

EXPERIMENTAL ASSESSMENT OF THE VIRULENCE OF FOUR SPECIES
OF VIBRIO BACTERIA IN PENAEID SHRIMP ^{a/}

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Direct intramuscular injection of Vibrio parahaemolyticus, V. alginolyticus, V. alginosus, and V. anguillarum into white shrimp (Penaeus setiferus) resulted in mortalities up to 100% depending on dosage (10^4 - 10^8 CFU/shrimp) and species of bacteria. In addition, 16% mortality occurred in brown shrimp (P. aztecus) when these animals were repeatedly fed white shrimp meat infected with V. parahaemolyticus at 10^8 - 10^9 CFU/food piece. Based on LD_{50} and LD_{98} data within 22 hr after injection, V. parahaemolyticus was found to be the most virulent species to white shrimp, followed by V. anguillarum, V. alginosus, and V. alginolyticus in that order. LT_{50} data of an injected bacterial dosage of either 1×10^7 or 2.5×10^7 CFU/shrimp also indicated the same order of virulence for those four organisms. The speed and/or pattern of dying of the shrimp in the experiments may indicate a toxin, or toxins, associated with Vibrio bacteria.

INTRODUCTION

Vibriosis has been implicated as a frequent mortality factor in juvenile and larval penaeid shrimp in culture (Sindermann, 1971; Lightner and Lewis, 1975). Several species of Vibrio bacteria, which often constitute part of the common flora in sea water, have been demonstrated in the laboratory as causative organisms of that disease (Vanderzant et al., 1970; Lewis, 1973; Lightner and Lewis, 1975). Since many aquaculture ventures are considering closed-system intensive culture of penaeid shrimp, it is important to evaluate the virulence of those bacteria in penaeids. Virulence information can help elucidate, directly or indirectly, questions concerning mode of infection, pathogenesis, and necessity of treatment and prophylaxis against infection.

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In the present study, we investigated the dosage-mortality and time-mortality relationships between four species of vibrios and white shrimp (Penaeus setiferus) through intramuscular injection. The bacteria used were V. alginolyticus, V. alginosus, V. anguillarum, and V. parahaemolyticus. Based on those mortality data, we determined and compared the virulence among the four bacteria. In addition, oral infection with V. parahaemolyticus was performed in brown shrimp (P. aztecus) to re-examine the possibility that that was one of the natural routes of infection. Other workers have reported conflicting results in their success to infect penaeids orally with V. parahaemolyticus (Vanderzant et al., 1970; Barkate, 1972).

Because we planned to follow and to observe the course of disease development in individual test shrimp, and at the same time to be able to establish several replicates for each dosage tested, efforts were spent to develop a bioassay system to attain those objectives.

MATERIALS AND METHODS

Test Shrimp and Aquarium Sea Water

Juvenile white shrimp (62-138 mm) and brown shrimp (54-83 mm) were purchased from a local bait camp. The clean and healthy-looking ones were held for at least one week in fiberglass laboratory tanks, equipped with under-gravel filters with air-lift pumps, before being randomly taken for use. White shrimp were used in injection experiments, while brown shrimp in oral infection tests. The shrimp were fed cooked shrimp meat.

Natural sea water was used in all the experiments. It was pumped in from the Gulf of Mexico and passed through a series of filtering and sedimenting devices to remove beach sand and particulates prior to storage in 28,000-gallon red-wood tanks lined with fiberglass. Those devices included in successive order a meshed-screened (90/in) cylindrical well-point buried under beach sand, a 30-in deep oyster-shell filter bed, and a sedimentation sump. Finally, in the Pathology Laboratory, the sea water was run through a set of diatom cartridge filters (pore sizes 5 and 1 μ) before being used in the experiments. The salinity of the water was adjusted to 20 ppt with tap water, and the water temperature was maintained at about 28⁰ C.

Challenging Organisms

Four Vibrio species and one gram-positive sphere, Micrococcus conglomeratus (strain GFC-76-48), were used in the experiments.

The micrococcus and two of the vibrios, V. alginolyticus (strain GFC-76-35) and V. parahaemolyticus (strain GFC-76-36), were isolated from postlarval white shrimp in our laboratory and identified by Dr. D. H. Lewis, Texas A&M University, College Station, Texas. The micrococcus was used as a positive control in the injection experiments assessing the pathogenicity and virulence of gram-negative vibrios. The other two Vibrio species were V. anguillarum (strain MS-670^{a/}) and V. anguillarum (strain MS-427^{a/}), which were obtained from Texas A&M University.

^{a/} Our culture code numbers were, respectively, GFC-76-5 and GFC-76-32.

All the bacteria were grown on tryptic-soy (T-S) agar plate (Difco^{a/}) with 2% NaCl added. After incubation at 28° C for 24 hr, the culture was harvested by sweeping it into a sterile NaCl solution (2%) with an L-shaped glass spreader. The bacterial suspension was pipetted up and down in a sterile test tube to break up clumps and aggregates. It was then diluted by 10-fold serial dilutions for plating out on T-S agar for titration of bacterial concentration.

For the preparation of toxic extract from V. parahaemolyticus, a harvested bacterial suspension (5.1×10^{11} CFU/ml) was passed through a disposable Nalgene filter (0.2 μ pore size) immersed in an ice bath. The filtrate was collected and utilized for injection as soon as possible.

For oral experimental use, V. parahaemolyticus suspended in a 2% NaCl solution was transferred to small pieces of pre-autoclaved brown shrimp meat (5 x 5 x 5 mm), one loopful per piece. The sterile shrimp meat was held in sets of petri-dishes (15 x 100 cm) with 10 pieces of meat per set. They were laid distant from each other on a piece of filter paper soaked with a sterile NaCl solution (2%). The inoculated meat was incubated at 28° C until being used at either 24 or 48 hr. Some shrimp pieces were not inoculated to serve as control.

^{a/} Trade names used in this publication do not constitute endorsement of commercial products.

Before the inoculated shrimp meat was fed to test shrimp, one piece from each of two sets of 10 pieces per set was transferred to a sterile 12-ml graduated, conical, tissue grinder (Belco Stock No. 1977-20012). A sterile NaCl solution (2%) was added to bring the total volume to 1 ml. The content was homogenized, and the homogenate was titrated for bacterial concentration on plate count agar (Difco). The average titer from the two pieces of meat was considered to represent the titer of V. parahaemolyticus on each piece of infected meat in two sets.

Bioassay System

Gallon-sized glass Mason jars were converted into experimental aquaria (Fig. 1). Flat-bottomed plastic food-serving bowls were used as covers. A 100-ml polypropylene Tri-Pour beaker (Sherwood #8889-206200) with a perforated bottom was seated in a 5.5-cm hole made in the bottom of the bowl. Fiberglass wool was placed inside the beaker to serve as filter.

The two vertical stems of an Eureka Baby-Saver sponge filter (sponge removed) assemblage^{a/} were each passed through a hole drilled in the bottom of the bowl so that the end that had the sponge was in the jar while the other end remained outside of the bowl cover. The free end of the larger filter stem was extended by means of elbow-shaped plastic connectors and rigid plastic tubes to form an assemblage, which had a short plastic tube positioned about 2 cm above the fiberglass wool inside the beaker.

^{a/} Eureka Products Co., 135 Jackson St., Newark, N. J. 07105.

When compressed air was introduced into the smaller filter stem, the sea water inside the glass jar was forced to recycle through the larger stem and the fiberglass filter. The system, with one shrimp in each jar, could maintain relatively clear aquarium water for 5 days or longer, if care was taken to remove excess food from the jar at the end of each work day.

The water temperature in our experiments was maintained at approximately 28° C by placing the aquarium jars collectively in a large water bath. The temperature of the bath water was regulated by means of submersible, thermostatically controlled, heating filaments and by circulating water pumps.

Injection Experiments

Each harvested bacterial suspension containing a test bacterial species was diluted by 10-fold dilution in 2% NaCl solution. A selected series of those diluted suspensions, usually from 10^{-2} to 10^{-5} , was separately injected intramuscularly into white shrimp by means of a 1 cc tuberculin syringe with a gauge 27 x $\frac{1}{4}$ inch hypodermic needle. Each animal received a 0.05-ml inoculum of one of the diluted suspensions, the site of injection being the junction between the lateral sides of the 4th and 5th abdominal segments. Injected shrimp were maintained individually in each aquarium jar. Each treatment was replicated from 6 to 10 times. Parallel positive controls with saline (2% NaCl) injection and negative controls (no injection) were also established.

A filtrate extracted from V. parahaemolyticus was also injected into test shrimp in a similar way. No dilution was made of the filtrate.

Oral Infection of Shrimp

Juvenile brown shrimp were individually isolated in aquarium jars and starved for 24 hours. Each of them was then fed with a piece of shrimp meat (5 x 5 x 5 mm) which sustained a 24-hr culture of V. parahaemolyticus. At three other 24-hr intervals, the shrimp were again fed with similar shrimp meats, respectively, containing 48-, 24-, and 48-hr growth of the same bacterium. After the last feeding of infected meat, the animals were maintained on non-infected meat. In control shrimp, they were fed only with non-infected meat. Thirty replicates were established for each group.

RESULTS

Injection Experiments

(a) Dosage-Mortality Response. Within 22 hours after each white shrimp was injected with 10^4 CFU or more of either of four Vibrio species (alginolyticus, algosus, anguillarum, and parahaemolyticus), up to 100%^{a/} of the animals died, depending on the dosages employed (Fig. 2). On the contrary, a gram-positive bacterium, Micrococcus conglomeratus, caused no mortality in white shrimp at a dosage of 1.9×10^7 CFU per animal.

Vibrio-induced mortality was dosage-dependent. From Figure 3, the median lethal dose (LD_{50}) of the Vibrio species in the test shrimp were respectively determined as 1.3×10^6 CFU/shrimp for V. parahaemolyticus, 2.2×10^6 for V. anguillarum, 9.1×10^6 for V. algosus, and 3.2×10^7 for V. alginolyticus. Similarly, the

^{a/} Corrected mortalities resulted from adjustments made against those in control groups (both saline-injection and non-injection) by means of Abbott's formula (Busvine, 1957).

respective LD_{98} for those bacteria in that same order were 1.1×10^7 , 7.2×10^7 , 1.3×10^8 , and 1.8×10^8 CFU/shrimp.

(b) Time-Mortality Relationship. The time-mortality relationship between Vibrio bacteria and white shrimp is shown in Figures 3 to 6. The speed of inducing mortality by a Vibrio species, as indicated by the steepness of the slopes of the regression curves, was obviously dosage dependent. Thus, at higher dosages, such as 10^8 CFU of V. parahaemolyticus, all the inoculated shrimp were dead within 6 hr (Fig. 3). At lower dosages, such as 10^6 CFU per shrimp, it took 22 hr for V. parahaemolyticus to kill 90% of the white shrimp. A similar relationship between dosage and speed of mortality existed in V. anguillarum, V. alginolyticus, and V. anguillarum (Figs. 4, 5, and 6). The bacterium-free filtrate extract from a culture of V. parahaemolyticus (5.1×10^{11} CFU/ml) produced 100% mortality within 4 hr (Fig. 3).

Values of median lethal time (LT_{50}) for different dosages were determined from the time-mortality curves (Figs. 3 to 6). Those of three of the Vibrio species (parahaemolyticus, anguillarum, and alginolyticus)^{a/} were plotted, and they showed that at a given dosage, the speed of causing mortality in white shrimp varied according to the bacterium species employed (Fig. 7). At a challenging dose of 1×10^7 CFU per shrimp, the LT_{50} was 7.2 hr for V. parahaemolyticus, 10 hr for V. anguillarum, and 50 hr (from extrapolation) for V. alginolyticus. The LT_{50} for V. alginolyticus was not available at 1×10^7 CFU/shrimp, but at a slightly higher dose of 2.5×10^7 CFU, it was determined as 11 hr (Fig. 5).

^{a/} V. alginolyticus was omitted from Figure 6 due to insufficient LT_{50} points as a result of less than 50% mortality at several lower dosages.

(c) Clinical Signs and Symptoms. Clinical manifestations in shrimp injected with V. alginolyticus and V. anguillarum were monitored. Shrimp which died within a few hours after inoculation with higher doses of bacteria usually displayed no clinical signs other than general weakness, lethargy, sluggishness in swimming, and/or quiescence while lying on either the ventral or lateral side of the body at the bottom of the aquarium, with or without leg movement; occasionally such animals might also be seen swimming on their side. Shrimp which were injected with less bacteria and which lived longer exhibited other types of clinical signs such as a white patch near the site of injection, mosaic white discoloration on abdominal segments, reddening of the pleopods and less frequently of the pereopods, and dorsal flexure usually between the 3rd and 4th abdominal segments. On two occasions, shrimp injected with V. alginolyticus appeared very unhealthy with pale patches all over the abdominal segments and had reddened legs. Our observations agreed with many of the clinical signs described for a natural septicemic bacterial disease of penaeid shrimp (Lightner and Lewis, 1975), although we often observed dorsal flexure between the 3rd and 4th instead of 2nd and 3rd abdominal segments, and that a white patch, which was not mentioned as a natural clinical sign, usually appeared around the site of injection. The white patch was perhaps related to injection of the Vibrio bacteria. In any case, we felt that the clinical signs were not specific to Vibrio infection. They were also frequently observed in shrimp dying from other causes. For diagnostic purposes, specific signs and symptoms of vibrosis and intoxication should be clearly defined in larval shrimp as well as in older shrimp.

(d) Molting. The frequency of molting in white shrimp was monitored in two separate experiments using V. alginolyticus as the challenging organisms. The results are summarized in Table 1. At dosages of 1.5×10^7 CFU/shrimp or more, no molting took place within 94 hr after injection. However, 5.6% and 25% of test shrimp molted either once or twice within 94 hr when they were respectively injected with 1.5×10^6 and 1.5×10^5 CFU per animal; no molting occurred in the saline-injected and non-injected groups.

TABLE 1
Molting of Juvenile White Shrimp (Penaeus setiferus)
After Injection of Vibrio alginolyticus

Dose of Injected Bacteria (CFU/Shrimp)	Within 94 hr After Injection of Bacteria	
	Molting Shrimp (%) ^{a/}	Mortality (%) ^{a/}
1.5×10^8	0	100 (within 22 hr)
1.5×10^7	0	87.5 (within 70 hr)
1.5×10^6	5.6	25 (within 70 hr)
1.5×10^5	25.0	0
Saline (2% NaCl)	0	6.3 (within 22 hr)
No Injection	0	10 (within 94 hr)

^{a/} Average of two experiments. One experiment was replicated 6 times and the other 10 times for every dosage applied. Each replicate contained one shrimp isolated in one aquarium jar.

Oral Infection Experiment

Attempts to infect brown shrimp with V. parahaemolyticus through repeated oral feeding produced about 16% mortality within 96 hr. The experiment revealed that the lethal effect of the inocula wore off very rapidly. Thus, following an initial injection of the bacterium, the corrected mortality rate of test shrimp rose to and leveled off at 8.4% within 23 hr. Mortality did not increase again until a second challenging dose from a 48-hr culture was administered, and then it leveled off at 15% within a total exposure time of 47 hr. (Fig. 8). A third feeding of infected shrimp meat failed to add to mortality, but a fourth feeding induced a further 1% corrected mortality to a total of about 16% within 96 hr. Thereafter, no more challenging bacteria were given, and no further death occurred in the "treatment" group.

DISCUSSION

Injection Experiments

(a) Virulence of Challenging Organisms. The dosage-mortality and time-mortality data showed that direct injection into white shrimp with the four tested vibrios could produce high mortalities depending on injected dose and time of exposure (Figs. 2 to 6). Failure of parallel injections with comparable doses of gram-positive M. conglomeratus to induce mortality indicated that the vibrios were intrinsically pathogenic to the shrimp. Based on the LD₅₀ and LD₉₈ values, V. parahaemolyticus was apparently the most virulent species since it had the smallest LD₅₀ (1.3×10^6 CFU/shrimp) and LD₉₈

(1.1×10^7 CFU/shrimp) among the four vibrios. For similar reasons, V. anguillarum was considered the next most virulent species to be followed by V. alginolyticus and V. anguillarum in that order.

With regard to the LT_{50} , V. parahaemolyticus also had the smallest value (7.2 hr at a dose of 1×10^7 CFU/shrimp) (Fig. 7). Vibrio anguillarum was the next smallest (10 hr) and V. alginolyticus again the next (50 hr). Since the LT_{50} indicates the speed of an organism to cause mortality, and since the length of time required to do so is inversely related to the virulence of the organism, therefore the sequential virulence of those three bacteria in injected white shrimp was in the order of V. parahaemolyticus, V. anguillarum, and V. alginolyticus.

The LT_{50} for V. alginolyticus was not available at the dose 1×10^7 CFU/shrimp, but it was determined as 11 hr at a slightly higher dose, 2.5×10^7 CFU/shrimp (Fig. 5). Theoretically then, at 1×10^7 CFU, V. alginolyticus should have a LT_{50} larger than 11 hr. That figure would place it after V. anguillarum, and probably before V. alginolyticus, in terms of virulence.

(b) Bacterial Toxin Mortality Factor. The very steep slopes of the time mortality curves at higher inoculation doses, particularly for those of V. parahaemolyticus and V. anguillarum (Figs. 3 and 4), may indicate that a toxic factor, or factors, were involved in the death of the shrimp. A similar postulate was presented by Vanderzant et al. (1970) when these workers attempted to infect brown shrimp by adding cultures of V. parahaemolyticus directly to aquarium water. In our present study, the toxin hypothesis was substantiated by injecting into white shrimp a bacterium-free filtrate extracted from a

24-hr culture of V. parahaemolyticus (5.1×10^{11} CFC/ml). The resulting time-mortality curve, showing 100% mortality within 4 hr, was comparable in slope characteristics to that derived from injecting a high dose, 1.4×10^8 CFU/shrimp, of V. parahaemolyticus (Fig. 3).

Results from further experimental studies on the pathogenic relationship between Vibrio toxins and penaeid shrimp are presented elsewhere (Leong et al., 1978). ^{unpublished data} The toxin mortality effect may explain why sometimes the challenging organism could not be recovered from moribund test shrimp.

(c) Molting. Table 1 shows that injection of V. alginolyticus into white shrimp apparently had a slight stimulating effect on molting. It was possible that V. alginolyticus induced molting by either stimulating the secretion of ecdysone (molting hormone) or inhibiting the production of a molt-inhibiting hormone. The absence of ecdysis at higher dosages of inoculation was perhaps due to the rapid death of the injected animals, preventing the completion of the molting processes.

Excess molting may not be favorable to the survival of the shrimp, since molting may render the animal more susceptible to environmental stresses and to invasion by opportunistic organisms. During the interim of separation of the old integument and the formation and hardening of the new one, the animal is deprived of an intimate layer of protective body covering, which has been regarded as the first line of defense against infection in arthropods. A review of the mode of invasion of Bacillus larvae, a pathogen of the honeybee, suggested that penetration of that bacterium into the gut tissues of host larvae took place when the gut peritrophic membrane sloughed off during metamorphic changes (Heimpel and Angus, 1963).

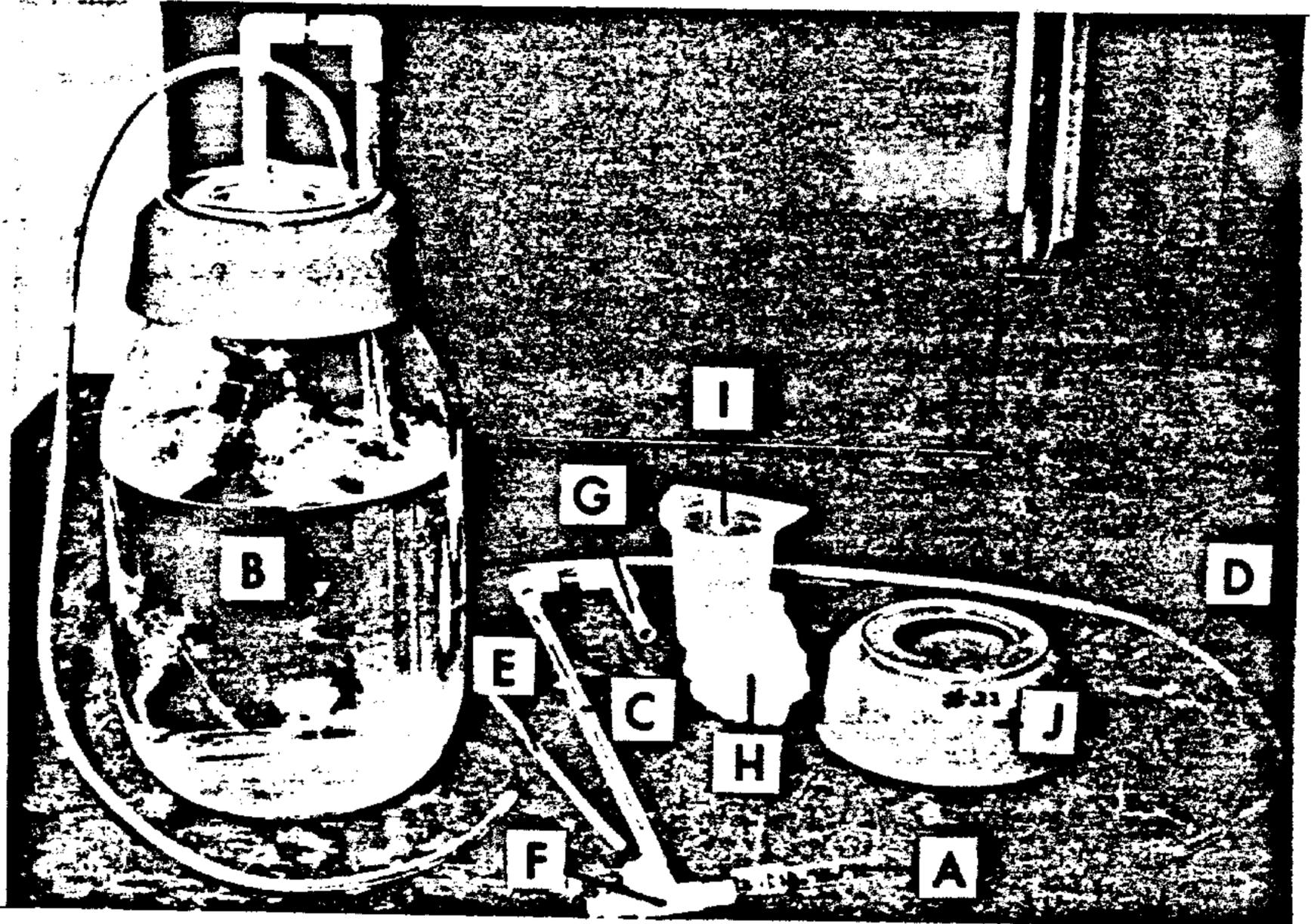


Fig. 1. Bioassay aquarium jar and components. Sponge-end "A" (sponge removed) of a Eureka sponge-filter unit is placed in aquarium jar "B", through which the aquarium water is taken in and moved by compressed air upward into plastic stem "C". (The compressed air comes in through Tygon hose "D" and the attached plastic stem "E" which is inserted into connector head "F".) The migrating aquarium water is discharged from plastic stem "G", and filtered through fiberglass wool "H" in Tri-Pour beaker "I" seated in jar cover "J" before returning to the aquarium jar. Please see text for more details.

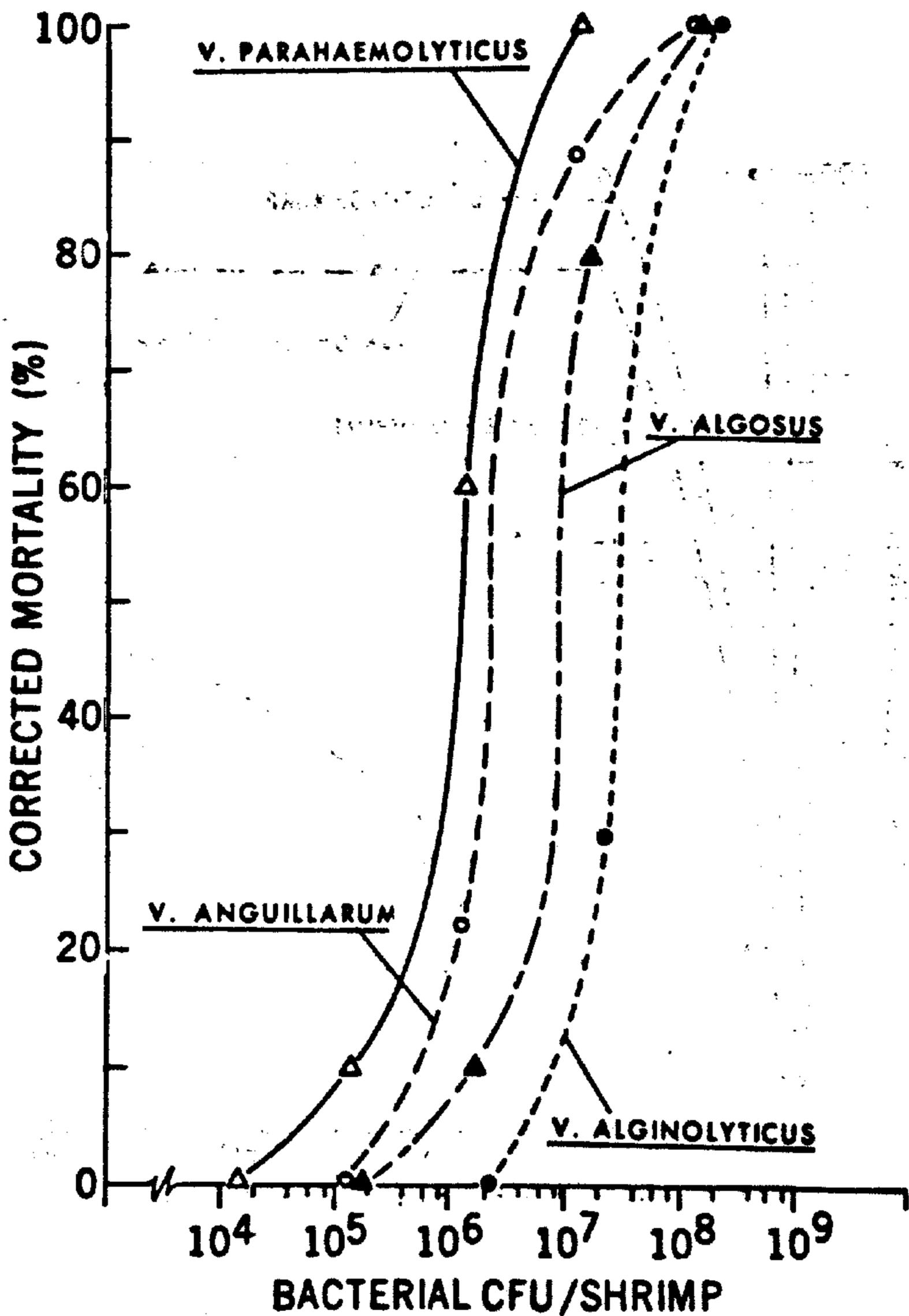


Fig. 2. Dosage-mortality response within 22 hours in white shrimp injected with four species of Vibrio bacteria.

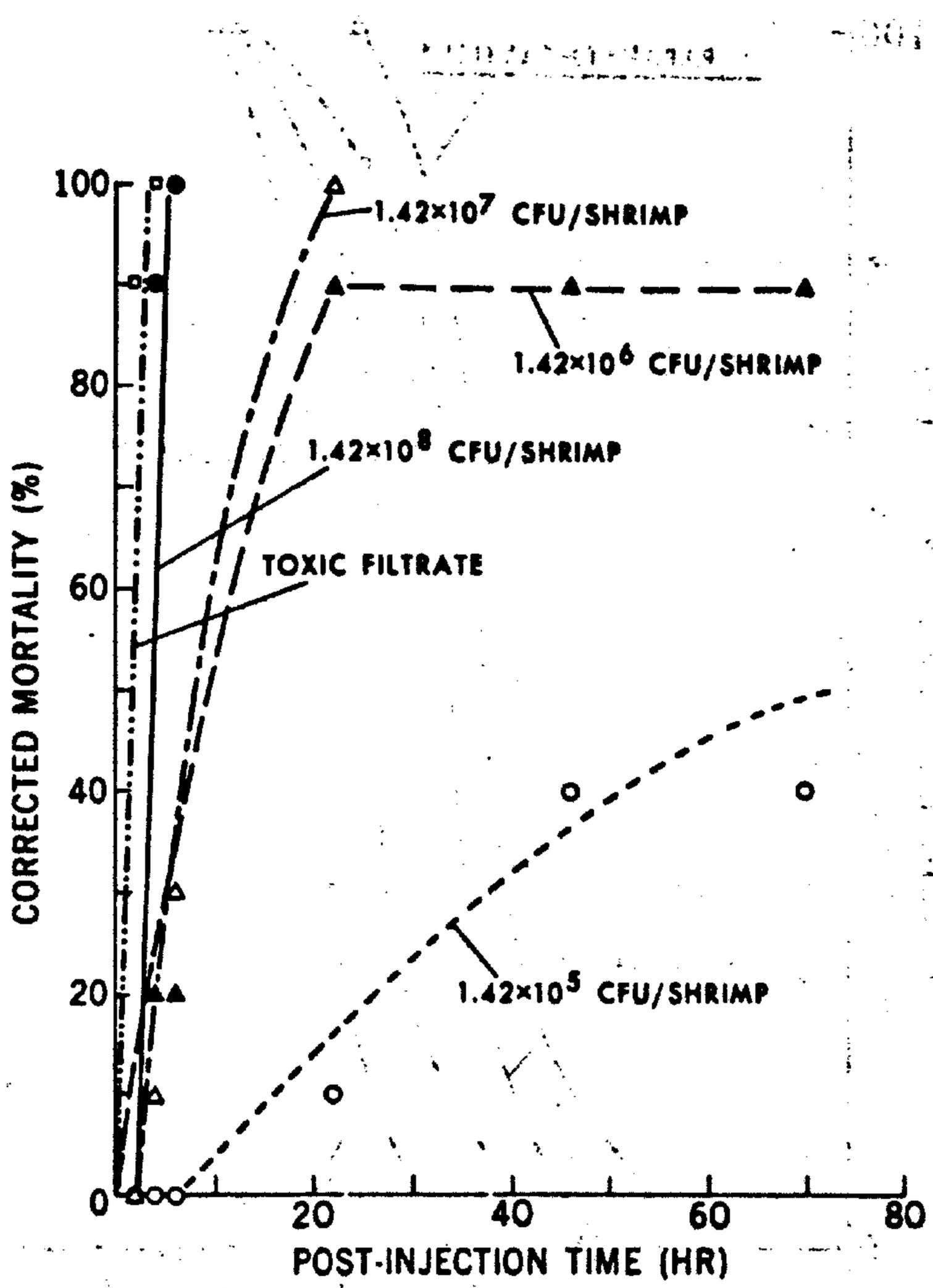


Fig. 3. Time-mortality response in white shrimp injected with Vibrio parahaemolyticus and a toxic filtrate from the same bacteria.

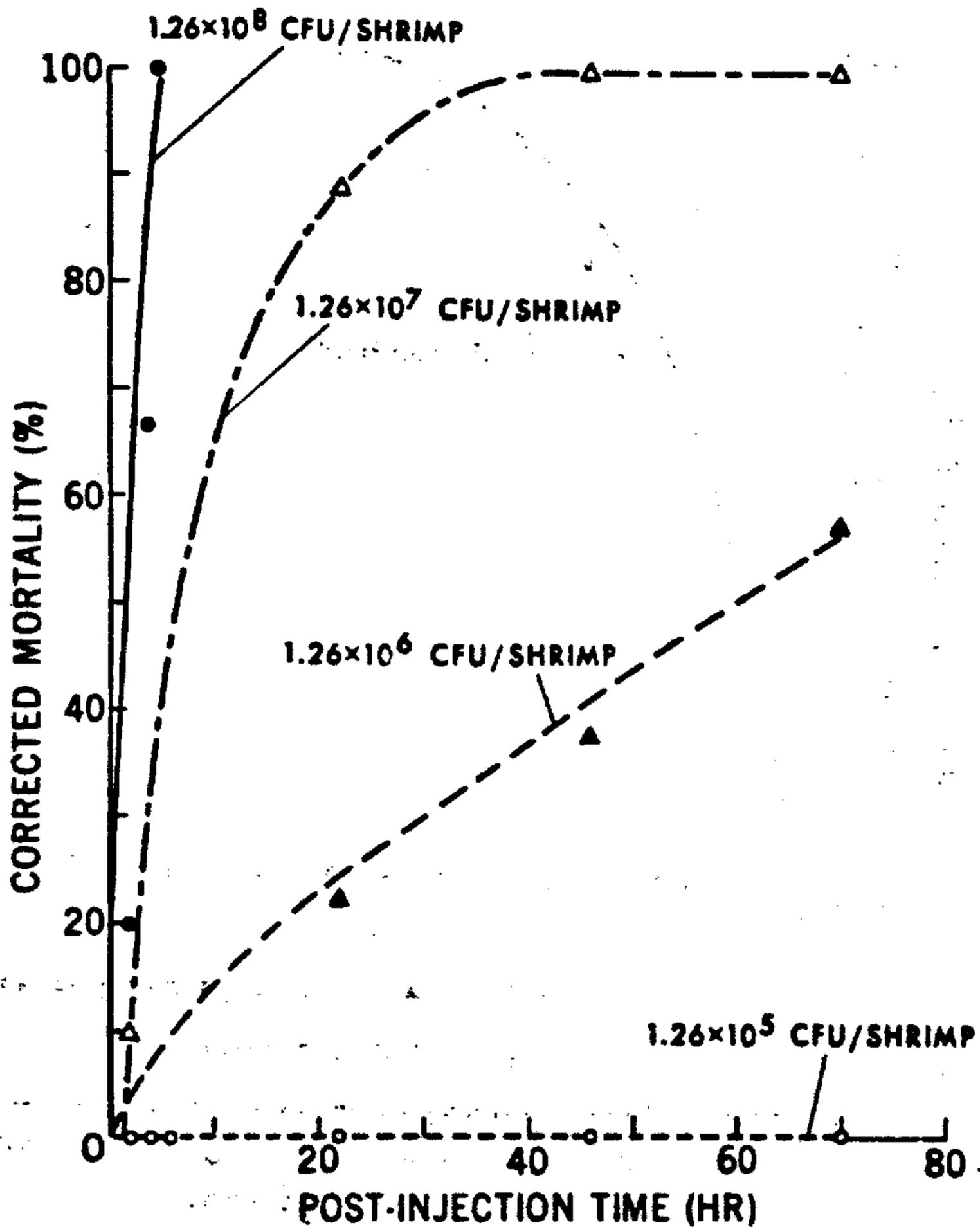


Fig. 4. Time-mortality response in white shrimp injected with Vibrio anguillarum.

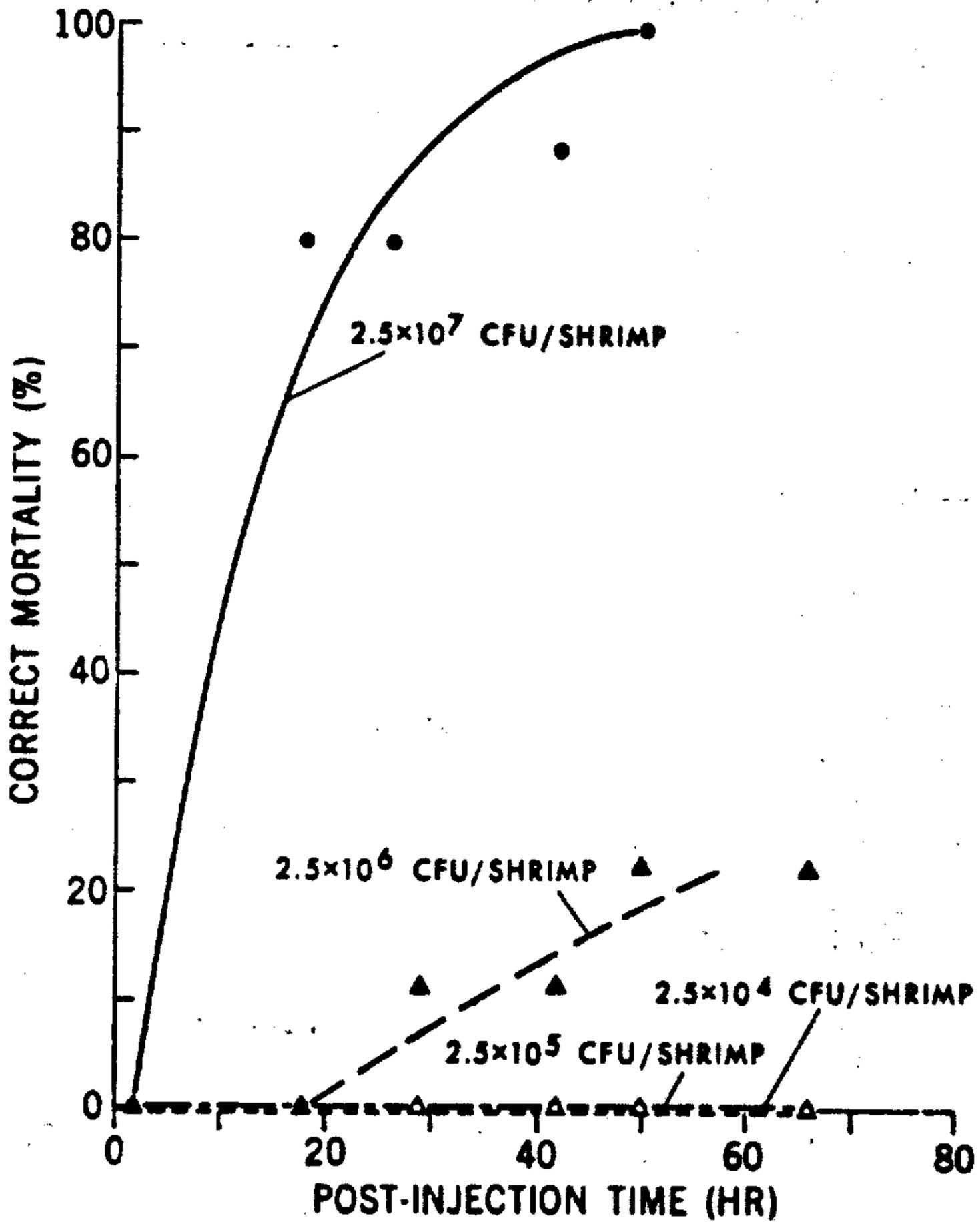


Fig. 5. Time-mortality response in white shrimp injected with Vibrio alginus.

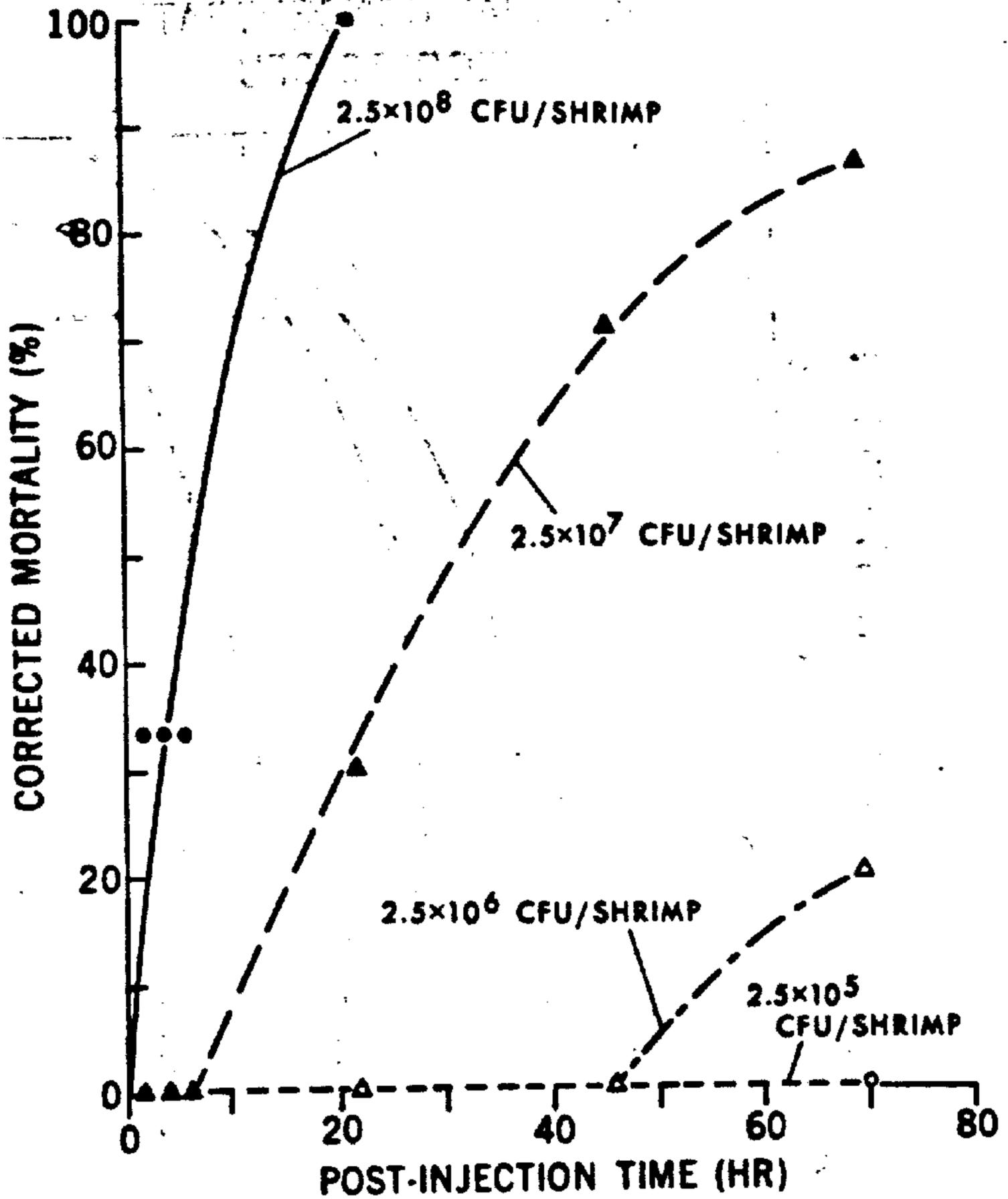


Fig. 6. Time-mortality response in white shrimp injected with Vibrio alginolyticus.

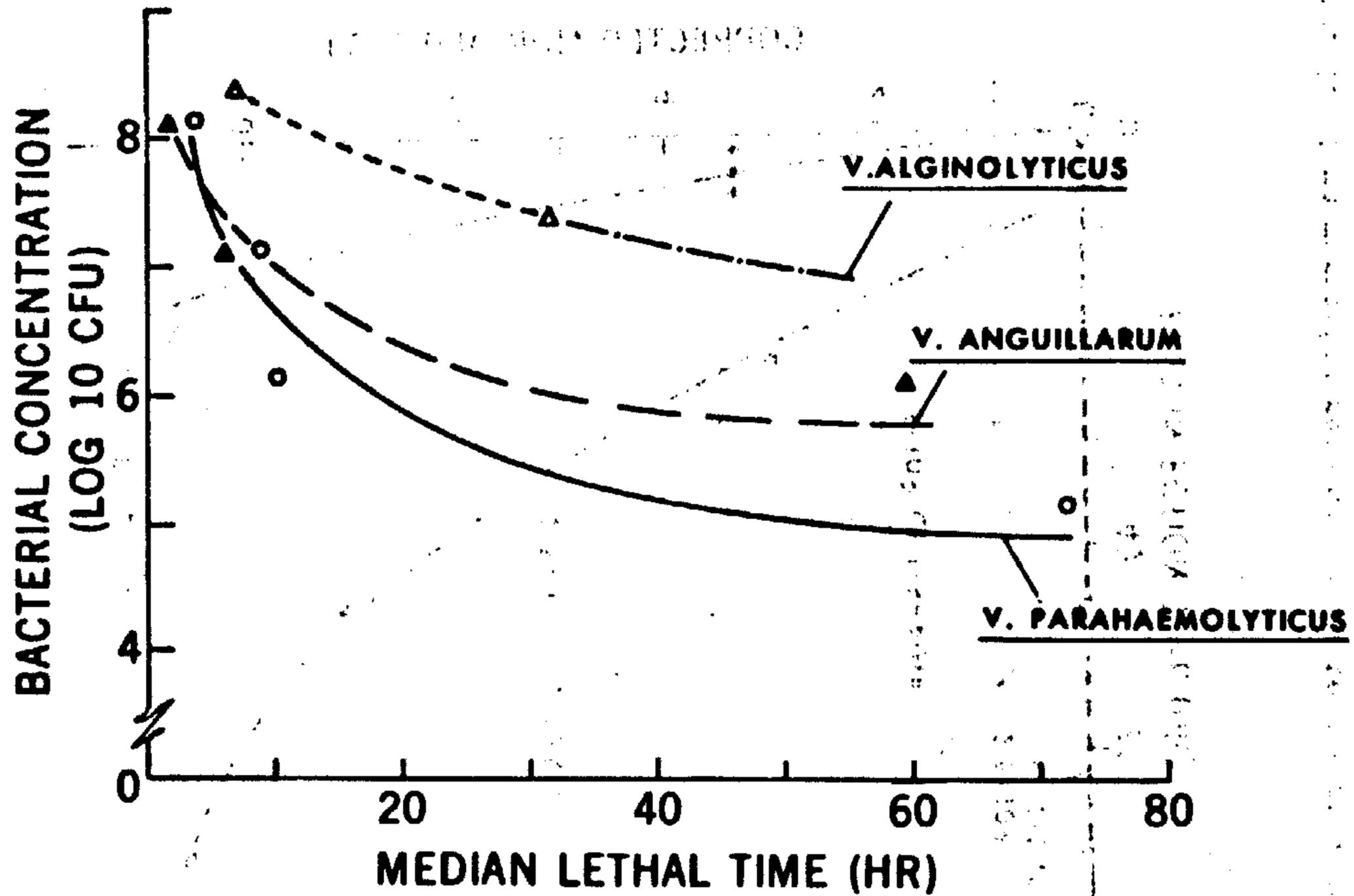


Fig. 7. Median lethal time (LT_{50}) for different doses of three species of Vibrio bacteria injected into white shrimp. Broken line (--- ---) in curve indicated extrapolated data.

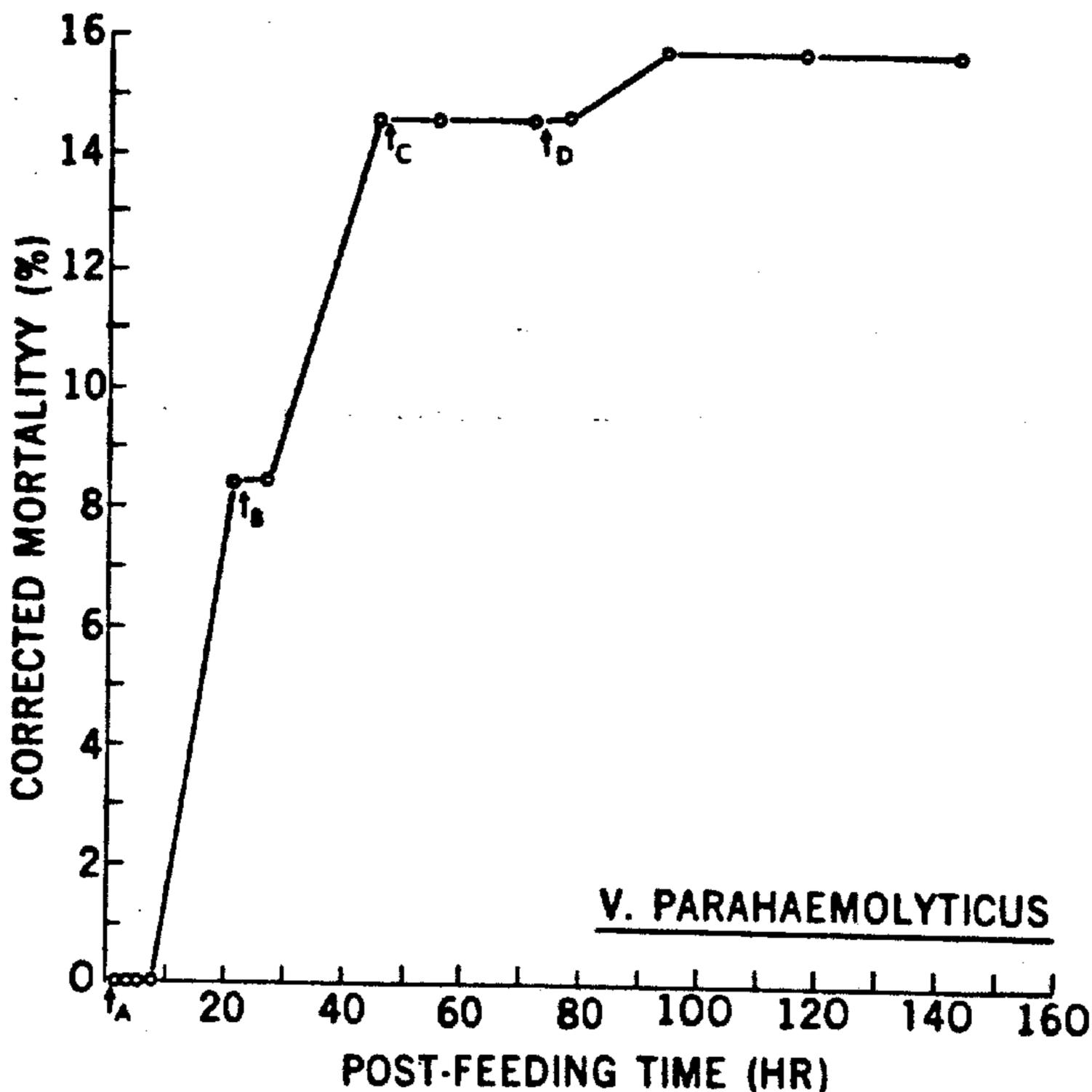


Fig. 8. Dosage-mortality response in brown shrimp fed with white shrimp meat supporting growth of Vibrio parahaemolyticus. Arrows indicate feeding time, and letter next to arrow indicates age of bacterial growth and dose of bacteria fed to each test shrimp: a = 1×10^9 CFU, 24-hr old; b = 2.9×10^8 CFU, 48-hr old; c = 5.4×10^8 CFU, 24-hr old; and d = 7.3×10^9 CFU, 48-hr old. After 94 hr, the shrimp were maintained on non-infected sterile shrimp meat as in control.

Oral Infection Experiment

The results from orally feeding brown shrimp with V. parahaemolyticus showed that this organism was slightly pathogenic to brown shrimp through the oral route, and that oral infection by vibrios in nature was possible. Such an infection may happen when a shrimp feeds on other shrimp or fish killed by vibriosis, or ingests vibrio-contaminated materials in the water. However, since only a relatively low mortality (16% ca.) in brown shrimp was caused by repeated ingestions of relatively high dosages of V. parahaemolyticus (from 10^8 to 10^9 CFU/shrimp) (Fig. 8), Vibrio bacteria are probably not highly virulent to juvenile penaeid shrimp in nature. They are more likely opportunistic organisms, causing mortalities only when the shrimp encounter unfavorable environmental conditions. This assumption is perhaps particularly true with V. alginolyticus, which displayed the lowest virulence to white shrimp in our studies. Vibrio alginolyticus as well as V. alginosus have been observed to be present in concentrations of from 10^2 to 10^4 CFU/ml in shrimp raceway water without causing significant loss to juvenile white shrimp (Leong et al., unpublished data). Experimentally, V. parahaemolyticus, when added directly to aquarium water to reach a final concentration of from 10^4 to 10^5 CFU/ml, was found to be either highly lethal to brown shrimp (Vanderzant et al., 1970) or harmless to juvenile pink shrimp and postlarval brown shrimp (Barkate, 1972). Piecing all the data together, it appeared that a Vibrio bacterium may become injurious to juvenile penaeid shrimp when its concentration reaches 10^5 CFU or more per ml. Therefore it is apparent that raceway or aquarium water should be kept from containing excess amounts of substrates, such as shrimp feed, which could sustain high concentrations of Vibrio bacteria.

Figure 8 showed that the lethal effect of V. parahaemolyticus subsided very rapidly when administered orally to brown shrimp. Mortality did not resume until repeated doses of bacteria were fed. Those events suggested that the death of the test shrimp probably resulted from the action of a toxin, or toxins, which were associated with V. parahaemolyticus and retained in the substrate shrimp meat, rather than the direct multiplication effect of that bacterium.

The lack of immediate mortality response in test shrimp following the feeding of the 3rd challenging dose of a 24-hr culture of V. parahaemolyticus (5.4×10^8 CFU/meat piece) indicated that the surviving shrimp either were intrinsically more resistant to the bacteria (or to their toxins) or have acquired a certain degree of immunity from the two previous injections. Penaeid shrimp have been reported to produce a hemolymph component which resembles vertebrate beta globulin within 48 hr after exposure to V. anguillarum (Lewis, 1973).

When a subsequent 4th feeding of 48-hr infected shrimp meat was made, the corrected mortality of the shrimp rose slightly again by 1%. Such increased mortality probably resulted from the use of a higher dose of challenging bacteria (7.3×10^9 CFU/meat piece) in the 4th feeding, overcoming the resistance of some of the surviving shrimp.

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