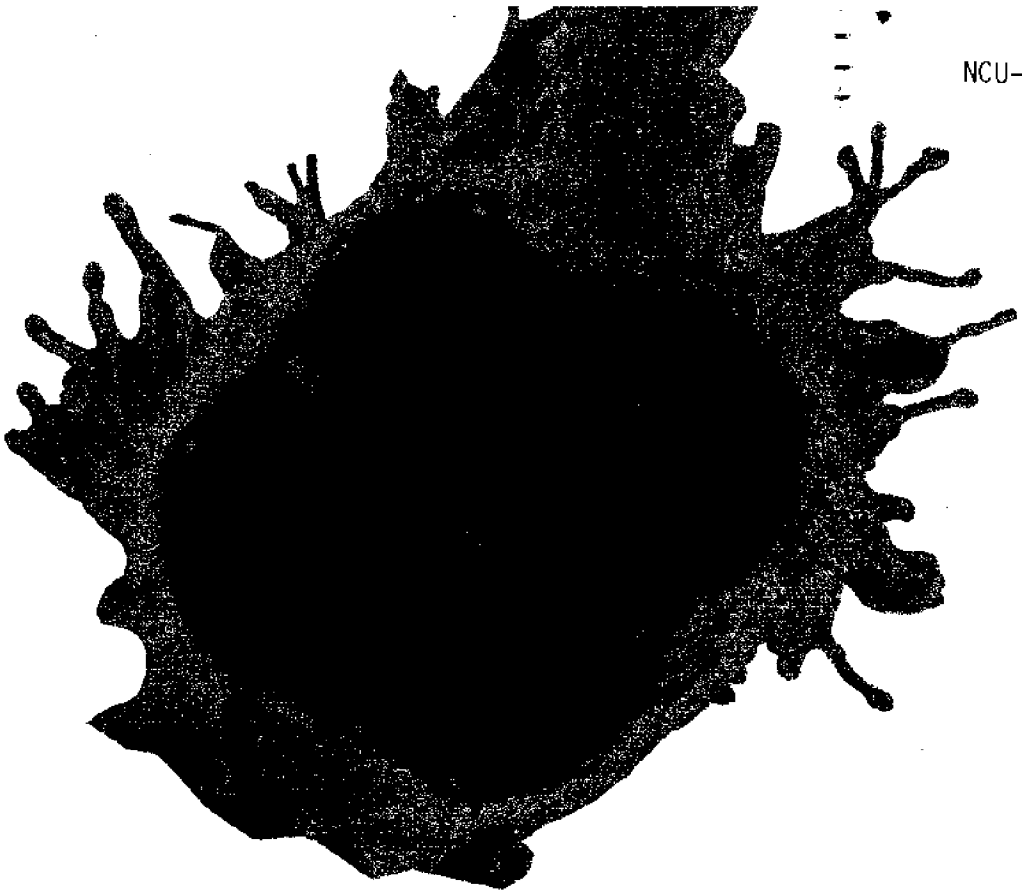

Chemical control of Lagenidium,
a fungal pathogen of marine crustacea

C.E. Bland
D.G. Ruch
B.R. Salser
D.V. Lightner

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CHEMICAL CONTROL OF LAGENIDIUM, A FUNGAL PATHOGEN OF MARINE CRUSTACEA

by

C. E. Bland and D. G. Ruch
Department of Biology, East Carolina University

B. R. Salser and D. V. Lightner
Environmental Research Laboratory
Tucson, Arizona

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University of North Carolina Sea Grant Program, 1235 Burlington Laboratories, North Carolina State University, Raleigh, North Carolina 27607

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ABSTRACT

The effects of twelve potentially fungitoxic compounds on growth and development of two strains of Lagenidium callinectes Couch were studied. The compounds tested were Benlate, Captan, Dichlone, Difolatan, DS 9073, Dyrene, malachite green, Manzate 200 (Dithane M-45), Terrachlor, Treflan, tribasic copper sulfate, and Vitavax. In vitro tests included determination of the minimum lethal concentration (LC_{100}) of each fungicide, the effects of the LC_{100} on length of motility, flagellar retraction or detachment, encystment and germination, and the length of time necessary for the LC_{100} of each fungicide to kill zoospores of L. callinectes. Various animal toxicity tests were conducted also with several of the fungitoxic compounds. In evaluating the total efficacy of these twelve compounds, only two, malachite green and DS 9073, appear to be of potential use as disease deterrents in mariculture. The problems associated with the use of each of these compounds are discussed.

INTRODUCTION

Although much is known concerning terrestrial and fresh-water fungi and the diseases which they cause, very little is known of their counterparts in the marine environment. Evidence of this is revealed in the fact that of the more than 50,000 species of fungi known to man, only 450-500 have been described from the sea with fewer than 25 of these being known pathogens. The purpose of this comparison is not to claim that the number of fungi and fungal pathogens in the sea is or ever will be equal to those known from terrestrial and freshwater habitats, but rather to dramatize the need for additional information concerning marine fungi. The need for this information is becoming increasingly evident with man's expanding exploitation of the sea through sea farming and other forms of mariculture. In such cases, in which marine animals and plants are raised under crowded situations with less than optimal environmental conditions, it appears that, just as in terrestrial farming, diseases caused by fungi and other pathogens will be a serious problem. In this regard, the present paper describes the results of an investigation into control of the fungus, Lagenidium, which is already causing serious disease problems in cultured marine crustacea.

The numerous, recent reports concerning the infection of larval marine crustacea with fungi of the genus Lagenidium (see Bland, 1975 for review) indicate the significance which this and similar pathogens may have in the future of crustacean culture. Originally described by Couch (1942) on ova of the blue crab, Callinectes sapidus, collected from Chesapeake Bay,

Virginia, strains of Lagenidium have now been recognized on and/or isolated from cultured shrimp larvae from Texas (Lightner and Fontaine, 1973), Florida (Barkate, personal communication), Mexico (Lightner, personal communication), Tahiti (Le Bittoux, personal communication), and Honduras (Herrin, personal communication); Dungeness crab larvae from Oregon (Armstrong and Buchanan, unpublished manuscript); and American lobster larvae from California (Nilson, Fisher and Schleser, unpublished manuscript). In many instances, mortalities attributed to this fungus reached a level of 100%. The question arises therefore, what if anything can be done to prevent or control the infection of cultured larval marine crustacea with the fungus, Lagenidium?

Since propagules, the infective agent, of Lagenidium are probably widely distributed in sea water (Sandoz, Rogers, and Newcombe, 1944; Rogers-Talbert, 1948; Scott, 1962; Fuller, Fowles, and McLaughlin, 1964; Gotelli, 1969; Bland, 1974), one approach to control might be the filtration and/or sterilization of all sea water used during the culture of larval crustacea. Although this procedure would be rather expensive, it could undoubtedly be highly effective were it not for the fact that eggs still have to be obtained from ovigerous females collected from the sea and therefore themselves represent possible carriers of the fungus.

Another possibility for control might be the maintenance of crustacean larvae under environmental conditions unfavorable to development of the fungus. This approach is impossible at present in that very little is known of the growth parameters of the various strains of Lagenidium. In this

regard, comparative physiological and nutritional studies of several strains of Lagenidium are now underway in the author's laboratory.

A third approach to the control of pathogenic fungi in aquaculture situations is the use of fungitoxic chemicals which would inhibit the growth of fungi but cause no harm to larval crustacea. Although there is at present virtually no information available concerning this subject, some limited success has been reported by Fisher, Nilson, Follett and Schleser (1975, unpublished manuscript) who used a malachite green dip combined with ultraviolet irradiation to successfully prevent Lagenidium infection of larvae of the American lobster, Also, Armstrong and Buchanan (1975, unpublished manuscript) found the compounds, Treflan and Captan to be effective in preventing spread of Lagenidium from infested hemp seeds (Cannabis sativa, a commonly used substrate for the growth of aquatic fungi) to uninfested seeds at concentrations which were non-toxic to larvae of the Dungeness crab. The purpose of the present paper is to present the results of an investigation into the effects of twelve potentially fungitoxic compounds on growth and development of two strains of Lagenidium and to describe also investigations into the toxicity of certain of these compounds to the larvae of several marine crustacea.

MATERIALS AND METHODS

Organisms Used, Culture Conditions and Zoospore Induction

Two strains of Lagenidium were used: L-1, isolated by Bland and Amerson (1973) from ova of crabs collected in the Newport Estuary, North Carolina, and L-3b, isolated by Lightner and Fontaine (1973) from larvae of the white shrimp, Penaeus setiferus, raised at the Gulf Coastal Fisheries Center, Galveston, Texas. Stock cultures of both strains were maintained on Cantino's PYG agar and broth (Difco Laboratories) made up in sea water of 30 ppt salinity (PYGS). These cultures were propagated by the procedure described by Bland and Amerson (1973). To obtain cultures for research, 50 ml of PYGS broth were inoculated with 60,000 spores* (spore numbers were determined by counts made with a hemacytometer) and grown at 25 C on a horizontal rotary shaker (109 rev / min) for 72-78 hr. Zoospore production was initiated by placing the mycelia of a 72-78 hr broth culture in sterile sea water. Release of zoospores occurred approximately 17-18 hr thereafter and continued over a 4-6 hr period.

Compounds Tested

The twelve compounds tested for effectiveness in controlling Lagenidium are listed in Table 1. These compounds were selected in order to compare the fungitoxicity of compounds from several different classes of fungicides.

*Previously determined to result in optimum growth and sporulation.

In Vitro Tests

To prepare the PYGS-fungicide broth, each fungicide was added at varying concentrations (ppm active component) to PYGS broth after autoclaving. (Because so many different concentrations were used in determining the minimal lethal concentration of every fungicide, they will not be listed here. These are, however, shown on the various dosage-response graphs.) The PYGS-fungicide medium was then poured into sterile 125 ml Erlenmeyer flasks, so that each flask contained exactly 50 ml (Although some of the fungicides were water soluble, it should be pointed out that most were not. When pouring media containing these non-water-soluble fungicides, constant agitation was used to insure a uniform suspension of the fungicide). Flasks were inoculated with 60,000 spores using an Aupette automatic syringe. After inoculation, the cultures were placed on a horizontal rotary shaker at 25 C for 72 hr. The LC_{100} of each compound was determined by dry weight analysis of 3 day old mycelia which were collected with a Buchner funnel on tared Whatman #1 qualitative 5.5 cm filter paper. Specimens were then oven-dried at 70 C for 2 days, placed in a desiccator for approximately 2 hr to allow cooling to room temperature, and weighed on an H16 Mettler balance.

The effect of the lethal concentration of each fungicide on zoospore motility as well as the length of time necessary for each fungicide to kill the spores were studied also. The latter was tested by placing 60,000 spores in 50 ml of PYGS broth containing the LC_{100} of a fungicide. Samples were withdrawn from this flask every 15 min for a total of 60 min and used

to inoculate 50 ml of PYGS broth. These cultures were placed on a horizontal rotary shaker for 72 hr to observe growth.

All results represent a composite of a minimum of 5 different tests.

Animal Toxicity Tests

The effects of each of the twelve fungicides tested on hatching and development of brine shrimp (Artemia salina) were tested by placing 0.05g of brine shrimp eggs in 50 ml of sterile sea water containing the concentration of the fungicide found to be lethal to zoospores of Lagenidium (L-1 isolate). These cultures were observed daily for a nine-day period.

Based on what were thought to be low LC_{100} concentrations, two compounds, Captan and Manzate 200 (Dithane M-45) were selected early in the study for further animal toxicity tests against penaeid shrimp larvae. In these tests, protozoeae I and mysis I larvae of Penaeus aztecus were exposed in sea water (30 ppt) to concentrations of each compound ranging from 0.1 ppm to 5 ppm.

In later tests, three compounds, Treflan, Terrachlor, and malachite green, were used in 24 hr static, bioassays on naupliar, protozoeal, mysis, and post larval stages of P. californiensis, P. stylirostris, and P. vanammei.

One additional test involved the use of malachite green at 0.006 ppm as a treatment for an epizootic in protozoea of P. stylirostris and P. californiensis due to a species of Lagenidium. Larvae used in this experiment were hatched at Puerto Peñasco, Mexico from spawners taken from a shallow bay (Cholla Bay) near Puerto Peñasco.

RESULTS

In Vitro Tests of Compounds

The relative effectiveness of each compound tested in controlling the growth of Lagenidium (L-1 and L-3b) was determined on the basis of its minimum lethal concentration, i.e. the lowest concentration at which no growth occurred. Results of these tests as well as the dosage response curve for each compound are given in Figures 1-12. Additionally, comparisons of the minimum lethal concentration of each compound tested are given in Figure 13 for strain L-1 and Figure 14 for strain L-3b. For both strains, the highest level of fungicidal activity was exhibited by malachite green while the lowest level was with tribasic copper sulfate. For all compounds tested, with the exception of tribasic copper sulfate and Treflan, the L-3b strain was more resistant than was the L-1 strain. Although Treflan was fungicidal to L-1 and L-3b at 5.0 ppm and 3.0 ppm respectively, this compound proved to be highly fungistatic at much lower concentrations (Fig. 7). For example, at a concentration of 1.0 ppm, the growth of L-1 in Treflan was reduced 92%, while the growth of L-3b was decreased 97%. However, at concentrations lower than 1.0 ppm, the effectiveness of Treflan quickly diminished.

The two compounds found most effective in reducing the swimming time of zoospores of both strains L-1 and L-3b were DS 9073 and Treflan (Tables 2 and 3). These chemicals at their minimal lethal concentrations caused complete immobilization of zoospores within 1 min. The only other fungicides that reduced the duration of zoospore motility significantly were Dichlone, Difolatan, Vitavax, and Captan. Other fungicides, although reducing the

duration of motility of both strains, were considerably less effective. It should be noted that malachite green, although having the lowest LC_{100}, had virtually no effect on the motile spores of L-1 at this concentration.

Since LC_{100} concentrations of both DS 9073 and Treflan both had an immediate effect on zoospore motility, tests to determine the effects of sublethal concentrations of each on the duration of motility of zoospores were conducted. DS 9073 (Fig. 15) at lower concentrations exhibited a greater influence on zoospore motility than Treflan (Fig. 16). At 0.1 ppm active component, the lowest concentration tested, this experimental bactericide/fungicide caused complete inactivation of L-1 zoospores within 3.5-4 min and L-3b zoospores within 4.5-5 min. As the concentration of this chemical increased, the duration of motility decreased. Immediate immobilization of L-1 zoospores occurred at concentrations of 1.0 ppm and higher, while L-3b zoospores required a concentration greater than 3.2 ppm. With Treflan, however, at 0.5 ppm active component, the lowest concentration tested for this compound, zoospores of L-1 remained motile for up to 24 min, while zoospores of L-3b swam for up to 27 min. When a concentration of 1.0 ppm was used, however, the duration of motility of zoospores of both strains was greatly decreased. At this concentration L-1 zoospores stopped swimming within 9-10 min and L-3b zoospores stopped swimming within 6-7 min. Immediate cessation of zoospore movement of both strains occurred in concentrations greater than 6.5 ppm. In sublethal concentrations of DS 9073 or Treflan, most zoospores would germinate.

Although most fungicides at the LC_{100} concentration inhibited germination (Tables 2 and 3), cysts of both L-1 and L-3b did germinate in lethal concentrations of malachite green, Terraclor, tribasic copper sulfate, and Vitavax.

Cysts of L-1 also germinated in Benlate. Even though germination did occur in these fungicides, the germ tubes were greatly distorted and extremely short, 14-96μ. After three days exposure, these short germ tubes were no longer growing and were devoid of cytoplasm, indicating that death had occurred.

The final in vitro test was to determine if zoospores would be killed when placed in the LC_{100} of each fungicide for exposures of 15 min to 1 hr (Table 4). In this, the most effective fungicide was DS 9073, which killed the zoospores of both L-1 and L-3b within the first 15 min exposure. The two dicarboximide fungicides, Captan and Difolatan, killed the zoospores of both strains when exposed for 30 min. The only other fungicides to render zoospores non-viable within 1 hr were Manzate 200 and Treflan. In both cases zoospores of L-1 would not germinate after being exposed to the LC_{100} of these compounds for 60 min.

Animal Toxicity Tests

Tests to determine the toxic effects of each fungicide used in this study on hatching and development of brine shrimp, revealed that three fungicides had no apparent effect on these biological processes. These fungicides, Captan, malachite green and Manzate 200, did not differ significantly from the control at the 0.01 level of significance either in the total number of brine shrimp hatched or the number surviving from day to day over the nine day test period (Table 5). Hysmith (personal communication) observed similar results when brine shrimp were placed in the LC_{100} (L-1 strain) of Captan or Manzate 200. The fungicides most toxic

to brine shrimp were Benlate, Vitavax, DS 9073, Treflan, and tribasic copper sulfate (Table 5). Benlate, and in many cases Vitavax, acted as an ovicide. In some instances the eggs simply burst, so that the immature brine shrimp were expelled from the egg cases and contained within a vesicle. These brine shrimp did not appear to be alive and no development beyond this stage was observed.

Toxicity tests involving the compounds Manzate and Captan against protozoeae I and mysis I larvae of P. aztecus showed fungitoxic levels of both these compounds to be highly toxic (55-100% mortality as opposed to controls of 10-35% mortality) to both protozoeae I and mysis I larvae.

Results of the three 24 hr bioassay-type experiments involving the compounds malachite green, Terrachlor, and Treflan against P. californiensis, P. stylirostris and P. vanammei are given in Tables 6, 7, and 8 respectively.

For malachite green, it appears that fungitoxic concentrations (0.006-0.012 ppm) of this compound have little or no effect on larval survival for either P. californiensis, P. stylirostris or P. vanammei. In fact, in several instances larvae treated with this compound exhibited higher survival percentages than the controls. Severe toxic effects of malachite green were not evident until a treatment level of 0.06 ppm was used with P. stylirostris. In the latter case, only the protozoea were sensitive to a 0.06 ppm concentration of malachite green whereas the nauplii and mysids were not affected until the concentrations reached 0.6 ppm. Malachite green concentrations of 0.6 ppm and above exhibited some toxicity to all larval stages.

For Terrachlor (fungitoxic level 1.3-4.3 ppm) all larval stages of P. californiensis showed tolerance at a treatment level of 1.3 ppm. Concentrations greater than this reduced greatly survival percentages. For P. stylirostris and P. vanammei, larval survival, however, was severely diminished by even the lowest fungitoxic level of Terrachlor.

With Treflan (fungitoxic level 3.0-5.0 ppm), a treatment level of 3.0 ppm had virtually no effect on larvae of P. californiensis whereas doubling this concentration to 6.0 ppm reduced survival of all larval stages tested by one-quarter to one-half the control. For P. stylirostris and P. vanammei however, the fungitoxic levels of 3.0 to 6.0 ppm reduced larval survival to 0%-20% of the control for all stages but the mysids of P. stylirostris where survival did not differ significantly from the control.

In the above, tests involving Terrachlor concentrations in excess 0.325 ppm and Treflan concentrations in excess 0.375 ppm resulted in aglutination of the algal foods during the protozoeal test series resulting in an associated high mortality.

Results of the use of malachite green in treating an epizootic in P. stylirostris and P. californiensis are given in Table 9. In these tests, malachite green at a concentration of 0.006 ppm was shown to be highly effective in arresting the epizootic. In the treated populations of both P. stylirostris and P. californiensis, there was no significant change in numbers between the pretreatment and 48 hr postreatment population. Small differences in population numbers between pre and postreatment were due to errors inherent in the method used in estimating larval populations and are

not significant. In hatch 28 of P. californiensis, however, in which malachite green treatment was omitted, therewere significant differences between the pretreatment and postreatment populations. It should be noted that in all of the tanks in which malachite green was used, Lagenidium sp was not detected after treatment.

DISCUSSION

In a paper concerning the vulnerability of fungal spores to fungicides, Byrde (1966) described the fungal spore in the following way: "The lone fungal spore, forced to remain unprotected on the leaf surface for at least several hours before it can penetrate the host, constitutes the most vulnerable stage in the life history of the pathogen." Essentially the same may be said of Lagenidium in its relationship to marine crustacean hosts. Therefore, the fungitoxic effects of a variety of compounds on zoospores of Lagenidium callinectes were tested. The following is a discussion of results obtained in the present study in relation to similar or related work of other investigators.

Tribasic Copper Sulfate: Tribasic copper sulfate, the only inorganic fungicide tested, was the least effective in that it required an excess of 150 ppm for control of Lagenidium. The relative ineffectiveness of this fungicide in controlling various diseases of fungal etiology has been described previously by Berquist, 1972; and Martin, 1969; and confirms the results obtained in the present study.

Even if tribasic copper sulfate had been found to be effective in controlling L. callinectes, the feasibility of its use would be in question.

This is due in part to the detrimental effects that heavy metal ions have on certain components of the environment and in part to the increasing use of the more efficient organic fungicides. In reference to the former, Topping (1973a,b) reported the accumulation of several heavy metal ions, including copper, in shellfish (lobster, crab and scallops) and fish collected from polluted Scottish waters. Thus, even had it been found effective, the use of this fungicide would be highly questionable at this time.

Benlate (benomyl) and Vitavax: The systemic fungicides, Benlate and Vitavax, have been found ineffective in the control of several important groups of fungal pathogens, including all the phycomycetes (Berquist, 1972; Bollen, 1971; Borum and Sinclair, 1967; Edgington, Khew, and Barrow, 1971; Edgington, Walton, and Miller, 1966; Edgington and Kelly, 1966). These findings were supported by the present study.

The use of Benlate as a mite ovicide was first reported by Delp and Klopping (1968a,b), and later by Upham and Delp (1973). Similar results were obtained in the present study when brine shrimp eggs were placed in solutions of Benlate. Although there have been no reports describing the possible ovicidal effects of Vitavax (oxathiin), in many cases results similar to those with Benlate were obtained in the present study when brine shrimp eggs were placed in solutions of Vitavax.

Dyrene, Dichlone and Terraclor: Since Dyrene was only introduced recently, its complete spectrum of control has yet to be fully realized (Lukens, 1969). However, the use of Dyrene, as well as Dichlone and

Terraclor, in the control of fungal diseases encountered in mariculture of marine crustacea is extremely questionable since all these compounds with the exception of Terraclor, require large concentrations for control of Lagenidium. For Terraclor, although the minimum lethal concentration is low, it has been shown that fungi can build up a tolerance to this compound (Georgopoulos, 1962). Another problem with all three of these fungicides is that they have only a moderate effect on zoospore motility (Tables 2 & 3). As such, even at high concentrations these compounds may not be able to check the spread of the disease.

Treflan: Since Treflan is an agricultural herbicide, little is known about its fungitoxic properties. However, Khurana and Singh (1972) found that Treflan greatly decreased the growth of the deuteromycete, Curvularia lunata, at 10 µg/ml (10 ppm). Similar results were obtained by Rodriguez-Kabana, Curl and Funderburk (1969) who reported that Treflan at 10 ppm reduced mycelial production of the deuteromycete, Sclerotium rolfsii. In both of these studies, mycelial growth was greatly reduced but not completely inhibited. A similar fungistatic response was obtained when spores of Lagenidium were exposed to low concentrations of Treflan. However, the toxicity of Treflan to cultured organisms could be a limiting factor to its use in mariculture. The LC_{50} of Treflan for fish in standard aquaria tests was 0,58 ppm for bluegills, 0.94 ppm for fathead minnows and 0.59 ppm for goldfish (Anonymous, 1973a). In the present study Treflan, at the LC_{100} level, was the most toxic chemical tested against brine shrimp larvae (Table 6).

However, because of the fungistatic nature of this chemical, it is possible that lower concentrations than required for fungitoxicity may reduce the growth of L. callinectes sufficiently for control and may be less toxic to marine organisms. In this regard, Armstrong and Buchanan (unpublished manuscript) report Treflan to be effective against transfer of Lagenidium from infected to uninfested hemp seed halves (Cannabis sativa) at concentrations of 0.005 ppm and 0.05 ppm for 48 hr and 96 hr tests respectively. These concentrations were found by the same investigators to be significantly lower than the LC_{50} concentration (0.3 ppm) of this compound for larvae of the Dungeness crab, Cancer magister. It appears, therefore, that Treflan may be effective in actual use at concentrations considerably lower than that necessary to bring about an LC_{100} of Lagenidium. This is undoubtedly due to the drastic reduction in time of zoospore motility which is accomplished by treatment with low concentrations of this compound. Additionally, Treflan may in some way actually inhibit the process of sporogenesis. Further investigations into this possibility are in order.

Captan and Difolatan: Although the dicarboximide fungicides, Captan and Difolatan, have a wide spectrum of fungicidal control, they are most effective against the phycomycetous pathogens (Lukens, 1969). Berquist (1972) found that Difolatan was second only to Dithane M-45 (Manzate 200) in controlling Phytophthora colocasiae. He found the minimal lethal concentration in vitro to be 9 ppm. There have been many other studies demonstrating the effectiveness of both Captan and Difolatan against fungal pathogens (Corbin, Lider, and

Roberts, 1968; Dancs, Csorba, and Pozsar, 1970; Hartill, 1968; Horsfall, 1956; McCallan, Miller, and Weed, 1954). The results of these studies were corroborated by the present study, in that, both Captan and Difolatan had low LC_{100}.

Manzate 200: Like Captan, Manzate 200 has a broad spectrum of fungicidal control. Its effectiveness is well documented (Owens, 1969). The use of the dicarboximide fungicides and Manzate 200 in marine mariculture is limited, since all of these compounds are toxic to marine crustacea. Costlow (personal communication) reported that LC_{100} concentrations (L-1 strain) of Manzate 200 or Captan were acutely toxic to both Callinectes sapidus zoeae and Menippe mercenaria megalopa. All deaths of C. sapidus zoeae occurred within 12 days and in this first stage of development, indicating that both fungicides restricted the molting process. The lethal effects of both these fungicides to larval stages of brown shrimp (Penaeus aztecus) were shown also in the present study. However, Hysmith (personal communication) found no mortality when protozoeae II stage larvae of P. aztecus were exposed to a 1 hr treatment with Manzate at 2.62 ppm. Since our in vitro tests indicate that spores of Lagenidium (L-1) are dead after a 1 hr exposure to the LC_{100} of Manzate, the use of this compound as a curative or preventive dip may be possible.

Malachite Green: Malachite green is used in fish hatcheries primarily as a curative or preventive dip for fungal diseases. In such treatments infected fish are dipped in a solution of malachite green, containing from 2 to 66 ppm, for 10 seconds to 5 minutes, depending on the severity of the

infection and the species of fish (Foster and Woodbury, 1936; O'Donnell, 1941; Scott and Warren, 1964). Sustained fish culture in this chemical is impossible, even at 2 ppm, because the dye penetrates to the inside of the gill filaments and results in death of the fish. However, Scott and Warren (1964) suggested that at concentrations lower than 2 ppm sustained fish culturing may be possible. In the present study, it was found that at concentrations of 0.006-0.01 ppm, the LC_{100} of malachite green for Lagenidium strains L-1 and L-3b, there is apparently no effect on either the hatching and development of brine shrimp or on larvae survival of P. californiensis, P. stylirostris and P. vanammei. Similarly, Johnson (1974) reported malachite green to be non toxic to larvae of P. duorarum when the compound was used at concentrations of 0.64 ppm and below for a 96 hr period. Thus, sustained culturing of marine crustacea in concentrations of malachite green sufficient to inhibit the growth of pathogenic fungi may be possible. In reference to this, since it appears that crustacea are only susceptible to infection with Lagenidium for a time period of about 7 days during their early larval development, it may be necessary to treat only during this period, after which the larvae could be transferred to non-treated water. Furthermore, it should be noted that solutions of malachite green lose their effectiveness in 2 to 3 days after mixture. This may be a plus factor in its use since once it kills the fungus it decomposes to what may be nontoxic derivatives. In related studies, Fisher, Nilson, Follett, and Schleser (unpublished manuscript) prevented infection of larvae of Homarus americanus by a strain

of Lagenidium by placing the larvae in a 5 min dip of 5 ppm malachite green prior to culture in water with ultraviolet irradiation. However, Armstrong and Buchanan (unpublished manuscript) found malachite green to be ineffective in controlling one strain of Lagenidium at concentrations which were non toxic to larvae of the Dungeness crab, Cancer magister. In summary, it appears that malachite green may have potential as a deterrent or cure for fungal diseases encountered in the culture of certain marine crustacea. However, before this compound can be used to treat diseases of food organisms much additional testing is in order and approval must be obtained from the U. S. Food and Drug Administration.

DS 9073: As mentioned earlier, the LC_{100} of the bactericide/fungicide DS 9073 caused immediate cessation of swimming of Lagenidium zoospores. This was true even at various sublethal concentrations (Fig. 16). Sonoda, Ogawa, Lyons, and Hanson (1970) found that there was a direct correlation between immobilization of zoospores by Difolatan and Dithane M-45 and the control of Phytophthora root and crown rot. They observed that increasing the concentration of the fungicide reduced motility time, which in turn, checked the spread of the disease. Since DS 9073 may possibly be toxic to marine organisms at the LC_{100} of the L-1 strain (not tested at this time), it is possible that sublethal concentrations of this compound, while decreasing the time of zoospore motility greatly, may be non toxic to marine animals. Further tests to determine this possibility are needed.

One point in favor of DS 9073 as an agent of disease control is that it is readily soluble in hot or cold water. Additionally, it has also been

shown to control the phycomycete Phytophthora citropthora at less than 50 ppm in vitro (Anonymous, 1973b).

SUMMARY

Results obtained in the present study indicate that only two, malachite green and DS 9073, of the twelve compounds tested may be of use in preventing or controlling infection of cultured marine crustacea with the fungus, Lagenidium. Of these two, malachite green has been found highly toxic to the fungus at concentrations which appear to be harmless to the larvae of certain crustacea. The other compound , DS 9073, although not tested for its toxicity to crustacean larvae, is of potential use because it is toxic to the fungus at relatively low concentrations and because it rapidly disables the infective agent of the fungus at concentrations much lower than the toxic level. A third compound, Treflan, although found to be unsuitable in the present study, may have application in certain situations as Armstrong (personal communication) has found it to be effective in controlling the growth of Lagenidium at concentrations which do not affect larval development in the Dungeness crab, Cancer magister.

In interpreting the above described results, it should be understood that the authors are not advocating the immediate, widespread use of such chemicals in crustacean culture. Indeed, before these compounds can be used for other than experimental purposes, additional studies must be conducted concerning their long term effects on crustacean development as well as the level at which such compounds are retained in the body tissues of crustacea. Additionally,

approval from appropriate governmental agencies will have to be obtained before any of these chemicals can be used in the culture of food organisms.

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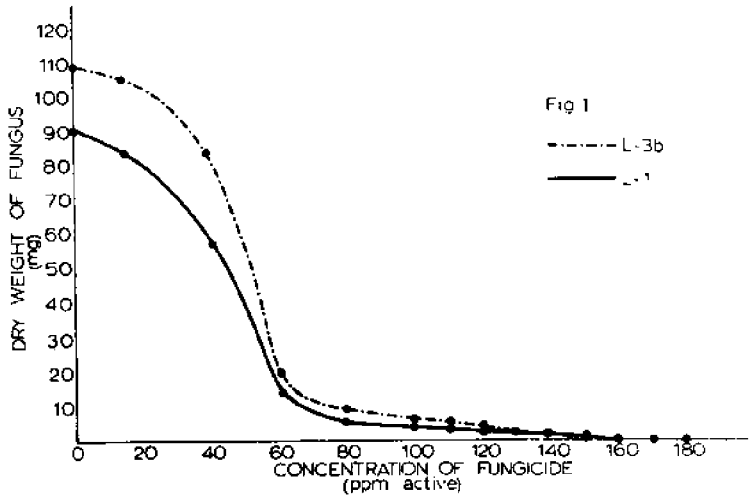
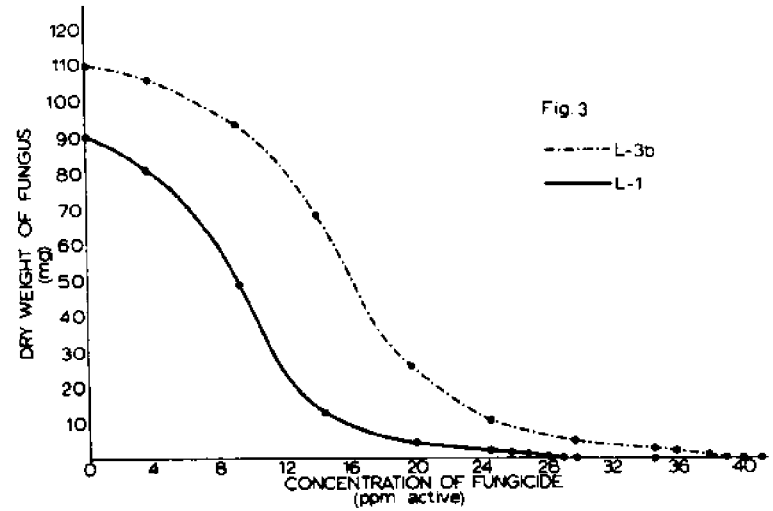
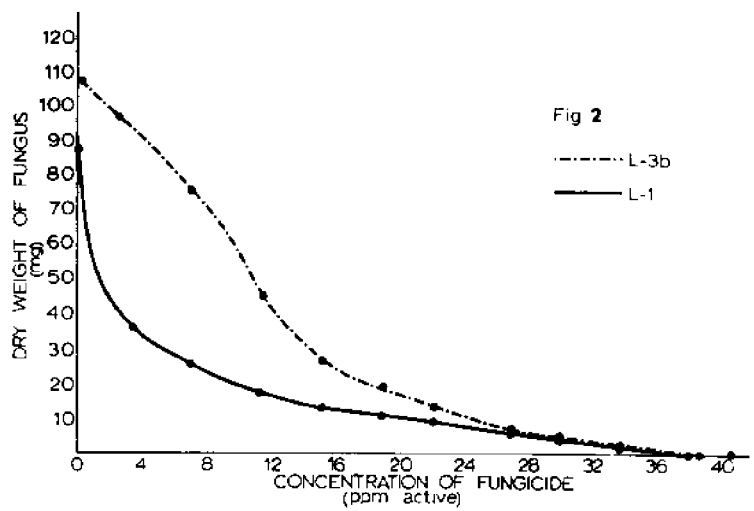
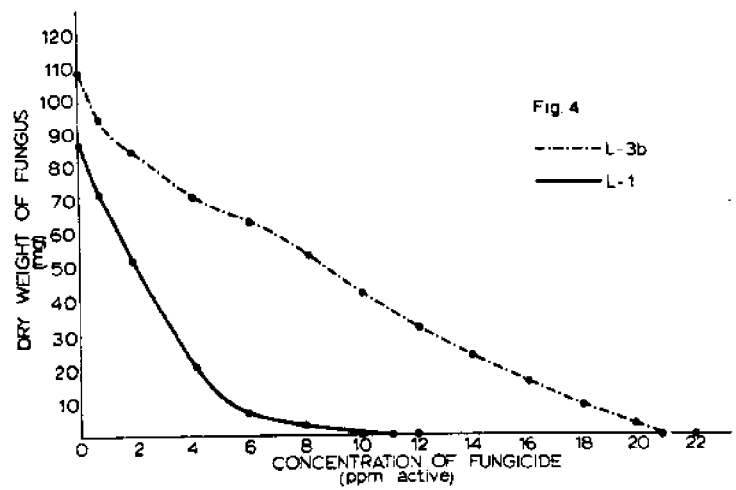
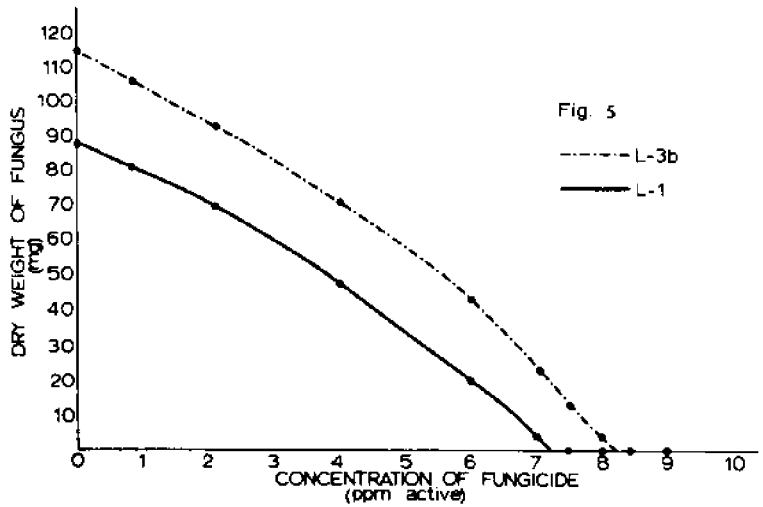


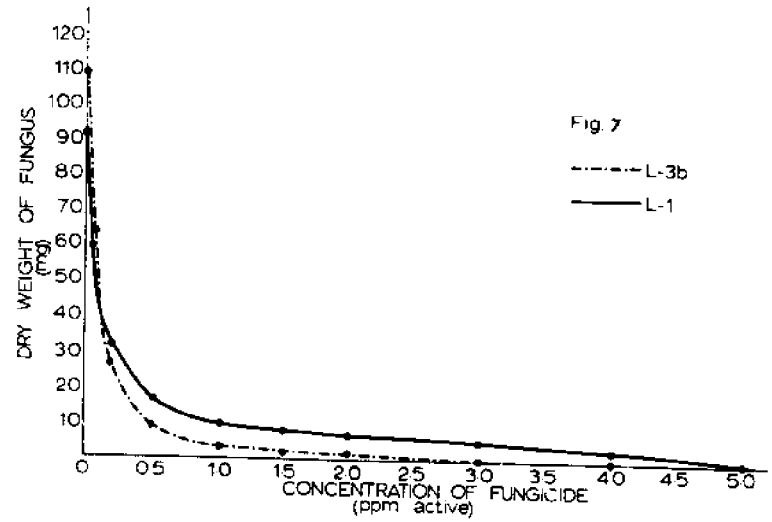


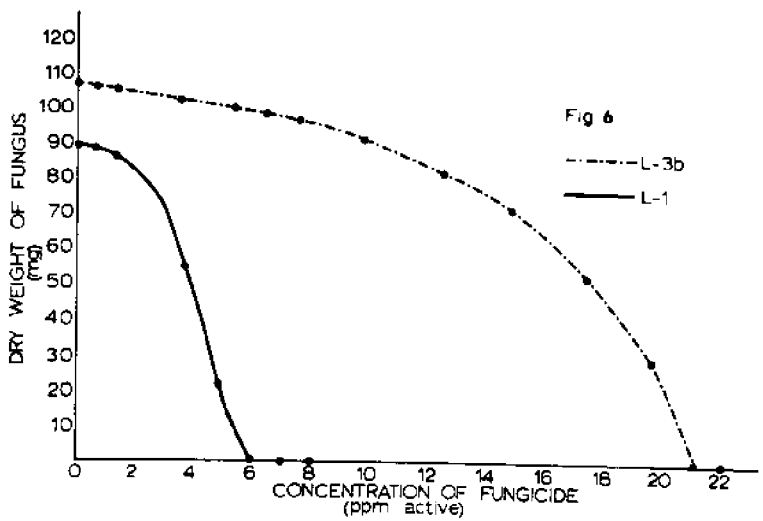


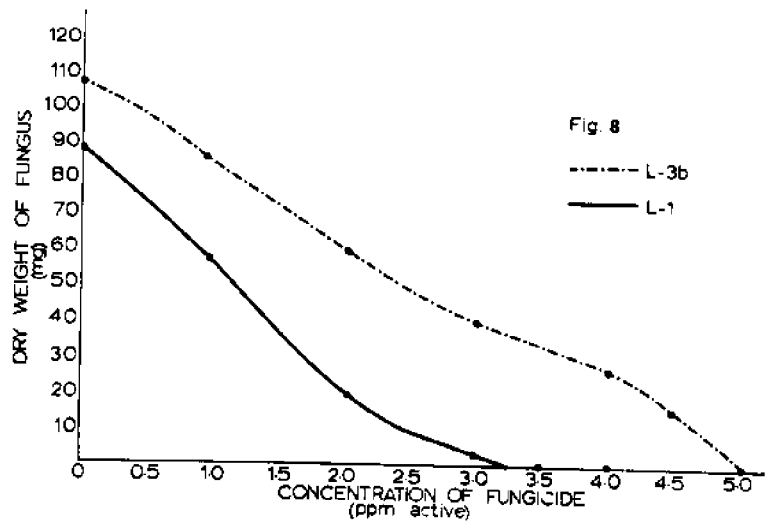


Figs. 1-4. Dosage response curves for Lagenidium strains L-1 and L-3B in compounds as follows: Fig. 1, tribasic copper sulfate; Fig. 2, Vitavax; Fig. 3, Benlate; Fig. 4, Dichlone.

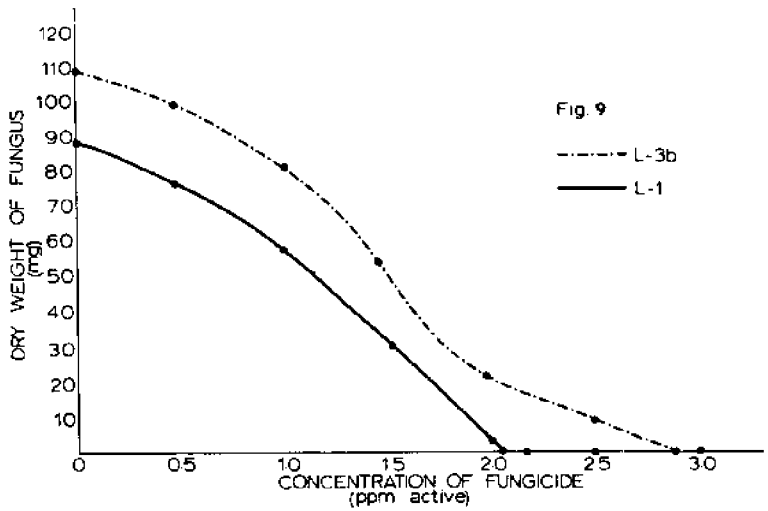


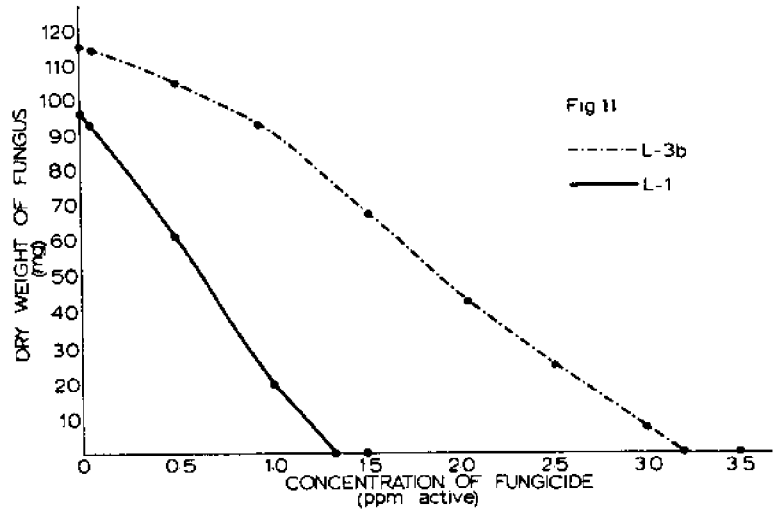


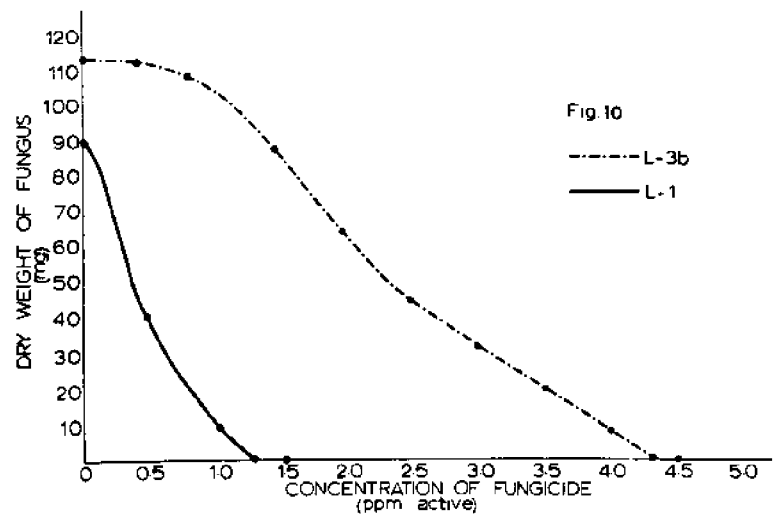


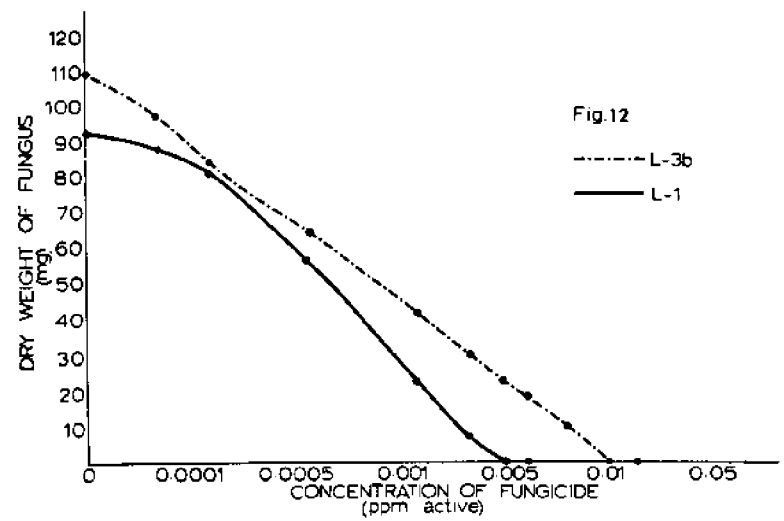


Figs. 5-8, Dosage response curves for Lagenidium strains L-1 and L-3B in compounds as follows: Fig. 5, Difolatan, Fig. 6, Dyrene; Fig. 7, Treflan; Fig. 8, Captan.

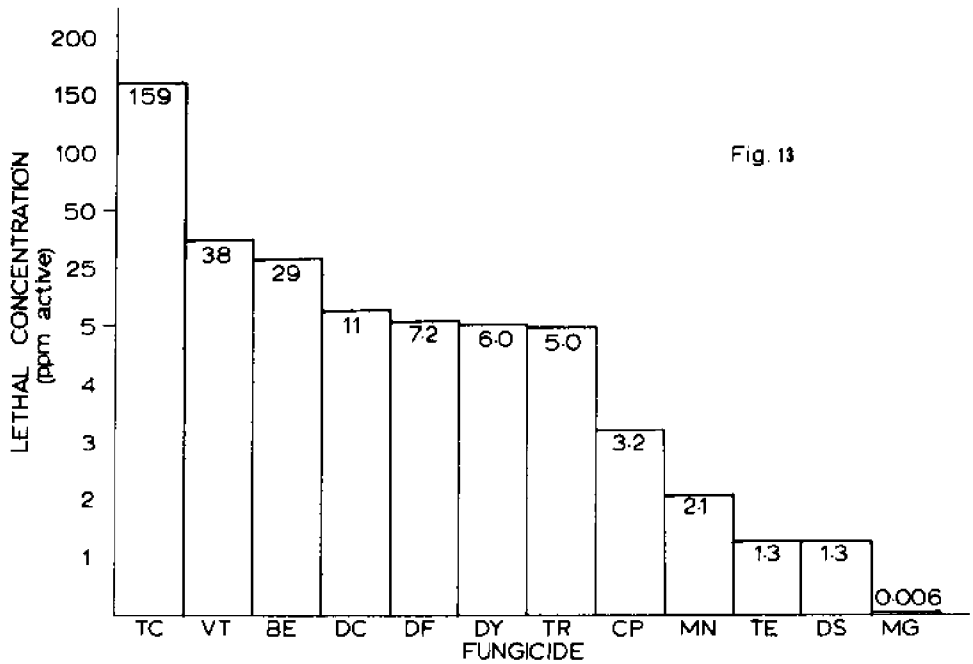








Figs. 9-12. Dosage response curves for Lagenidium strains L-1 and L-3B in compounds as follows: Fig. 9, Manzate 200 or Dithane M-45; Fig. 10, Terrachlor; Fig. 11, DS 9073; Fig. 12, malachite green.



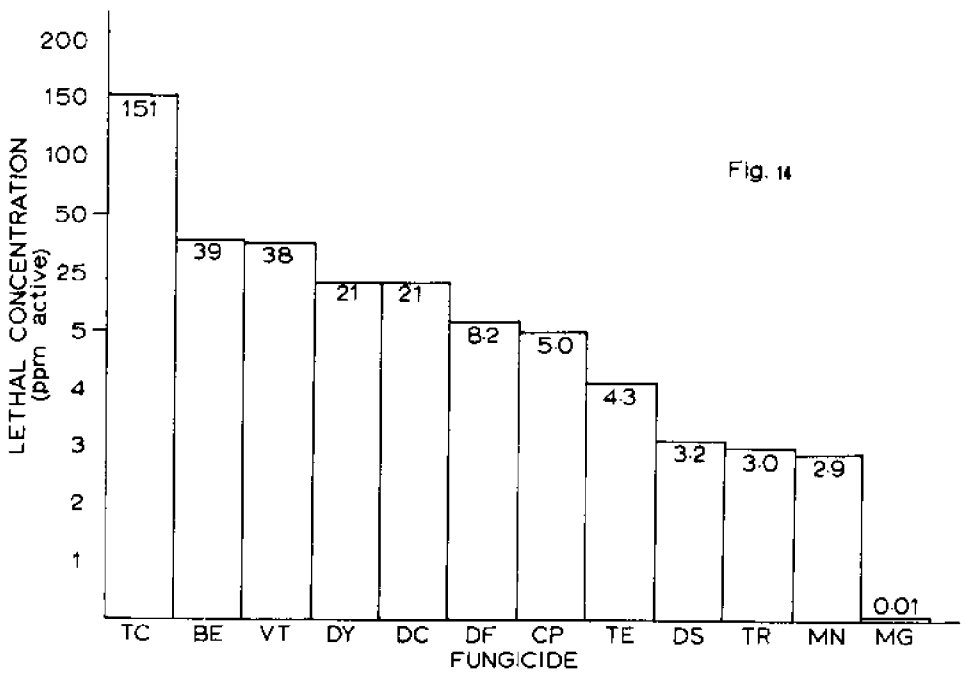
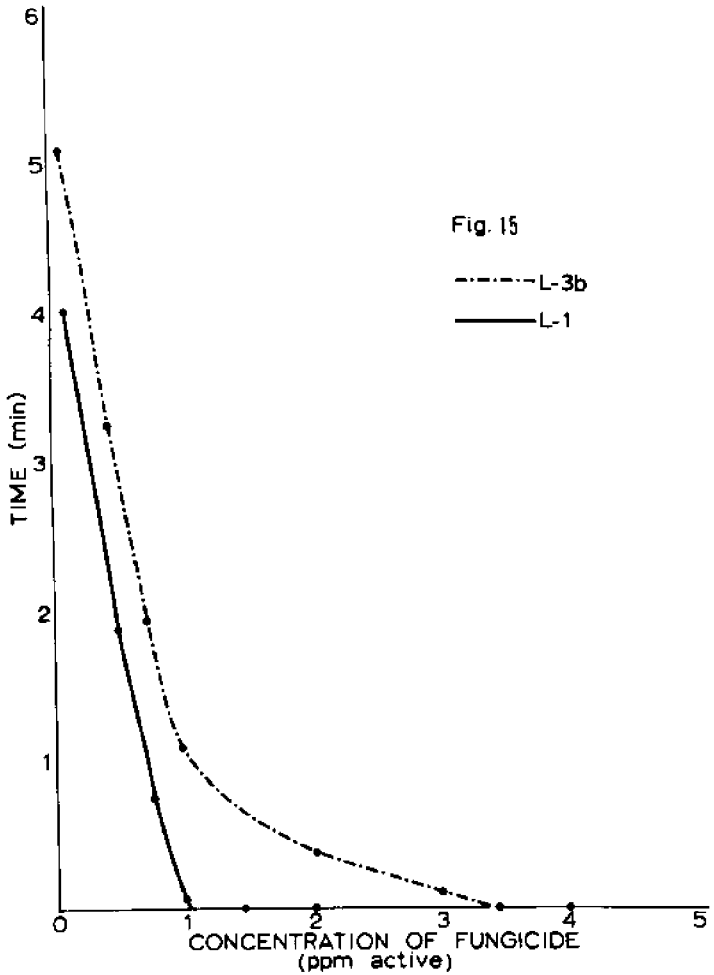
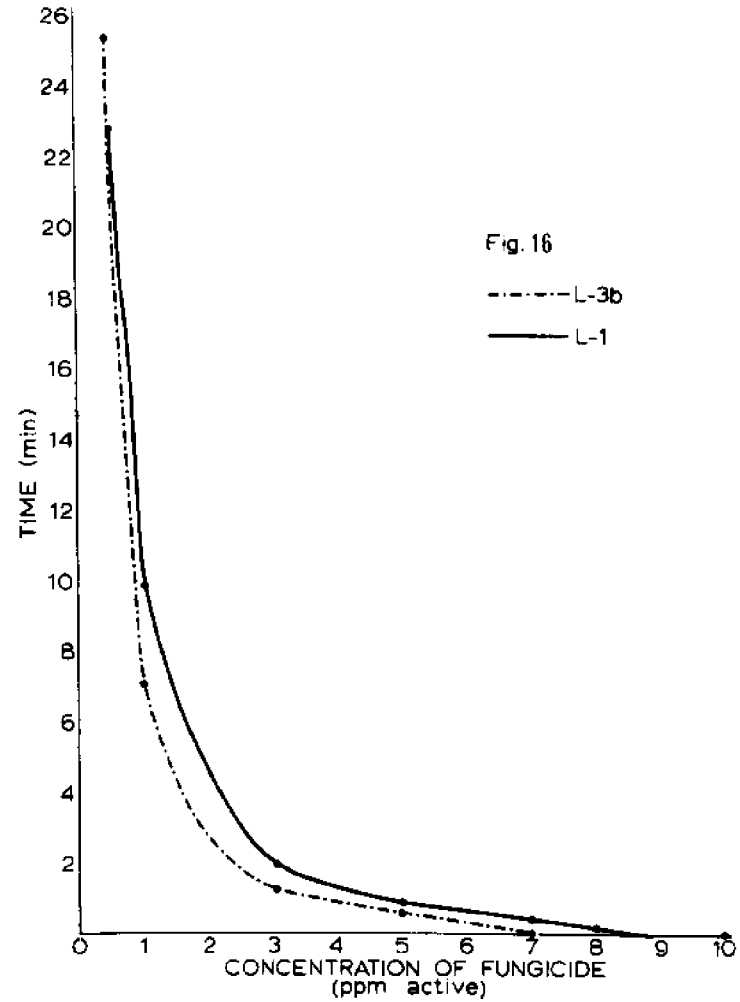


Fig. 13 & 14. Comparison of the minimum lethal concentration (LC_{100}) of each compound tested for Lagenidium strain L-1 (Fig. 13) and L-3B (Fig. 14). TC, tribasic copper sulfate; BE, Benlate; VT, Vitavax; DY, Dyrene; DC, Dichlone; TR, Treflan; CP, Captan; MN, Manzate or Dithane M-45; TE, Terrachlor; DS, DS 9073; MG, malachite green.





Figs, 15 & 16, Effect of sublethal concentrations of DS 9073 (Fig. 15) and Treflan (Fig. 16) on the length of motility of Lagenidium zoospores.

Table 1. Fungicides Tested

COMMON NAME	CHEMICAL NAME	COMPANY
1. BENLATE	METHYL 1-(BUTYLCARBAMOYL) - 2 - BENZIMIDAZOLE- CARBAMATE	E. I. DUPONT
2. CAPTAN	N[(TRICHLOROMETHYL)THIO] CYCLOHEXENE 1,2-DICARBOXIMIDE	STAUFFER CHEMICAL CO.
3. DICHLONE	2,3-DICHLORO-1,4-NAPHTHOQUINONE	FISHER CHEMICAL CO.
4. DIFOLATAN	CIS-N[(1,1,2,2-TETRACHLOROETHYL)THIO]-4-CYCLOHEXENE -1,2-DICARBOXIMIDE	CHEVRON CHEMICAL CO.
5. DS 9073	EXPERIMENTAL BACTERICIDE/FUNGICIDE	ICI AMERICA INC.
6. DYRENE	2,4-DICHLORO-6-(O-CHLOROANILINO)-S-TRIAZINE	CHEMAGRO CHEMICAL CO.
7. MALACHITE GREEN	THE CHLORIDE OF TETRAMETHYL-DI-p-AMINOTRIPHENYLCARBINOL	FISHER CHEMICAL CO.
8. MANZATE 200 DITHANE M-45	A COORDINATION PRODUCT OF ZINC ION AND MANGANESE ETHYLENEBISDITHIO - CARBAMATE	ROHM AND HAAS, AND E. I. DUPONT
9. TERRACLOR	PENTACHLORONITROBENZENE	OLIN CHEMICAL CO.
10. TREFLAN	a,a,a-TRIFLUORO-2,6-DINITRO- N,N-DIPROPYL-p-TOLUIDINE	ELI LILLY AND CO.
11. TRIBASIC COPPER SULFATE		CITIES SERVICE CO.
12. VITAVAX	5,6-DIHYDRO-2-METHAL-1,4-OXATHIIN-3- CARBOXANILIDE	UNIROYAL CHEMICAL CO.

Table 2. Effects of selected fungicides at minimal lethal concentration on development of the zoospores of Lagenidium callinectes isolate L-1 in sterile sea water

Fungicide	Duration of motility (min)[o]	Encyst	Germinate	Flagella*
Benlate	21-25	Yes	Yes	A,B,C
Captan	16-21	No	No	A,B,C
Dichlone	7-10	Yes	No	A,B,C,D,E
Difolatan	9-12	No	No	A,B,C
DS 9073	0-0.2	No	No	A,B,C
Dyrene	46-51	Yes	No	A,B,C
Malachite Green	108-112	Yes	Yes	A,B,C
Manzate 200	19-34	Yes	No	A,B,C
Terraclor	33-41	Yes	Yes	A,B,C
Treflan	0.5-1	No	No	A,B,C
Tribasic Copper Sulfate	22-27	Yes	Yes	A,B,C
Vitavax	12-15	Yes	Yes	A,B,C
Control	102-136	Yes	Yes	A,B,C,D,E

[o]Time required for complete inactivation of the motility of the zoospores of L. callinectes.

*Methods of flagella retraction or detachment: A is vesicular detachment; B is vesicular retraction; C is simple casting-off of flagella; D is straight drop; E is straight-in retraction. Underlining denotes type of flagella retraction or detachment most frequently observed.

Table 3. Effects of selected fungicides at minimal lethal concentration on development of the zoospores of Lagenidium callinectes isolate L-3b in sterile sea water

Fungicide	Duration of motility (min)[o]	Encyst	Germinate	Flagella*
Benlate	19-23	Yes	No	A,B,C
Captan	3-5	No	No	A,B,C
Dichlone	3-6	Yes	No	A,B,D,E
Difolatan	4-6	No	No	A,B,C
DS 9073	0-0.2	No	No	A,B,C
Dyrene	36-39	Yes	No	A,B,C
Malachite Green	37-41	Yes	Yes	A,B,C
Manzate 200	12-13	Yes	No	A,B,C
Terraclor	22-29	Yes	Yes	A,B,C,D
Treflan	1-1.5	Yes	No	A,B,C
Tribasic Copper Sulfate	61-67	Yes	Yes	A,B,C
Vitavax	9-12	Yes	Yes	A,B,C
Control	93-114	Yes	Yes	A,B,C

[o]Time required for complete inactivation of the motility of the zoospores of L. callinectes.

*Methods of flagella retraction or detachment: A is vesicular detachment; B is vesicular retraction; C is simple casting-off of flagella; D is straight drop; E is straight-in retraction. Underlining denotes type of flagella retraction or detachment most frequently observed.

Table 4. Time of mortality for zoospores of *Lagenidium callinectes* when exposed to lethal concentrations of selected fungicides in PYGS

Fungicide	Exposure time for L-1 (min)				Exposure time for L-3b (min)			
	15	30	45	60	15	30	45	60
Benlate	+	+	+	+	+	+	+	+
Captan	+	-	-	-	+	-	-	-
Dichlone	+	+	+	+	+	+	+	+
Difolatan	+	-	-	-	+	-	-	-
DS 9073	-	-	-	-	-	-	-	-
Dyrene	+	+	+	+	+	+	+	+
Malachite Green	+	+	+	+	+	+	+	+
Manzate 200	+	+	+	-	+	+	+	+
Terraclor	+	+	+	+	+	+	+	+
Treflan	+	+	+	-	+	+	+	+
Tribasic Copper Sulfate	+	+	+	+	+	+	+	+
Vitavax	+	+	+	+	+	+	+	+

+ indicates growth

- indicates no growth

Table 5. Effect of lethal concentrations of selected fungicides on hatching and development of brine shrimp

Fungicide	Day 2			Day 3			Day 4			Day 6			Day 7			Day 9		
	A*	D*	%*	A	D	%	A	D	%	A*	D*	%*	A	D	%	A	D	%
Benlate	0	0	--	0	0	--	0	0	--	0	0	--	0	0	--	0	0	--
Captan+	16	0	100%	68	8	89%	87	6	94%	94	9	91%	69	16	81%	46	48	49%
Dichlone	17	0	100%	92	3	97%	66	18	79%	58	26	69%	55	32	63%	28	51	35%
Difolatan	15	0	100%	66	14	79%	37	47	44%	24	94	20%	15	80	16%	7	101	6%
DS 9073	11	0	100%	59	19	76%	27	37	42%	20	50	29%	6	68	8%	4	67	6%
Dyrene	13	0	100%	73	10	88%	82	18	82%	46	50	48%	26	64	29%	28	69	29%
Malachite Green+	17	0	100%	89	0	100%	92	5	95%	91	10	90%	75	20	79%	36	44	45%
Manzate 200+	13	0	100%	61	0	100%	79	10	89%	85	10	89%	60	16	79%	39	56	41%
Terraclor	16	0	100%	86	0	100%	53	21	72%	44	45	49%	17	79	18%	11	66	14%
Treflan	18	1	95%	49	32	60%	21	62	25%	10	69	13%	4	64	6%	2	70	3%
Tribasic Copper Sulfate	1	0	100%	7	4	64%	11	2	85%	27	10	73%	23	16	59%	16	19	46%
Vitavax	1	0	100%	11	5	69%	9	18	33%	6	25	19%	2	32	6%	2	41	5%
Control	19	0	100%	100	0	100%	86	10	90%	110	7	94%	67	22	75%	48	63	43%

*A is the number of brine shrimp alive in 3 mls of sea water; D is the number of brine shrimp dead in 3 mls of sea water; % is the percent survival.

+Does not differ significantly at the 0.01 level from the control.

Table 6: Bioassay experiments - *Penaeus californiensis*

Treatment in Mg/L.	Nauplius		Protozoea		Mysis		Postlarval	
	Temp. Range	% Surv.	Temp. Range	% Surv.	Temp. Range	% Surv.	Temp. Range	% Surv.
Malachite Green								
.0006	28.0-22.1	92	28.2-22.0	96	28.0-23.0	94	28.2-20.3	84
.003	28.0-22.1	98	28.2-22.2	76	28.0-23.0	92	28.2-20.3	78
.006	28.0-21.8	82	28.2-22.0	96	28.0-22.5	94	28.2-20.0	90
.012	28.0-21.5	92	28.2-22.2	86	28.0-22.4	96	28.2-19.8	96
.06	28.0-21.5	84	28.2-21.9	90	28.0-22.5	96	28.2-19.7	92
.6	28.0-21.5	0	28.2-21.8	68	28.0-22.0	2	28.2-19.9	98
Terraclor								
.1625	28.0-22.0	100	28.2-21.9	40	28.0-23.0	94	28.2-20.0	70
.325	28.0-22.0	94	28.2-22.0	68	28.0-22.5	100	28.2-20.0	100
.65	28.0-21.9	86	28.2-22.3	94	28.0-23.0	98	28.2-20.3	96
1.3	28.0-21.8	94	28.2-22.3	92	28.0-22.8	98	28.2-20.2	96
2.6	28.0-21.6	60	28.2-21.8	86	28.0-22.2	30	28.2-19.8	92
5.2	28.0-21.5	84	28.2-21.9	66	28.0-22.0	8	28.2-19.5	74*
Treflan								
.1875	28.0-22.0	90	28.2-22.0	62	28.0-22.9	98	28.2-20.2	92
.375	28.0-22.0	90	28.2-22.2	70	28.0-23.0	94	28.2-20.3	88
.75	28.0-22.0	92	28.2-22.3	92	28.0-23.0	98	28.2-20.3	86
1.5	28.0-21.5	86	28.2-21.9	84	28.0-22.4	90	28.2-19.8	98
3.0	28.0-21.5	92	28.2-21.9	92	28.0-22.2	90	28.2-19.8	94
6.0	28.0-21.6	18	28.2-21.8	64	28.0-22.0	40	28.2-19.7	20*
Controls	28.0-21.75	82 Min.	28.2-22.0	76 Min.	28.0-22.73	70 Min.	28.2-19.98	80 Min.
Average		91.6		86		89		90.5

Table 7: Bioassay experiments - *Penaeus stylirostris*

Treatment in Mg/L.	Nauplius		Protozoea		Mysis	
	Temp. Range	% Surv.	Temp. Range	% Surv.	Temp. Range	% Surv.
Malachite Green						
.0006	28.0-27.8	100	28.0-27.4	90	28.0-26.8	100
.003	28.2-27.3	82	28.0-27.4	90	28.0-26.8	72
.006	28.2-27.8	84	28.0-27.4	100	28.0-26.8	100
.012	28.2-27.7	100	28.0-27.4	100	28.0-26.8	98
.06	28.2-27.8	86	28.0-27.4	0	28.0-26.8	98
.6	28.2-27.8	0	28.0-27.4	0	28.0-26.8	0
Terraclor						
.1625	28.2-27.8	46	28.0-27.3	20	28.0-26.8	100
.325	28.2-27.6	32	28.0-27.4	0	28.0-26.8	100
.65	28.2-27.8	0	28.0-27.4	0	28.0-26.8	100
1.3	28.2-27.8	16	28.0-27.4	0	28.0-26.8	0
2.6	28.2-27.8	0	28.0-27.4	0	28.0-26.8	8
5.2	28.0-27.8	0	28.0-27.4	0	28.0-26.8	16
Treflan*						
.1875	28.2-27.3	84	28.0-27.4	80	28.0-26.8	100
.375	28.2-27.8	60	28.0-27.4	0	28.0-26.8	98
.75	28.2-27.6	68	28.0-27.4	0	28.0-26.8	100
1.5	28.2-27.8	20	28.0-27.4	0	28.0-26.8	100
3.0	28.2-27.8	0	28.0-27.4	0	28.0-26.8	92
6.0	28.2-27.8	0	28.0-27.4	0	28.0-26.8	88
Controls	28.2-27.3	72 Min	28.0-27.3	68 Min	28.0-26.8	96 Min
Average		90.6		86		98

*Terraclor concentrations of 0.325 ppm and greater and Treflan concentrations of 0.375 ppm and greater resulted in aglutination of the algal foods during the Protozoeal test series resulting in an associated heavy mortality problem.

Table 8. Bioassay experiments - Penaeus vanammei

Treatment in Mg/L	Nauplius		Protozoea		Mysis		Postlarvae	
	Temp. Range	% Surv.	Temp. Range	% Surv.	Temp. Range	% Surv.	Temp. Range	% Surv.
Malachite Green								
.0006	28.2-27.6	100	28.2-27-8	88	27.8-27.0	90	27.4-26.3	62
.003	28.2-27.6	92	28.2-27.8	86	27.8-27.0	68	27.4-26.3	80
.006	28.2-27.6	96	28.2-27.8	82	27.8-27.0	70	27.4-26.3	54*
.012	28.2-27.6	78	28.2-27.8	74	27.8-27.0	64	27.4-26.3	58*
.06	28.2-27.6	94	28.2-27.8	56	27.8-27.0	60	27.4-26.3	72
.6	28.2-27.6	24	28.2-27.8	34	27.8-27.0	30	27.4-26.3	54
Terraclor								
.1625	28.2-27.6	84	28.2-27.8	98	27.8-27.0	64	27.4-26.3	76
.325	28.2-27.6	62	28.2-27.8	94	27.8-27.0	98	27.4-26.3	66*
.65	28.2-27.6	70	28.2-27.8	78	27.8-27.0	46	27.4-26.3	44
1.3	28.2-27.6	60	28.2-27.8	10	27.8-27.0	2	27.4-26.3	0
2.6	28.2-27.6	28	28.2-27.8	0	27.8-27.0	50	27.4-26.3	44
5.2	28.2-27.6	0	28.2-27.8	0	27.8-27.0	6	27.4-26.3	0
Treflan								
.1875	28.2-27.6	62	28.2-27.8	92	27.8-27.0	90	27.4-26.3	54
.375	28.2-27.6	80	28.2-27.8	64	27.8-27.0	72	27.4-26.3	80
.75	28.2-27.6	78	28.2-27.8	74	27.8-27.0	70	27.4-26.3	50
1.5	28.2-27.6	36	28.2-27.8	66	27.8-27.0	72	27.4-26.3	40
3.0	28.2-27.6	20	28.2-27.8	12	27.8-27.0	16	27.4-26.3	0
6.0	28.2-27.6	4	28.2-27.8	2	27.8-27.0	18	27.4-26.3	0
Controls								
Min.	28.2-27.6	74	28.2-27.8	80	27.8-27.0	64	27.4-26.3	48
Avg.	28.2-27.6	86.8	28.2-27.8	85.6	27.8-27.0	80.6	27.4-26.3	*63

*NOTE: The results in the postlarvae section were quite inconsistent because of jumpouts and insufficient food.

Table 9: Results from the use of malachite green in treating epizootic due to a Lagenidium sp.

Hatch No.	Species	Pre-Treatment Population	Stage of *Lagenidium* Appearance	Population After Treatment		Treatment
				24 hour	48 hour	
26	*P. stylirostris*	99,100	Late PI	102,200	90,000	.006 mg/L
*27	*P. stylirostris*	155,100	Late PI	158,500	156,900	.006 mg/L
28	*P. californiensis*	48,400	Late PI	27,692	12,666	none
29	*P. californiensis*	275,757	Lage PI	288,500	263,700	.006 mg/L

*The population 12 hours prior to the treatment was 248,500; thus mortality attributed to pre-treatment *Lagenidium* sp. infections was 90,000 shrimp or approximately 18%.