algal feeding when reared beyond the usual 10-day experimental period in all cases grew to metamorphosis with negligible mortality within 20 days at 24 C. In all such instances, of course, it required a longer time for these larvae to reach metamorphosis than for larvae receiving algal food supplements, but untreated, unfed larvae in parallel cultures never progressed beyond 140μ in length.

Combistrep also increased growth of clam larvae receiving live flagellates as food (Fig. 1). The increase in the growth increment averaged about 25μ at 12 days of age at optimal Combistrep concentrations. This was about a 25% increase in growth over larvae fed live flagellates without Combistrep. The optimum concentrations of Combistrep appeared to be higher in cultures receiving algal foods than in those not receiving the algae.

Growth of oyster larvae was increased nearly 100% by the addition of Combistrep at concentrations between 200 and 300 ppm

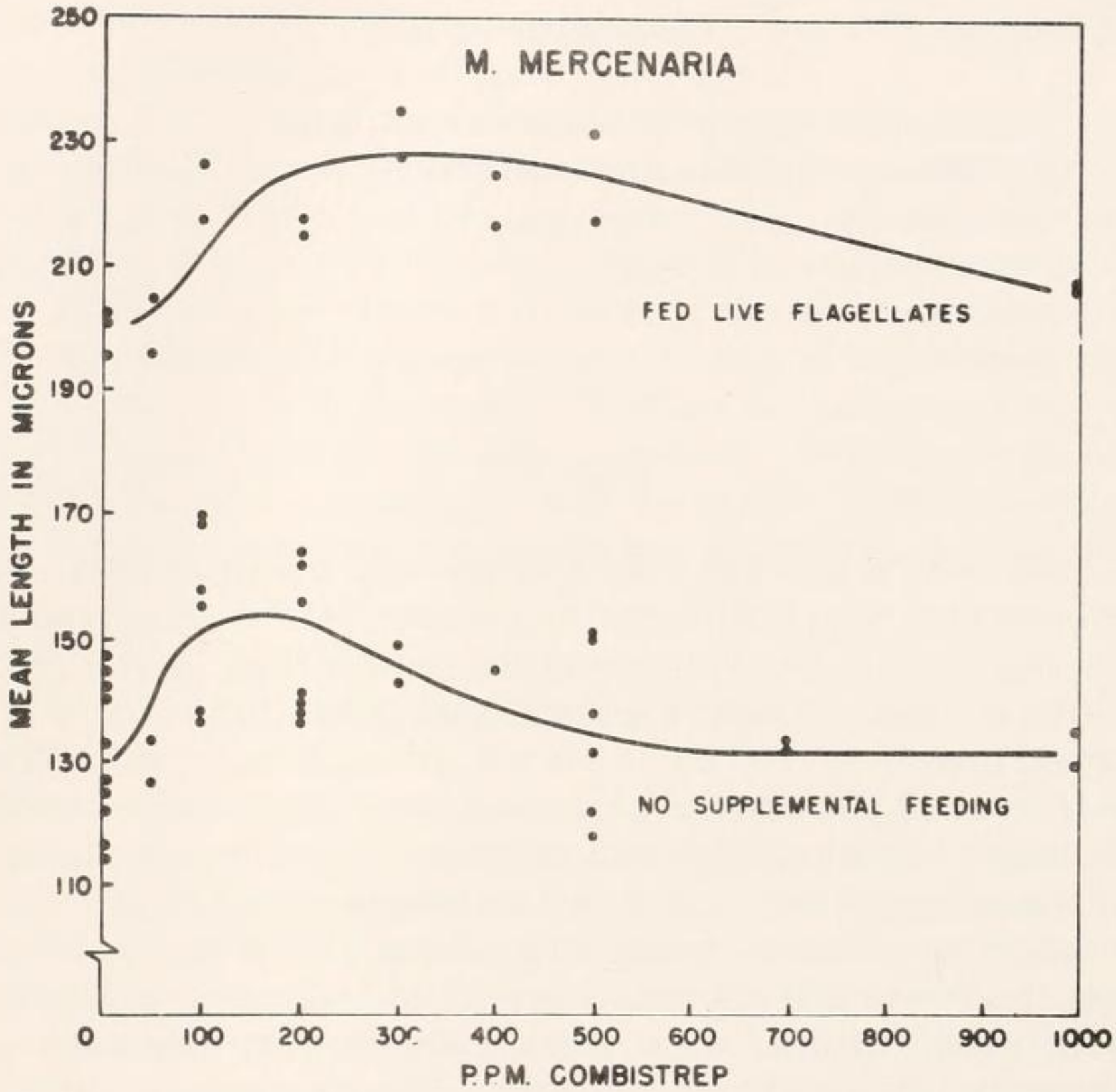

Fig. 1. Mean lengths of clam, M. mercenaria, larvae at 12 days of age receiving several dosage rates of Combistrep, with and without supplemental algal feeding. Data are a composite of several experiments which included Combistrep-treated cultures, together with suitable untreated controls. Each point represents a mean length of 50 larvae from a single population of approximately 10,000 larvae.

(Fig. 2). Figure 2 represents a composite of Combistrep-treated cultures, some of which received algal food supplements while others did not. Controls not receiving Combistrep are included in all cases, however. Because of the sensitivity of oyster larvae to variations in algal food quality, it is often difficult to distinguish larvae receiving algal food from those not receiving the food. The composite of mean lengths of fed and unfed larvae, however, clearly shows the value of Combistrep in increasing growth rates.

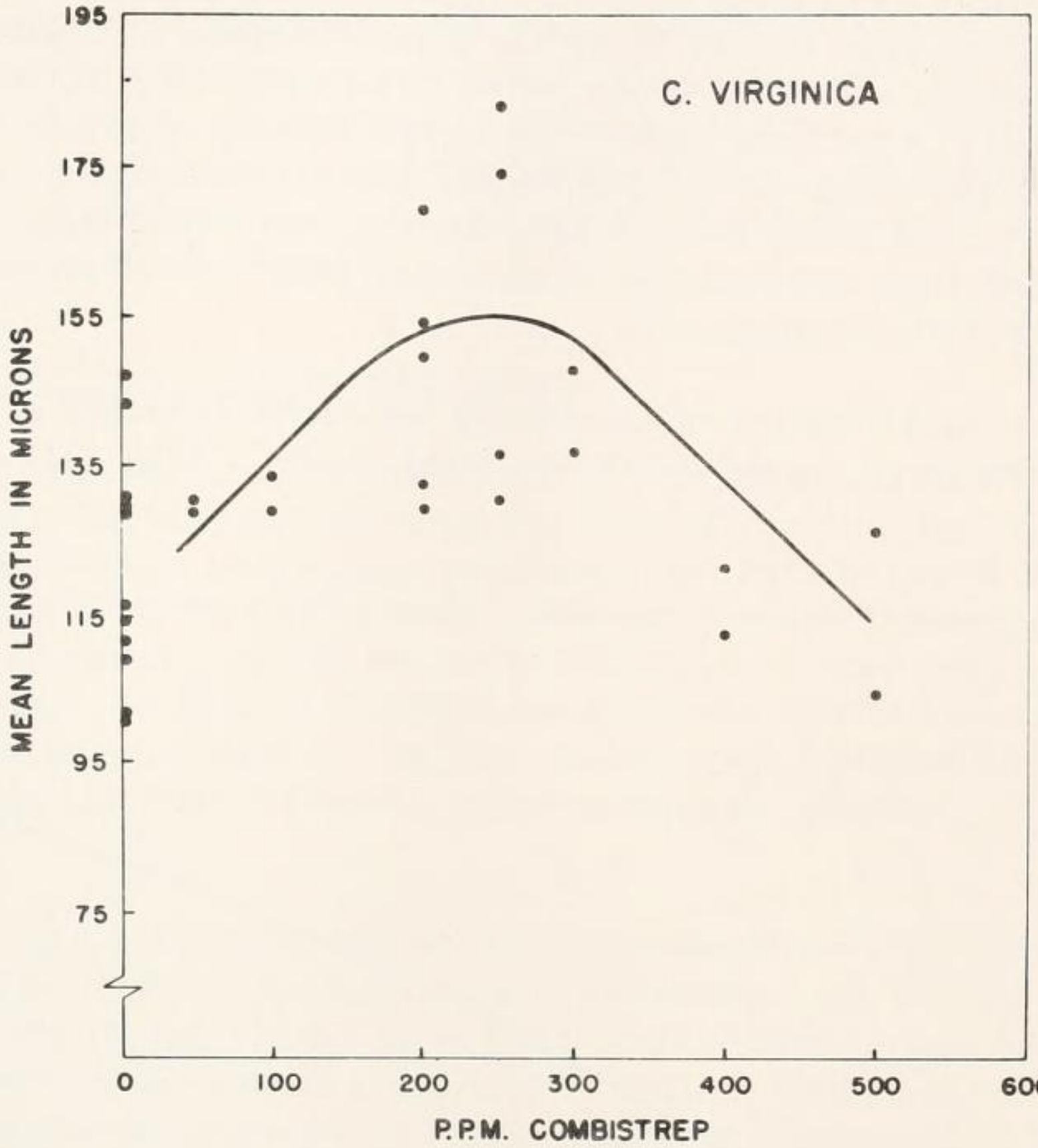

Fig. 2. Mean lengths of oyster, C. virginica, larvae at 14 days of age receiving several dosage rates of Combistrep. Data are a composite of several experiments which included Combistrep-treated cultures, together with suitable untreated controls. Each point represents a mean length of 100 larvae from a single population of approximately 10,000 larvae.

Effects of Combistrep on Bacterial Populations

It was noticed in the above experiments that, although Combistrep did help to prevent mortalities in cultures of clam and oyster larvae, the bacterial population of larval cultures treated with Combistrep and some other antibiotics became actually higher than bacterial populations in untreated cultures. Experiments were then designed to determine the effect of different concentrations of Combistrep on bacterial populations in sea water.

One-liter cultures of filtered ultraviolet-light-treated sea water were set up and given several different concentrations of Combistrep but without larvae and algal food. After 48 hours at 24 ± 1 C samples for plate counts of bacteria were taken. The 48-hour cultures were plated at several dilutions using standard plating techniques on Trypticase Glucose Yeast Extract Agar made up with sea water. Bacterial colonies were counted; no attempt was made to determine the species of bacteria represented.

Increasing dosages of the sterile antibiotic resulted in almost directly proportional increases in bacterial numbers (Fig. 3). This

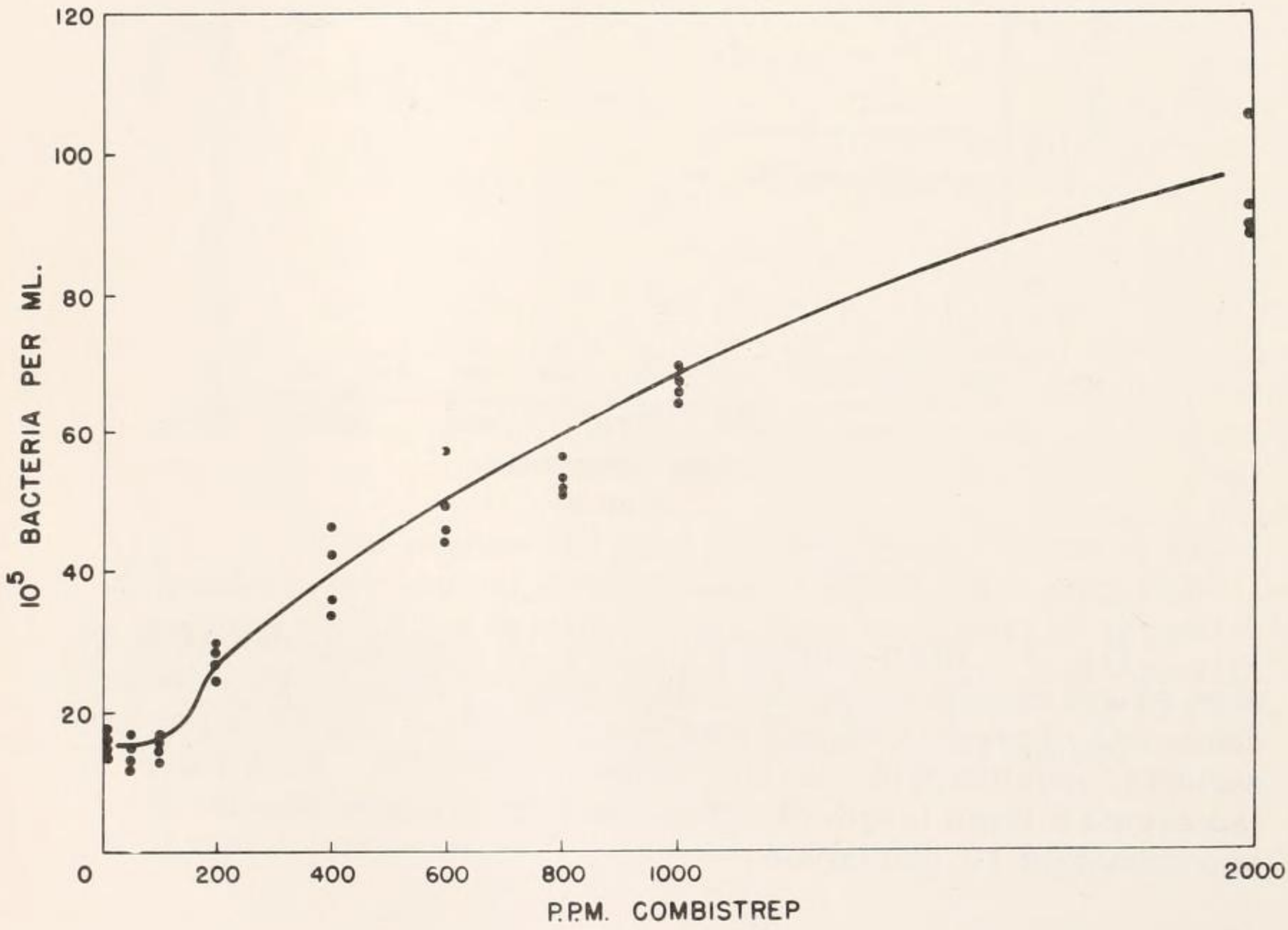

Fig. 3. Numbers of bacteria per ml of filtered sea water 48 hours after application of several concentrations of Combistrep. Duplicate sea water cultures were used at each test concentration. Points represent each of duplicate plate counts made of each sea water culture.

effect was not noted at the lowest concentrations (50 to 100 ppm). At concentrations of Combistrep that have been especially beneficial to larvae (200 to 400 ppm), there was approximately a two-fold increase in bacterial numbers over Combistrep-free cultures. Although the actual number of bacteria differed somewhat from experiment to experiment, the percentage increase with increasing dosages of Combistrep remained relatively constant.

Effect of Bacteria on Growth of Larvae

At this point it was important to determine whether the bivalve larvae were deriving benefit from the Combistrep directly or possibly from the increased bacterial populations that resulted from its addition to sea water. To do this we were eventually able to compare growth of larvae in aseptic and in bacterized Combistrep-treated cultures. Before we had developed methods for obtaining sterile larvae for aseptic cultures, however, several experiments were run in an attempt to correlate the rate of growth of larvae with the number of bacteria present.

It was postulated that if bacteria here were important in growth, then larvae receiving Combistrep and sea water with a fully developed bacterial population should grow more rapidly than larvae receiving Combistrep and sea water which were sterile. Consequently, non-sterile clam larvae receiving Combistrep were reared in sea water (1750 larvae in 500 mls) pretreated as follows:

1. Autoclaved and aged one week with sterile Combistrep added at the time of addition of water to cultures of larvae (sterile water supply).

2. Autoclaved, then aged one week after Combistrep plus a non-sterile sea water inoculum had been added (fully developed initial bacterial populations).

Larvae in control cultures, not receiving Combistrep, were reared in sea water with the following pretreatment:

1. Autoclaved, then aged one week.

2. Autoclaved, then aged one week after a non-sterile sea water inoculum had been added.

3. Autoclaved with live flagellate food added at the time of feeding to cultures of larvae.

The water in each culture was completely renewed every 24 hours and temperatures held to 20 C to minimize buildup of bacteria in treatments receiving the initially sterile water supplies. Duplicate cultures were used to test each treatment in each of the three replicate experiments. Results are shown as mean lengths of larvae after eight days of culture (Table 1). Mortality of larvae in all cases was negligible and thus is not included.

In the first experiment larvae grown in the presence of Combistrep with a fully developed initial bacterial population reached a mean length of 149.65μ, while those grown with Combistrep and sea water which were initially sterile reached only 117.90μ. Control cultures, i.e., those reared in sterile sea water and those kept in aged water plus the bacterial inoculum, also showed poor growth. Although the 149.65μ mean length was appreciably less than the 175.15μ mean length attained by larvae receiving live flagellates as food, it represents a significantly faster rate of growth than was achieved in control cultures.

The second experiment (Table 1), although conducted in exactly the same manner as the first, produced quite different results. In this trial 500 ppm Combistrep actually retarded clam growth, both when given with a sterile water supply and with a fully developed bacterial population. Since 500 ppm Combistrep is above an optimum dosage for clam larvae (Fig. 1), variable results might be expected. Growth was not reduced in a control culture within this experiment given only 250 ppm Combistrep. In a third trial Combistrep concentrations were adjusted to 250 ppm, which is a more nearly optimum concentration for clam larvae.

At 250 ppm (Experiment 3, Table 1) Combistrep-treated sea water with a fully developed initial bacterial population again produced good growth of clam larvae. Larvae kept in this water attained a mean length of 166.53μ, a length not much smaller than the 189.05μ achieved by larvae receiving live flagellate food. Larvae receiving sterile Combistrep and sterile sea water showed some growth (135μ mean length), while larvae kept in the sterile sea water and those kept in non-sterile sea water without Combistrep showed very little growth (121.20 and 116μ mean lengths, respectively).

Plate counts were made immediately after a change of sea water and again 24 hours later in this experiment to determine the typical numbers of bacteria at 0 and at 24 hours with each treatment (Table 2).

Table 1. The effect of several sea water treatments on the growth of non-sterile clam larvae. Larvae were cultured for 8 days at 20 C with water supplies renewed every 24 hours.

Sea water culture supply	Mean length (μ)		
	Exp. #1	Exp. #2	Exp. #3
Autoclaved (sterile)	113.00 ± 0.84[1]	121.25 ± 1.30	120.20 ± 0.83
Autoclaved + Combistrep (500 ppm)	117.90 ± 0.76	118.25 ± 0.94	No trial
Autoclaved + Combistrep (250 ppm)	No trial	126.65 ± 1.40	135.00 ± 1.18
Autoclaved + non-sterile inoculum (aged)	122.90 ± 1.09	125.55 ± 1.42	116.00 ± 0.77
Autoclaved + non-sterile inoculum + Combistrep (500 ppm) (aged)	149.65 ± 1.09	119.15 ± 1.66	No trial
Autoclaved + non-sterile inoculum + Combistrep (250 ppm) (aged)	No trial	No trial	166.53 ± 2.73
Autoclaved + flagellate food supplement	175.15 ± 1.68	190.35 ± 2.21	189.05 ± 2.64

[1]Indicates 95 per cent confidence limits, ± 1.98 SEm

Table 2. Numbers of bacteria present in non-sterile larval clam cultures receiving different treatments (Experiment 3, Table 1). Counts were made at times of water renewal (0 hours) and at the end of each change cycle (24 hours).

Sea water culture supply	Bacteria per ml	
	0 Hours	24 Hours
Autoclaved	19,000—21,000	550,000—580,000
Autoclaved + Combistrep	33,000	4,000,000
Autoclaved + non-sterile inoculum (aged)	50,000—100,000	65,000—150,000
Autoclaved + non-sterile inoculum + Combistrep (aged)	100,000—180,000	145,000—500,000

Although bacterial numbers were low at 0 hours in the cultures receiving the sterile water (19,000 to 33,000 bacteria per ml), there was a rapid buildup in the cultures by 24 hours (550,000 to 4,000,000 bacteria per ml). On the other hand, numbers of bacteria were initially high in cultures receiving the non-sterile water treatments (50,000 to 180,000 per ml) and showed a slower rate of increase in these cultures (65,000 to 500,000 per ml) at 24 hours. The aged sea water that had received the non-sterile inoculum plus Combistrep had considerably more bacteria per ml, both at 0 and at 24 hours, than the aged sea water that had the bacterial inoculum only. These results are in general agreement with experiments in which we determined the effect of Combistrep on the number of bacteria in sea water as previously described.

In subsequent experiments bacteria-free larvae were used to test the effect of Combistrep in aseptic vs. non-sterile cultures. Fertilized clam eggs collected on sterile stainless steel screens were washed several times with autoclaved sea water. These eggs were then permitted to develop into 48-hour veliger larvae in the trivalent antibiotic solutions described by Guillard (1959). Five

to ten of these sterile larvae were then transferred aseptically to 10 ml of autoclaved sea water in each of 16 test tubes for each of the following treatments:

1. No treatment (sterile control)

2. 250 ppm sterile Combistrep (Combistrep only)

3. 0.1 ml non-sterile sea water (bacteria only)

4. 250 ppm sterile Combistrep + 0.1 ml non-sterile sea water (Combistrep + bacteria).

After sterile larvae had been added cultures were held 11 days at room temperature. Sterility tests in sea water-thioglycollate were run on treatments 1 and 2 (above) at the end of the experimental culture period. Larvae in all vials were then killed and length measurements were taken of all larvae that had survived the test period.

Of several such experiments to measure growth of clam larvae receiving Combistrep under septic and aseptic conditions, only one was successful. In this experiment many of the culture tubes of the sterile groups remained sterile to the end of the test period, permitting valid measurement of the effect of treatment. Larval survival in this experiment was variable, but due to the low initial larval density survival did not appear to affect growth rates. The results expressed as mean lengths of larvae are listed in Table 3. In addition to the four original experimental treatments, two additional categories developed. These were the mean lengths of larvae from several cultures of the two originally sterile treatments which proved non-sterile by the end of the culture period. Although all larvae grew poorly, differences in mean lengths between treatments were highly significant statistically. An analysis of variance gave an F value of 21.82, indicating an overall difference of means significant far greater than the 99% confidence level (Snedecor, 1962).

Tests of significance of differences between individual treatments within this experiment showed that (a) mean length of sterile larvae (102.82μ) was not significantly different (at the 99% confidence level) from sterile larvae receiving Combistrep (103.68μ); (b) sterile larvae (102.82μ) did not differ significantly from those receiving the non-sterile inoculum but no Combistrep (105.54μ); (c) larvae receiving Combistrep plus the non-sterile inoculum (110.84μ) were significantly larger (at the 99% confidence level) than either those receiving Combistrep under sterile conditions (102.82μ) or those that had received only the non-sterile inoculum (105.54μ).

Table 3. Mean lengths of clam larvae after 11 days of culture receiving several different treatments

Treatment	Mean length (μ)
1. Sterile	102.82
2. Sterile + 250 ppm Combistrep	103.68
3. Bacterized non-sterile	105.54
4. Non-sterile + 250 ppm Combistrep	110.84
1a. Sterile (contaminated)	106.14
2a. Sterile + 250 ppm Combistrep (contaminated)	108.53

Also notable was the fact that the larvae receiving the sterile-Combistrep treatment, which accidentally became contaminated (108.53μ), were significantly larger than the other replicates within the treatment which remained sterile to the end of the culture period (103.68μ).

DISCUSSION AND CONCLUSIONS

The mechanism by which Combistrep induces greater bacterial populations in sea water can only be speculated upon at this time. Experiments thus far have only measured increase in gross numbers of bacteria without regard to possible species selection. It is possible that Combistrep is acting to inhibit certain toxin-producing species, thus allowing greater total numbers. The possibility of the minimal quantity of citrate present in dilute Combistrep acting as an energy source seems remote.

The series of experiments in which non-sterile clam larvae were exposed to Combistrep-treated sea water both sterile and with fully developed bacterial populations initially present indicated that the rate of growth of larvae was associated with the number and

probably the species of bacteria present and suggested that the larvae were using these bacteria as foods. In the two experiments in which Combistrep was beneficial to larvae (Exps. 1 and 3, Table 1), those larvae cultured in Combistrep-treated water with high initial populations of bacteria present in each case showed markedly greater growth than those receiving the initially-sterile water plus Combistrep. Although clam cultures receiving Combistrep with low initial bacterial numbers contained appreciable populations of bacteria by the end of the 24-hour water change cycle, there undoubtedly were significantly fewer bacteria present in these cultures throughout most of the 24-hour change cycle than in those receiving Combistrep with initially high bacterial populations. The fact that larvae in cultures receiving the bacterized aged water without Combistrep showed poor growth even though there were considerable bacterial populations present, throughout, would indicate that Combistrep was selecting and accelerating the growth of only certain beneficial species of bacteria.

The experiment using bacteria-free larvae showed that clam larvae in bacterized Combistrep-treated cultures grew, whereas those kept in sterile Combistrep-treated cultures and those kept in sterile, non-treated cultures showed little or no growth. This again demonstrated that it was the bacteria associated with the Combistrep treatment, not the Combistrep, itself, that caused the more rapid growth of bivalve larvae. Again, larvae in bacterized cultures that did not receive Combistrep showed less growth than in similar cultures containing Combistrep. This supports the above contention that the Combistrep-induced bacteria are perhaps of preferential utility to the larvae.

It is conceivable that the increased numbers of bacteria may at times be too great (over 500 ppm Combistrep). Such a biological mass might nullify beneficial effects by the creation of adverse environmental conditions for the larvae, such as reduction of dissolved oxygen, creation of toxic metabolites, etc. The variable results measured at 500 ppm Combistrep between experiments (Table 1) may be the result of this.

These studies, of course, do not show beyond all doubt that bacterial populations are utilized directly as food by bivalve larvae but do very definitely associate increased and probably selected bacterial populations with larval growth. As we learn more about these relationships, it may be possible to control bacterial populations to such an extent that they may become generally useful in future shellfish hatcheries, possible circumventing the presently difficult culture of relatively fastidious live algal food cells.

SUMMARY

1. Routine addition of the proprietary antibiotic formulation, "Combistrep" (dihydrostreptomycin-streptomycin sulfates) to cultures of clam or oyster larvae has consistently resulted in 25 to 100 per cent increases in the rate of growth of the larvae.

2. The addition of sterile Combistrep to filtered ultraviolet-light-treated sea water has produced an increase in numbers of bacteria roughly proportional to the concentration of Combistrep.

3. Tests with non-sterile Combistrep-treated cultures showed that clam larvae grew faster in Combistrep-treated cultures with a high initial bacterial count than in cultures with a low initial count.

4. In bacteria-free cultures the addition of Combistrep did not increase the rate of growth of clam larvae.

5. All data indicate that the increased rate of growth of larvae receiving Combistrep treatment is associated with the increase in numbers of a possibly selected group of bacteria.

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