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ABSTRACT

Laddie M. Crisp, Jr. BIOSYSTEMATICS AND CONTROL OF LAGENIDIUM, HALIPHTHOROS AND FUSARIUM: FUNGAL PATHOGENS OF MARINE CRUSTACEA. (Under the direction of Dr. Charles E. Bland) Department of Biology, East Carolina University, July 1982.

In an effort to control diseases of cultured marine crustacea caused by the fungi *Lagenidium callinectes, Haliphthoros milfordensis* and *Fusarium solani*, isolates of each species were subjected to toxicity testing with a number of experimental fungicides and ozone. Three fungicides, R-41-782 (Tavolek), IL-780 (ICI Americas), and EL-131 (Eli Lilly and Co.), were shown to be most effective, with LC_{100}'s of less than 2 ppm. for both *L. callinectes* and *H. milfordensis*. For *F. solani* the LC_{100} for all fungicides exceeded 50 ppm. Ozone was highly effective also and caused zoospore death within 8 minutes after ozonation.

Another aspect of this study involved the fact that differences among various isolates of *Lagenidium callinectes* Couch have caused some investigators to question the validity of *L. callinectes* as a single species. Therefore, morphological and physiological characters for eight isolates were evaluated in an attempt to quantify differences, if any, among the isolates that might facilitate their recognition as one or more species. Although results of the morphological evaluation were inconclusive, clustering analysis of physiological characters grouped the isolates in distinct subgroups, with the isolates in each subgroup noted to occur in similar geographic regions. Although the physiological differences

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were consistent among the subgroups, such differences were not deemed sufficient for recognition of separate species within the *L. callinectes* "complex" because of the overall morphological similarity of the isolates.

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BIOSYSTEMATICS AND CONTROL OF *LAGENIDIUM*, *HALIPHTHOROS* AND *FUSARIUM*: FUNGAL PATHOGENS OF MARINE CRUSTACEA

A Thesis

presented to

the Faculty of the Department of Biology

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Laddie M. Crisp, Jr.

July 1982

BIOSYSTEMATICS AND CONTROL OF *LAGENIDIUM*, *HALIPHTHOROS* AND *FUSARIUM*: FUNGAL PATHOGENS OF MARINE CRUSTACEA

by

Laddie M. Crisp, Jr.

APPROVED BY:

DIRECTOR OF THESIS ______________________
Dr. Charles E. Bland

COMMITTEE MEMBER ______________________
Dr. Charles R. Coble

COMMITTEE MEMBER ______________________
Dr. Graham J. Davis

COMMITTEE MEMBER ______________________
Dr. Donald B. Jeffreys

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

____
Dr. Charles E. Bland

DEAN OF GRADUATE SCHOOL

____
Dr. Joseph G. Boyette

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DEDICATION

This thesis is dedicated to my loving parents who have endured so much through its making and, as in other endeavors, have served as a source of inspiration.

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INTRODUCTION

With the human population growing exponentially and land area available for cultivation decreasing, man is looking more to the sea for a source of nutrition. As such, mariculture (farming of the sea) is slowly emerging as a potential means of food production. However, for mariculture to become economically feasible in the United States and other areas, the use of a minimum amount of space and water is essential (Lightner, Fontaine, and Hanks, 1975). Major difficulties involve handling, containing, and providing adequate nutrition of organisms, avoiding build-up of toxins, and maintaining suitable stocking densities. Stress of the cultured organisms in any of these areas increases their susceptibility to diseases and may result in death of some or, in certain instances, all of the specimens of a given cultured organism. In crustacean culture, current methodology requires that eggs be obtained from gravid females collected from the wild and subsequently allowed to spawn in captivity. Although spawning takes place in water freed of potential pathogens, the females and their ova often serve as harbors of contagion. In such cases disease causing organisms may include bacteria, fungi, viruses and protozoa (Sinderman, 1977). Among the fungi, _Lagenidium callinectes_ Couch, _Haliphthoros milfordensis_ vishniac and _Fusarium_ sp. have proved to be significant pathogens of cultured marine crustacea (Sinderman, 1974).

Lagenidium callinectes, a holocarpic, coenocytic, filamentous marine phycomycete (class: Oomycetes, order: Lagenidiales) was first

described by Couch (1942) from ova of the blue crab Callinectes sapidus. Rogers-Talbert (1948) showed this fungus to be only a peripheral parasite of the egg mass (sponge) of crabs and likely of little or no significance in reducing the population of wild crabs. Recently, this fungus has been isolated from cultured penaeid shrimp (Cook, 1971; Lightner and Fontaine, 1973; Barkate, Laramore, Hirnon and Persyn, 1974; Aquacop, 1975), Dungeness crabs (Armstrong, Buchanan and Caldwell, 1976) and both the American lobster, Homarus americanus, and European lobster, Homarus gammarus (Nilson, Fisher and Shleser, 1976). Occurring primarily on larvae, the fungus has little effect on post-larval stages of either shrimp or lobster (Lightner and Fontaine, 1973; Nilson et. al., 1976). In larvae, however, infection develops rapidly and may cause high levels of mortality. Lightner (1974) artificially infected four thousand Stage I and Stage II brown shrimp, Penaeus aztecus, larvae with spores of L. callinectes and effected a ninety-seven percent mortality within a ninety-six hour period. Evidence of infection is first observed in the thorax, abdomen, eyestalks and swimming appendages. However, within twenty-four hours the fungus may spread throughout the larva, converting all tissue into hyphae (Lightner and Fontaine, 1973). Upon death of the larva, unbranched discharge tubes grow through the carapace, form vesicles, and release monophonetic, secondary type zoospores, the agents of infection (Lightner and Fontaine, 1973).

Currently nine isolates of Lagenidium callinectes have been obtained and are deposited at the Department of Biology, East Carolina University. For these isolates, work by previous

investigators has indicated that differences exist regarding their salinity tolerances, growth rates, sensitivity to various fungicidal compounds, and even morphology (Lightner and Fontaine, 1973, Ruch, 1974; Nilson et. al., 1976; Daniels, 1977).

Haliphthoros milfordensis, a holocarpic, coenocytic, filamentous marine phycomycete (class: Oomycetes, order: Lagenidiales) was described by Vishniac (1958) from ova of the oyster drill, Urosalpinx cinerea. This organism is parasitic also on larvae of two species of cultured lobster, Homarus americanus (the American lobster) and Homarus gammarus (the European lobster), and on two species of shrimp, Penaeus setiferus (the white shrimp) and Penaeus dourarum (the pink shrimp) (Bland and Emerson, 1974, Fisher, Nilson, and Shleser, 1975; Tharp and Bland, 1977). A major pathogen of cultured lobster, this fungus has been shown under experimental conditions to cause mortality as high as forty-four percent within a twenty-two day period under experimental conditions (Fisher et. al., 1975). Although it is mainly a pathogen of larvae and juveniles, hyphae of H. milfordensis are often first detected on gills and appendages, appearing later in other parts of the larvae. Infection often accompanied by severe melanization, may result in death due to incomplete ecdysis at midmolt. Death is otherwise due to tissue replacement by hyphae (Fisher et. al., 1975). Like L. callinectes, the infective agent of H. milfordensis is its biflagellate, secondary type zoospore (Overton, 1979).

Fusarium sp., probably F. solani of the class Deuteromycetes, may be extremely pathogenic to cultured adult shrimp and lobsters

(Egusa and Ueda, 1972; Johnson, 1974; Lightner, 1975; Lightner and Fontaine,1975; Laramore, Barkate and Persyn, 1977). This organism was first reported as the causative agent of "Black Gill Syndrome" (Egusa and Ueda, 1972), a condition characterized by heavy melanization of the gills and resultant impaired oxygen uptake, often resulting in death. Infection has been reported to occur also in antennae, eyes, segments of walking legs, and other parts of the abdomen and thorax of shrimp (Lightner, 1974; Lightner and Fontaine, 1975; Johnson, 1978). The importance of *Fusarium* as a pathogen was demonstrated during the summer of 1974 at the University of Arizona Shrimp Culture Project at Puerto Penasco, Mexico, where during a two month period, only 600 of 6,000 shrimp survived an outbreak (Lightner, 1975). The infective agent of *Fusarium* appears to be one or both types of nonmotile spores (conidiospores) produced by this organism.

Currently, control measures for these and other pathogens fall into two categories: (1) a prophylactic measure involving improvement of water quality or (2) the use of chemical control. For the first, improvement of water quality by increasing oxygen level, decreasing toxic metabolites, and/or sterilization reduces susceptibility to diseases. In addition, water filtration, replacement, and/or u.v. irradiation may be used (Cook, 1969; anonymous, 1971; Mock and Murphy, 1971; Serling, Ost, and Ford, 1974; Hand, 1975). Chemicals used in the second category may be either therapeutic or prophylactic.

Recently, ozone, an allotrope of oxygen, has been investigated as a means to control diseases in aquaculture systems (Benoit and

Matlin, 1966; Straub, 1975; Anonymous, 1978). In this regard, ozone has been shown to effect a 99.9% mortality of *Vibrio alginolyticus* within five minutes at a concentration of only 2.6 ppm. (Danald, Ure, and Lightner, 1979).

The use of fungitoxic compounds which inhibit growth of fungal pathogens while causing no harm to the cultured organisms is now an established procedure in aquaculture. The use of such compounds is usually as a preventative dip for gravid females brought in from the wild or as a therapeutic path for organisms already showing symptoms of disease. Since relatively few compounds containing these properties have been registered by the government for use with food organisms, there is a continual search for effective new compounds which may be approved (Hatai, Nakajima, and Egusa, 1974; Ruch, 1974; Armstrong, Buchanan and Caldwell, 1976; Bland, Ruch, Salser and Lightner, 1976; Fisher, Rosemark and Shleser, 1976; Daniels, 1977; Abrahams and Brown, 1977).

Since isolate differences within strains of the species *L. callinectes* have been established, and because of the significant and continuing need for a means to control fungal diseases of cultured marine crustaecae, research was warranted in both of these areas. This research reported herein had, therefore, two major goals or facets: First, to quantify morphological and physiological differences among the isolates of *Lagenidium callinectes* -- an essential factor if reevaluation of the species is to occur. Secondly, to evaluate several experimental fungicides as well as the use of ozone as a means to control the fungal pathogens, *Lagenidium callinectes*, *Haliphthoros milfordensis* and *Fusarium solani.*

MATERIALS AND METHODS

Organisms Used

The three organisms used in this study were Lagenidium callinectes, Haliphthoros milfordensis and Fusarium solani, all of which were obtained from the mycological culture collection of Dr. C. E. Bland, Department of Biology, East Carolina University. Table 1 provides collection data for all isolates and indicates the isolates selected for use in various aspects of the studies described herein.

Culture Methods

Stock cultures of L. callinectes, H. milfordensis and F. solani were maintained on PYGS agar media which was composed of Bacto-peptone (1.25 gms.), yeast-extract (1.25 gms.), D-glucose (3.00 gms.), agar (15.00 gms.) and sea water (1.00 l. at a salinity of approximately 33 ppt.).

Both culures of L. callinectes required for in vitro studies were obtained initially by transferring agar scrapings from stock cultures to 125 ml. erlenmeyer flasks, containing 50 mls. of PYGS broth (prepared as PYGS agar w/o agar).

After growth for 72 hrs. on a rotary shaker, hyphae were separated from the culture media via a sterile nylon filter and the media was replaced with sterile sea water. Sporulation occurred within 4-18 hrs.

Subcultures in PYGS broth were prepared via zoospore transfer involving inoculation with 60,000[1] zoospores. These were allowed to grow on a rotary shaker (109 revolutions/min.) as described

Table 1. Tabulation of isolates, source, host and tests performed

Key to Abbreviations of Tests Performed

FO - Toxicity testing with Imazalil Nitrate, R-27-180, R-41-400, R-41-782, E.-131, Il-780, Algal extracts: 1, 2, 3, 8, 9, 10
F1 - Toxicity testing with R-41-782
F2 - Toxicity testing with EL-131
F3 - Toxicity testing with IL-780
OZ - Toxicity testing with Ozone
CA - Clustering Analysis
SD - Spore Demensions
VD - Vesicle Diameter
HC - Hyphal Characterization

ORGANISM:	STRAIN:	HOST:	SOURCE:	ISOLATED BY:	TEST PERFORMED:
Lagenidium callinectes					
	L-1	*Callinectes sapidus*	Newport Estuary, N.C.	Bland & Amerson (1973)	F1, F3, OZ, CA, SD, VD, HC
	L-3B	*Penaeus setiferus*	Galveston, Tx.	Lightner & Fontaine (1973)	F0, F1, F2, F3, CA, SD, VD, HC
	L-MF	Penaeid shrimp	Panama City, Fl.	Bland (1976)	CA, SD, VD, HC
	L-815	*Penaeus stylirostris*	Puerto Penasco, Mexico	Lightner (1976)	F2, OZ, CA, SD, VD, HC
	L-16	*Penaeus monodon*	Iliolo City, Philippines	Gacutan (1978)	F1, F2, F3, CA, SD, VD, HC
	L-CE	*Penaeus monodon*	Iliolo City, Philippines	Gacutan (1978)	CA
	L-F2	*Pandalus platyceros*	Bodega Bay, Ca.	Fisher (1975)	CA
	D-1	*Cancer magister*	Newport, Or.	Armstrong (1975)	CA
	L-G	Algal drift	Seattle, Wa.	Gotelli (1973)	
Haliphthoros milfordensis					
	H-222	*Homarus americanus*	Bodega Bay, Ca.	Fisher	F1, F2, F3, OZ, CA
	F-130	*Homarus americanus*	Bodega Bay, Ca.	Fisher	CA
	F-143	*Cancer magister*	Bodega Bay, Ca.	Fisher	CA
	H-2	*Penaeus setiferus*	Newport Estuary, N.C.	Tharp & Bland	CA
	ARO-575	Algal Drift	North Sea, Germany	Bahnweg	CA
	P-78-1	*Limulu polyphemus*	U.S.	N/A	CA
Fusarium solari					
	No Strain Designation	N/A	N/A	N/A	F1, F2, F3, OZ

previously. Zoospore concentration was determined with an American Optics-Spencer Brightline Hemocytometer.

Broth cultures of *H. milfordensis* were prepared in a manner similar to those of *L. callinectes*, except that the culture medium required for *H. milfordensis* consisted of an infusion of shrimp in sea water. The infusion was prepared by autoclaving 7-8 headless shrimp in one liter of sea water (approximately 33 ppt. salinity) for 15 minutes. After autoclaving, two grams of glucose were added to the infusion. After removal of the shrimp via filtration through cheesecloth, the filtrate was dispensed into 125 ml. erlenmyer flasks and reautoclaved. Broth cultures of *H. milfordensis* were initiated and perpetuated by the same methods used for *L. callinectes*.

Non-cycling broth cultures of *F. solani* were produced by the following method: cultures were allowed to grow on PYGS agar in 125 ml. erlenmyer flasks for twelve days. On the twelfth day conidia were washed from the plate in 50 mls. of sterile sea water. *In vitro* studies were carried out in 125 ml. erlenmyer flasks containing 50 mls. of PYGS broth and an inoculum of 60,000 spores.

Fungicides Studied

In an effort to find new compounds effective in controlling the fungal pathogens, several experimental fungicides were selected for *in vitro* toxicity testing. The compounds used and the supplier for each are given in Table 2. All compounds were tested initially

[1]Number determined previously by Ruch (1974) to yield optimum growth and sporulation under the culture conditions employed.

against the L-3b isolate of L. callinectes, with those showing the lowest LC_{100} (Lethal Concentration for 100% death) being selected for further testing against H. milfordensis, F. solani and at least one other isolate of L. callinectes.

In vitro Tests

The in vitro tests used in fungicide and ozone toxicity determinations were designed to establish the effect of varying concentrations of fungicides and ozone on growth of the organisms studied. All fungicide tests were run in triplicate and were in 125 ml. erlenmeyer flasks containing 50 mls. of PYGS broth containing a fungicide at a given concentration. Following inoculation of flasks with 60,000 spores, growth was for 72 hrs. on a rotary shaker. At this time, hyphae were collected with a Buchner funnel on a preweighted, Whatman #3, qualitative, 5.5 cm. filter paper. The filter paper with the mycelial mass was then placed in an oven for drying at 70°C for 48 hrs. After cooling to room temperature in a dessicator, the filters with hyphae were weighed on an H16 Mettler balance. The effectiveness of the fungicides was determined by comparing concentration of fungicide versus growth as indicated by dry weight of hyphae. The lowest concentration of fungicides required to produce no growth was considered the LC_{100}.

To establish the fungicidal effect of ozone, a special test apparatus was constructed (Fig. 1). In this apparatus, air pumped by a Wisa ozone resistant pump (Wisa, West Germany) was passed via nalgene aquarium tubing to a clamp-type regulator, dried by passage through a $CaCO_3$ drying tube, and then introduced into a Sander S-1

Table 2. List of fungicides and their supplier

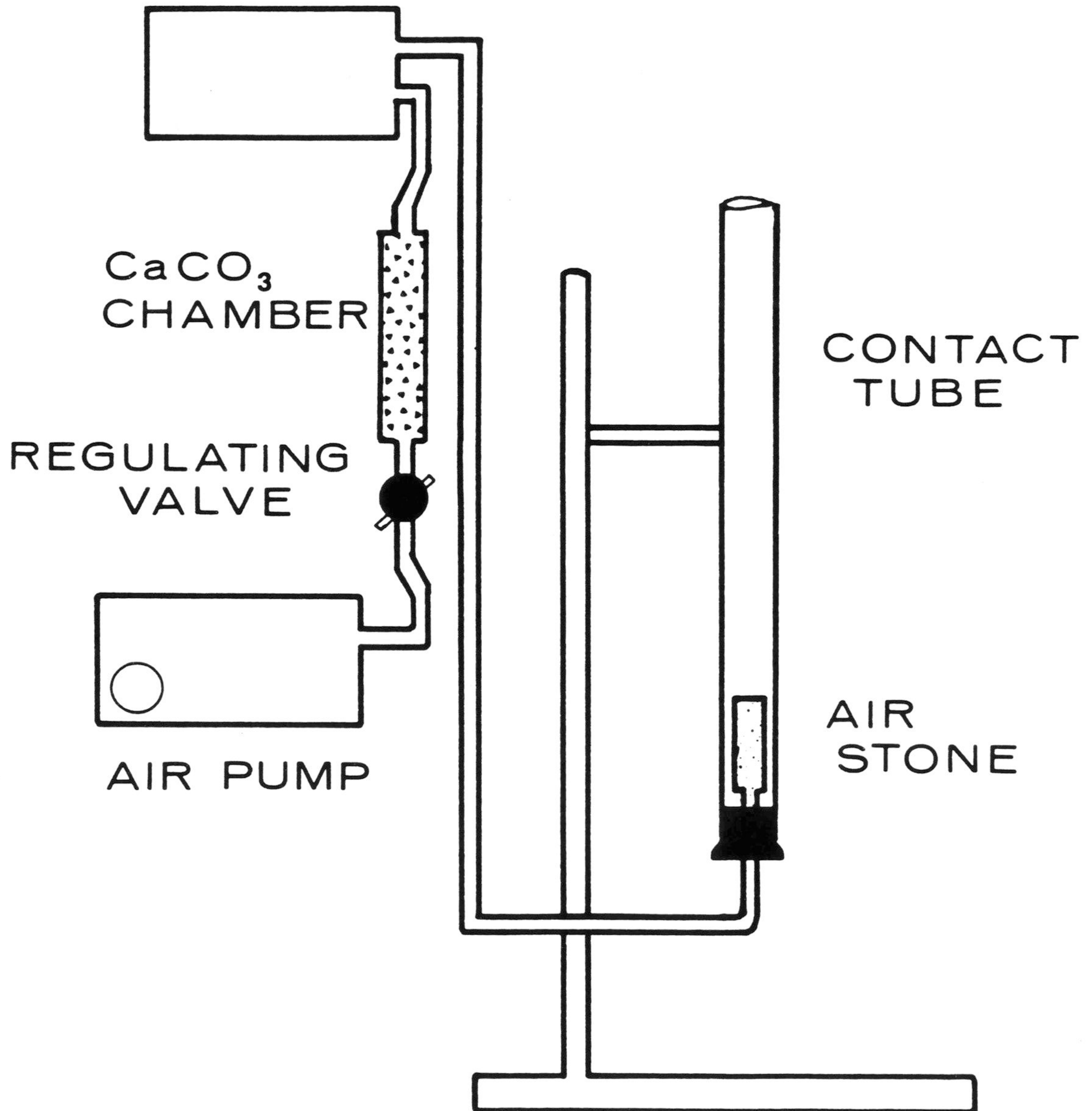
Fungicide	Supplier
EL-131	Elanco (subsidiary of Eli Lilly Company) Indianopilis, Ind.
IL-780 (formerly DS 9073)	ICI United States Wilmington, Del.
R-27-180	Tavolek (subsidiary of Johnson & Johnson Corp.) Redmond, Wa
R-41-400	Tavolek (subsidiary of Johnson & Johnson Corp.) Redmond, Wa
R-41-782	Tavolek (subsidiary of Johnson & Johnson Corp.) Redmond, Wa
Imazalil nitrate	Tavolek (subsidiary of Johnson & Johnson Corp.) Redmond, Wa
Algal extract #1	Skidaway Institute of Oceanography Savannah, Ga.
Algal extract #2	Skidaway Institute of Oceanography Savannah, Ga.
Algal extract #3	Skidaway Institute of Oceanography Savannah, Ga.
Algal extract #8	Skidaway Institute of Oceanography Savannah, Ga.
Algal extract #9	Skidaway Institute of Oceanography Savannah, Ga.
Algal extract #10	Skidaway Institute of Oceanography Savannah, Ga.

ozonizer (Sander, West Germany). The ozonized air was then bubbled through sea water in a vertical contact tube constructed from a 3.5 ft. X 1 inch in diameter section of glass tubing. All tests involved spore suspensions in 125 mls. of sea water with a spore concentration of 15,000 spores/ml. After bubbling non-ozonized air through the spore suspension for two minutes, a 1 ml. aliquot of the suspension was aseptically removed and placed as a control in 50 mls. of PYGS broth. The ozonizer was then activated. At three minute intervals, 1 ml. aliquots were aseptically removed and innoculated in PYGS broth for determination of growth. Sampling was terminated after the twenty-one minute exposure aliquot had been taken. All flasks, including the controls, were placed on a rotary shaker for 72 hrs. Dry weight analysis (technique described previously) provided data relative to spore survival and hyphal growth following exposure to ozone at various time intervals.

Ozone Concentration Determination

Ozone concentration as a function of time was determined by the iodometric procedure specified in Standard Methods for the Examination of Water and Wastewaters (1976). For this, 125 ml. samples of sea water were exposed to ozone in the contact tube for 0, 3, 5, 10, 15, and 20 minutes. Ozone from each sample was then passed into 200 mls. of potassium iodide (KI) solution by use of a standard gas washing bottle, absorbers and nitrogen gas. This transfer of ozone to the KI solution facilitated release of free iodine. After this transfer, ten milliliters of 1 N sulfuric acid was added to the ozone/KI solution to stabilize the complex. The acidified KI was

Figure 1. Ozone apparatus



OZONE GENERATOR
CaCO3
CHAMBER
REGULATING
VALVE
AIR PUMP
CONTACT
TUBE
AIR
STONE

then titrated with standardized .005 N Sodium thiosulfate until the yellow color of the liberated iodine had almost disappeared. Two milliliters of starch indicating solution was then added to impart a blue color. Titration was continued to the endpoint. A blank of pure KI solution was titrated also to eliminate the effect of impurities which might have reduced some of the iodine. Concentrations of the ozone in the sample were calculated from the following formula:

$$\text{Mg. of ozone/L. (ppm.)} = \frac{\text{(ml. of titrant for sample - ml. of titrant for blank)} \times .005 \times 24{,}000}{125 \text{ ml.}}$$

Physiology, Nutrition and Clustering Analysis

In this investigation, data from a comparative study of physiology and nutrition of several isolates of *L. callinectes* and *H. milfordensis* (Bahnweg, 1980) were subjected to computer analysis designed to group isolates into two or more assemblages or subgroups (clusters) on the basis of a set of attributes they share (Davies, 1971; Sneath and Sokal, 1973). From 125 tests involving modes of carbon nutrition, nitrogen nutrition, vitamin dependancy, pH tolerance, and salinity/temperature tolerance, 64 significant data items are selected for analysis via a "Clustering Analysis Program" which was developed and written by Dr. J. F. Reynolds, Department of Botany, North Carolina State University, Raleigh, North Carolina. In this program, unlike conventional taxonomy, all attributes or data items are given equal weight.

Morphological Evaluation

Spore Size: Koch (1968) stated that "since spore size is constant when standardized media and standardized conditions are used, this criterion may be used in classification." Therefore, morphometric analysis was used to determine if variations occurred between different isolates (Table 1) of L. callinectes. For this, spores form 72 hr. cultures, with a swarm period of less than 10 hrs., were first fixed in 1.5% glutaraldehyde, and subsequently photographed at 500X using a Nikon AFM camera on a Zeiss WL microscope. Contact prints of the spores were then measured for maximum diameter and maximum length. Approximately forty spores of each isolate were measured. Means and standard deviations for each of the isolates were determined for spore length and width. An analysis of variance was performed as well as a Student Newman Keuls Test if a significant difference among the isolates was established.

Vesicle Size: Size of spore vesicle is a characteristic often used in species descriptions of fungi and in keys for fungal identification (Ainsworth, Sparrow and Sussman, 1973, Coker, 1923; Sparrow, 1960). To determine if consistent variations in vesicle size occurred between isolates of species of L. callinectes, vesicles from selected isolates (Table 1) were measured for optimum diameter. Mature vesicles were obtained from seventy-two hour cultures which had been induced to sporulate as described previously. Maturity of vesicles was indicated by the presence of actively swarming zoospores. Vesicle diameter was determined in the manner described for measurement of spore dimension, except that phase contrast optics

were used to enhance visibility of the vesicle. Means and standard deviations were established for each of the isolates. Approximately, forty vesicles from each isolate were measured. An analysis of variance was performed as well as a Student Newman Keuls Test if a significant difference among the isolates was established.

Hyphal Characteristics: In recent papers by Trinci (1973), Gull (1975) and Ho (1977), hyphal characteristics have been used in categorization of species, as well as in the differentiation of strains of the same species. These hyphal growth characters, which are often species and strain specific include: hyphal diameter, angle of branching, degree of apical dominance, ratio of branch length to branch order, and ratio of numbers of branches to specific branch order. A complete list of hyphal characters used in this investigation along with a description of the characters is given in Table 3. Hyphal measurements were made from photographs of 12 to 48 hr. hyphae grown on thin layered PYGS agar coated slides which were prepared by the following method: Clean microscope slides were placed in standard Coplin stain jars filled with PYGS agar and autoclaved for 15 mins. at 121° C. After autoclaving, the Coplin jars were placed in an ultraviolet hood to cool. After fifteen minutes of cooling, the slides were aseptically removed and allowed to drain, thus permitting only a thin layer of agar to remain on the slide. The slides were then placed in moisture chambers fashioned from petri dishes, filter papers, and blocks of glass. Approximately twenty hyphal systems were measured from each of the isolates studied. The mean and degree of variation were established for each

Table 3. Definition of hyphal growth characters

HYPHAL GROWTH CHARACTERS

Apical Segment Length --	The length of the terminal segment of leading hypha, from the growing apex to the first node where the youngest primary branch appears.
Internodes --	The length of a segment of the leading hypha found between one primary branch to the next primary branch.
Apical Segment Ratio --	The ratio between the length of "Apical Segment" and the mean length of the internodes.
First Order Branch Length --	The mean length of first order branches defined as a branch which does not give rise to further branching.
Second Order Branch Length --	The mean length of second order branches defined as a branch which gives rise only to first order branches.
Third Order Branch Length --	The mean length of third order branches defined as branches which give rise to first and second order branches.
Hyphal Growth Unit --	The total length of all hyphal extensions divided by the total number of all hyphal tips.
Leading Hyphal Diameter --	The diameter of the leading hypha behind the first node.
Primary Hyphal Diameters --	The mean diameter of a primary branch at the first node.
Hyphal Segment Ratio --	The ratio between the leading hyphal diameter and primary hyphal diameter.
Branch Angle --	The angle between a leading hypha and its primary branch.

character within each species. An analysis of variance was performed as well as a Student Newman Keuls Test if a significant difference among the isolates was established.

RESULTS

Fungicide Tests

In the in-vitro tests, fungicide effectiveness was determined on the basis of the LC_{100} for each, the LC_{100} being the lowest concentration of fungicide completely inhibiting growth. For the L-3b isolate of *L. callinectes* the following LC_{100}'s were obtained:

Imazalil nitrate---------------------------5.5 ppm.

R-27-180------------------------------------4.0 ppm.

R-41-400------------------------------------9.0 ppm.

R-41-782------------------------------------1.6 ppm. (Fig. 2)

EL-131---------------------------------------0.8 ppm. (Fig. 6)

IL-780 (previously DS 9073)----------------0.9 ppm. (Fig. 10)

Algal Extract #1------------greater than 40.0 ppm.

Algal Extract 2------------greater than 40.0 ppm.

Algal Extract 3------------greater than 40.0 ppm.

Algal Extract 8------------greater than 40.0 ppm.

Algal Extract 9------------greater than 40.0 ppm.

Algal Extract 10-----------greater than 40.0 ppm.

Three compounds, R-41-782, EL-131 and IL-780, had an LC_{100} of less than 2.0 ppm and thus were selected for further testing against two other isolates of *L. callinectes*, one isolate of *H. milfordensis* and one isolate of *F. solani*. Results of these tests established the following LC_{100}'s.

Compound	Isolate	LC_{100}	Figure
R-41-782	L-16 (*L. callinectes*)	0.75 ppm.	No. 3
R-41-782	L-1 (*L. callinectes*)	1.0 ppm.	No. 4
R-41-782	H-222 (*H. milfordensis*)	1.3 ppm.	No. 5
R-41-782	*F. solani*	>50.0 ppm.	N/A
EL-131	L-16 (*L. callinectes*)	0.3 ppm.	No. 7
EL-131	L-815 (*L. callinectes*)	0.1 ppm.	No. 8
EL-131	H-222 (*H. milfordensis*)	2.3 ppm.	No. 9
El-131	*F. solani*	>50.0 ppm.	N/A
IL-780	L-16 (*L. callinectes*)	2.0 ppm.	No. 11
IL-780	L-1 (*L. callinectes*)	1.0 ppm.	No. 12
IL-780	H-222 (*H. milfordensis*)	1.5 ppm.	No. 13
IL-780	*F. solani*	>50.0 ppm.	N/A

From these results, the fungicide El-131, with a LC_{100} of less than 1.0 ppm., appears to be most effective against the *Lagenidium* isolates, whereas the fungicides R-41-782 and Il-780, with LC_{100}'s of 1.3 ppm. and 1.5 ppm. respectively, appear more effective against the H-222 isolate of *H. milfordensis*.

Ozone Toxicity Tests

Results of the ozone concentration determination (Fig. 14) indicated that there is no significant concentration build-up in the sea water during the first five minutes of ozonation. Starting at the six to eight minute period, ozone concentration increases linearly to a concentration of 2.8 ppm. at termination of the experiment (20 minutes).

In testing the effectiveness of ozone as a control agent, *L.*

callinectes (isolates L-815, Fig. 15; and L-1, Fig. 16) appeared resistant during the first five minutes of treatment, yet at eight minutes following treatment 100% spore mortality occurred. The LC_{100} for L. callinectes is, therefore, approximately 0.8 ppm. of ozone.

When testing the effect of ozone on F. solani similar results were obtained (Fig. 17). In this case, ozone appeared to have no effect during the first four minutes of treatment. However, after five minutes of treatment 100% spore mortality occurred, establishing an LC_{100} for F. solani of approximately 0.1 ppm.

The effect of ozone in the control of H. milfordensis could not be tested due to malfunction of the ozonation apparatus. It should be noted that when spore suspensions of either L. callinectes or F. solani were checked by microscopic observation, only small amounts of cellular debris remained after the "total death" period.

Clustering Analysis of L. callinectes

Clustering analysis of the isolates L-1, L-3b, L-MF, L-815, L-16, L-CE, L-F2 and D-1 (Table 1) of L. callinectes resulted in the grouping of the isolates into four clusters (Fig. 18): isolates L-3b, L-815 and L-MF at the 0.62 clustering level; isolates D-1 and L-F2 at the 0.61 clustering level, L-16 and L-CE at the 0.64 clustering level, and a single membered cluster of isolate L-1. As the analysis progressed, two of the groups merged into one at the 0.93 clustering level, including the isolates L-1, D-1, and L-F2. At the 0.97 clustering level the L. callinectes isolates L-3b, L-815 and L-MF merged with the isolates L-1, L-F2 and D-1 to form a single group. At the 1.0 clustering level isolates L-CE and L-16 merged with all

others to form one.

For comparison purposes and in order to determine true clusters of most similar organisms, a second analysis was performed including, not only the eight isolates of *L. callinectes*, but also six isolates of *H. milfordensis*. In this analysis (Fig. 19) all six isolates of *H. milfordensis* joined to form one cluster at the 0.73 clustering level. Unlike *H. milfordensis* the four previously described clusters for isolates of *L. callinectes* retained their identity at the 0.73 clustering level. It should be noted that none of the four *L. callinectes* clusters merged before joining the *H. milfordensis* species cluster.

Morphological Evaluation of Isolates of *L. callinectes*

Zoospore dimensions for selected isolates of *L. callinectes* were recorded as follows:

Isolate	Mean Length (SD)	Mean Width (SD)
L-1	13.7μ (1.3μ)	10.0μ(1.7μ)
L-3b	11.3μ (1.5μ)	9.9μ (1.4μ)
L-MF	13.5μ (1.6μ)	11.3μ (1.2μ)
L-815	11.7μ (1.0μ)	9.9μ (0.9μ)
L-16	13.0μ (1.6μ)	9.7μ (1.4μ)

An analysis of variance at the 0.05 level indicated that there is no significant difference in zoospore length and width among the isolates.

Vesicle diameter for five selected strains of *L. callinectes* were recorded as follows:

Isolate	Mean Diameter	SD
L-1	50.7μ	(5.6μ)
L-3b	42.6μ	(6.2μ)
L-MF	47.3μ	(11.2μ)
L-815	47.4μ	(4.7μ)
L-16	48.8μ	(5.6μ)

An analysis of variance of the raw data at the 0.05 significance level indicated a significant difference among the isolates. Based on this, results were evaluated in a Student Newman Keuls Test to establish which isolate(s) were of significant difference. This test proved inconclusive and no judgment could be made as to which isolate(s) were different.

Results of hyphal characterization of *L. callinectes* isolates L-1, L-3b, L-815, L-MF and L-16 are given in Table 4. Based on an analysis of variance for each of these characters at the 0.05 level of significance, differences among the isolates were found only for the characters: apical segment lengths, first order hyphal length, and hyphal growth unit. A Student Newman Kuels Test was performed to discern any grouping which could be made from these characters. Results of this analysis indicated two populations from both the first order hyphal length character and hyphal growth unit character, with the apical segment lengths character giving inconclusive results. The grouping indicated one population being composed of the L-1 and L-3b isolates with the other being L-MF, L-815 and L-16 isolate. When hyphal characters were being measured, it was observed that the isolate L-815 produced multiple germ tubes 73% of the time

with L-16 producing multiple germ tubes 44%, L-1 13% and L-3b only 10% of the time.

Figure 2. Dosage-response of *Lagenidium callinectes*, L-3b isolate to R-41-782

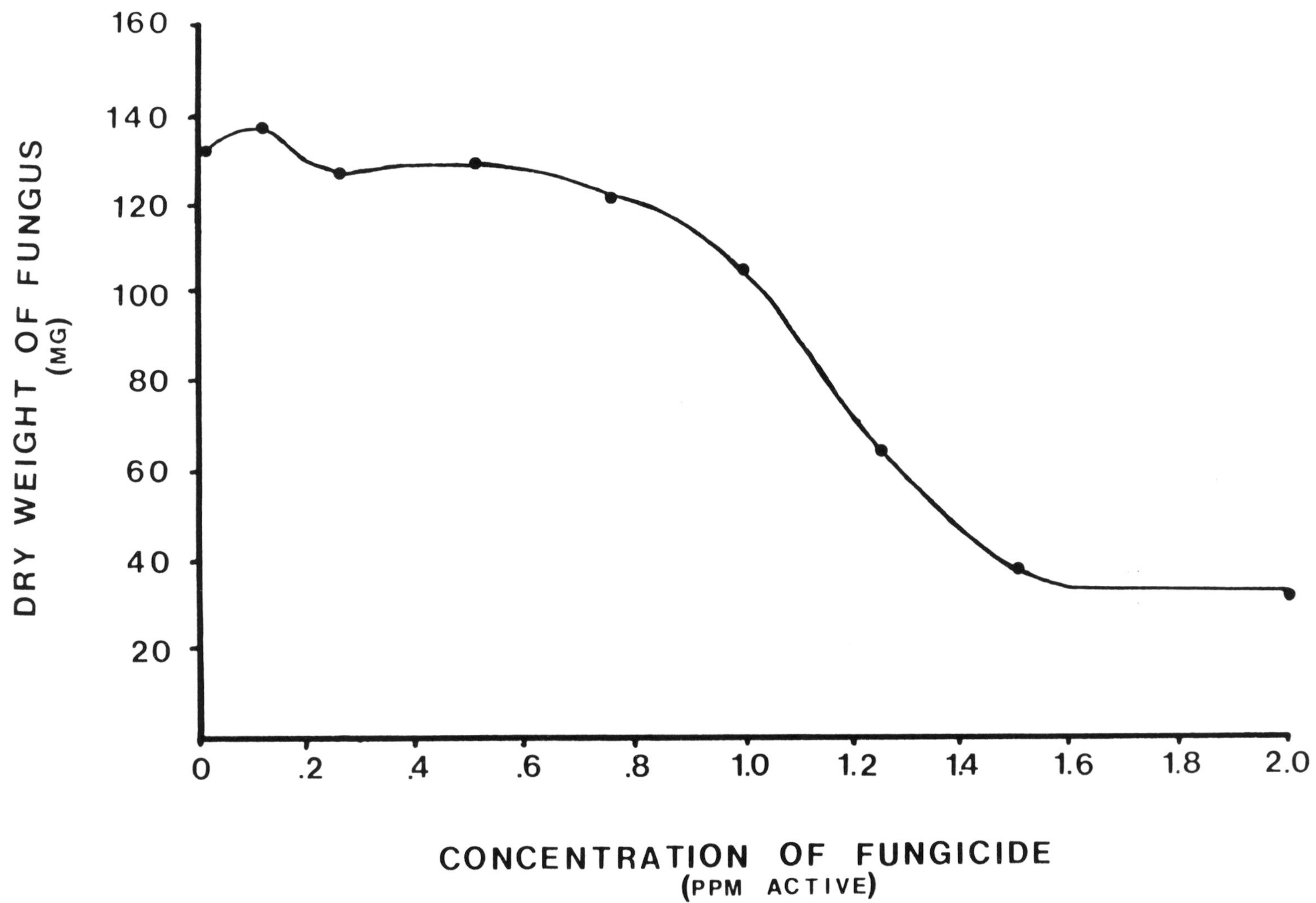

DRY WEIGHT OF FUNGUS
(MG)
160
140
120
100
80
60
40
20
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 3. Dosage-response of *Lagenidium callinectes*, L-16 isolate to R-41-782

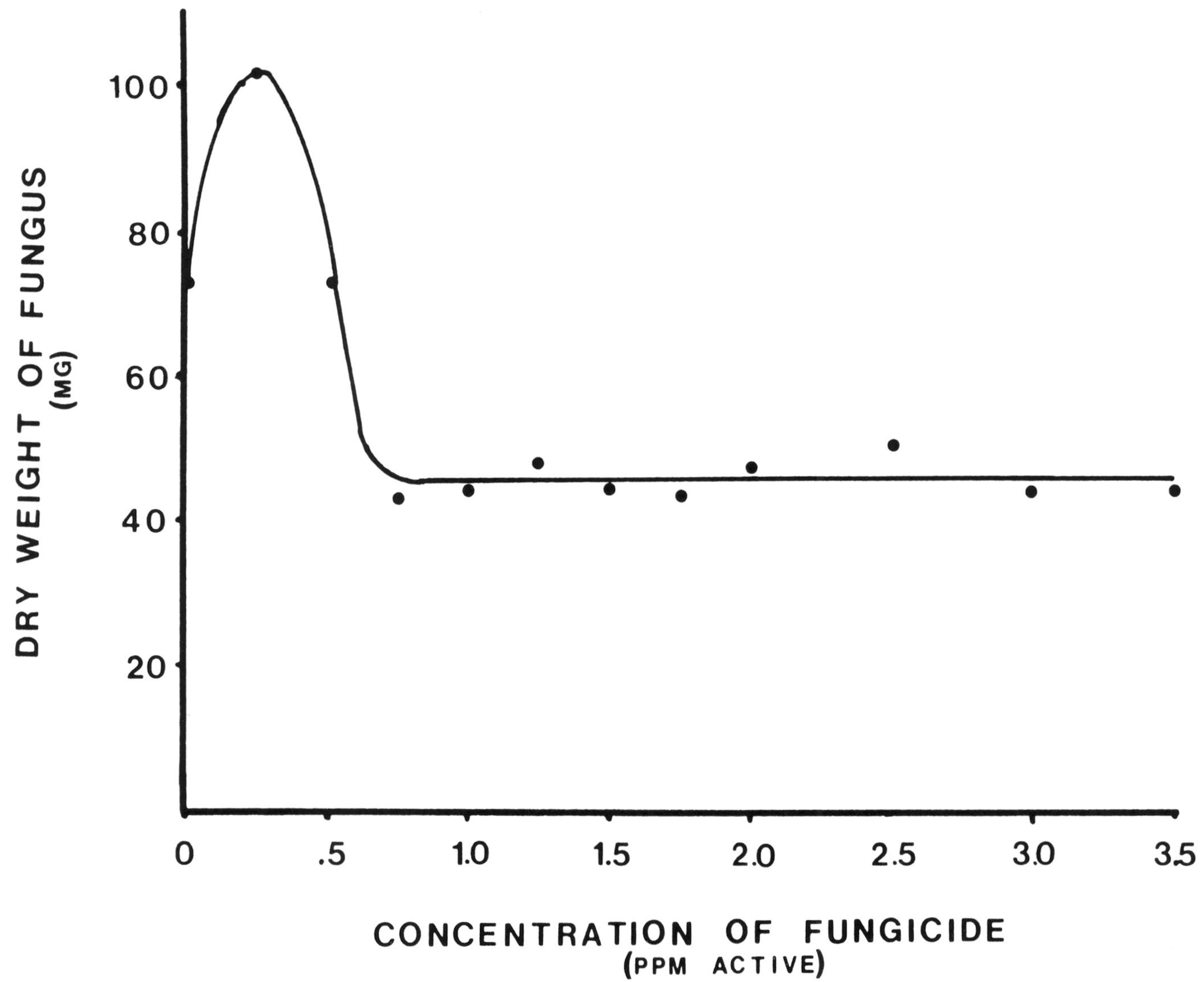

DRY WEIGHT OF FUNGUS
(MG)
100
80
60
40
20
0
.5
1.0
1.5
2.0
2.5
3.0
3.5
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 4. Dosage-response of *Lagenidium callinectes*, L-1 isolate to R-41-782

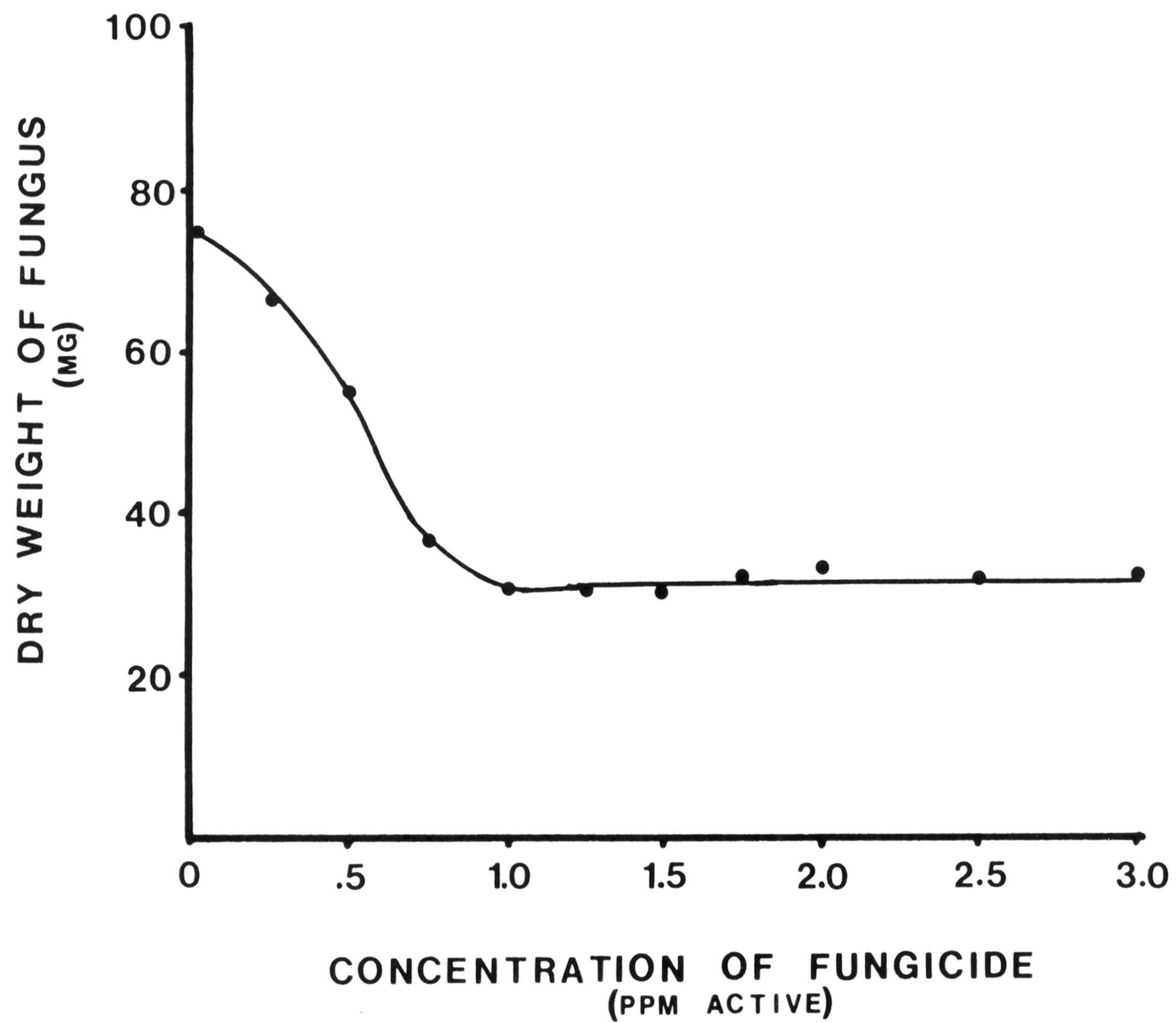

DRY WEIGHT OF FUNGUS
(MG)
100
80
60
40
20
0
.5
1.0
1.5
2.0
2.5
3.0
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 5. Dosage-response of *Haliphthoros milfordensis*, H-222 isolate to R-41-782

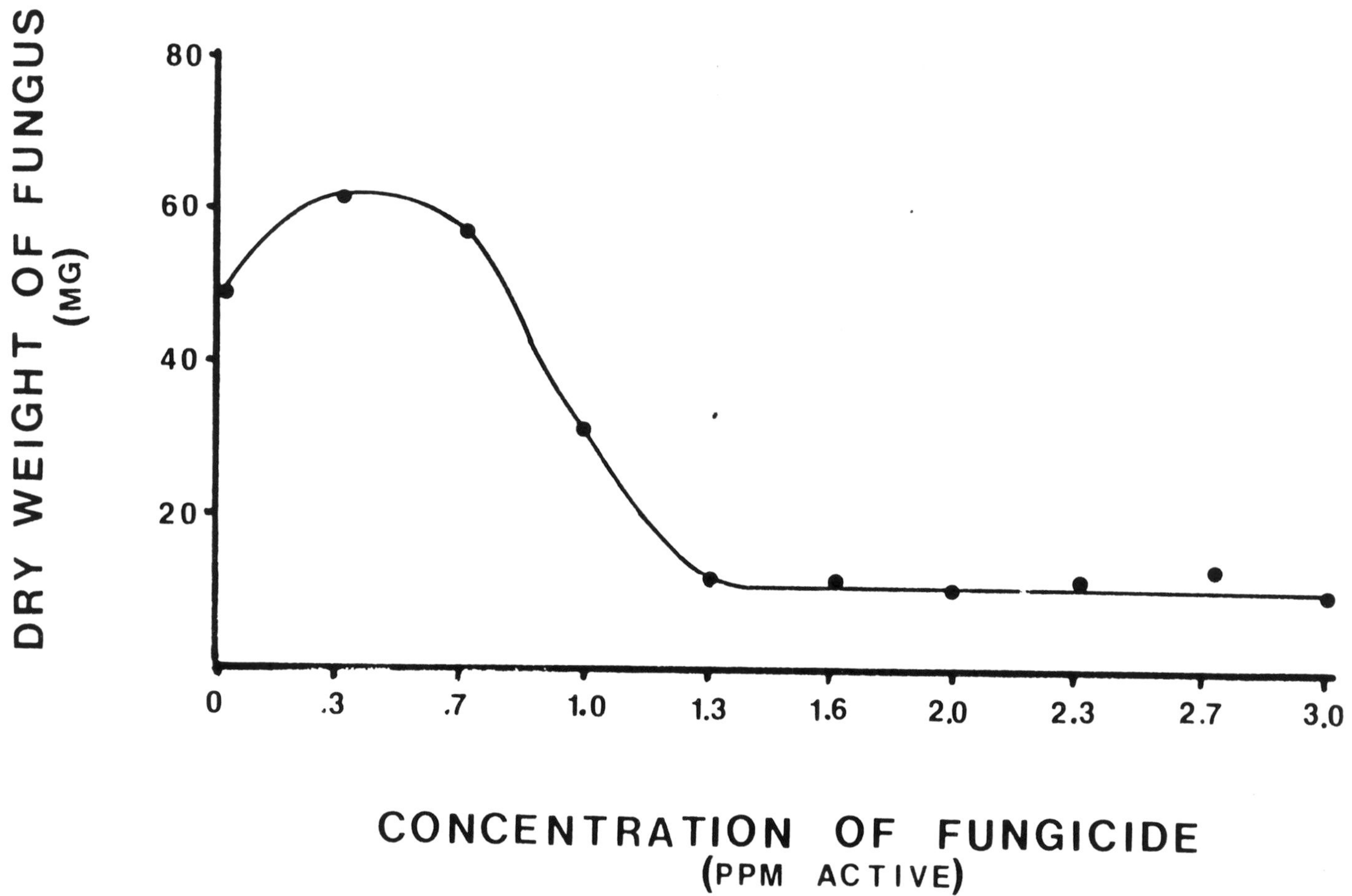

DRY WEIGHT OF FUNGUS
(MG)
80
60
40
20
0
.3
.7
1.0
1.3
1.6
2.0
2.3
2.7
3.0
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 6. Dosage-response of *Lagenidium callinectes*, L-3b isolate to EL-131

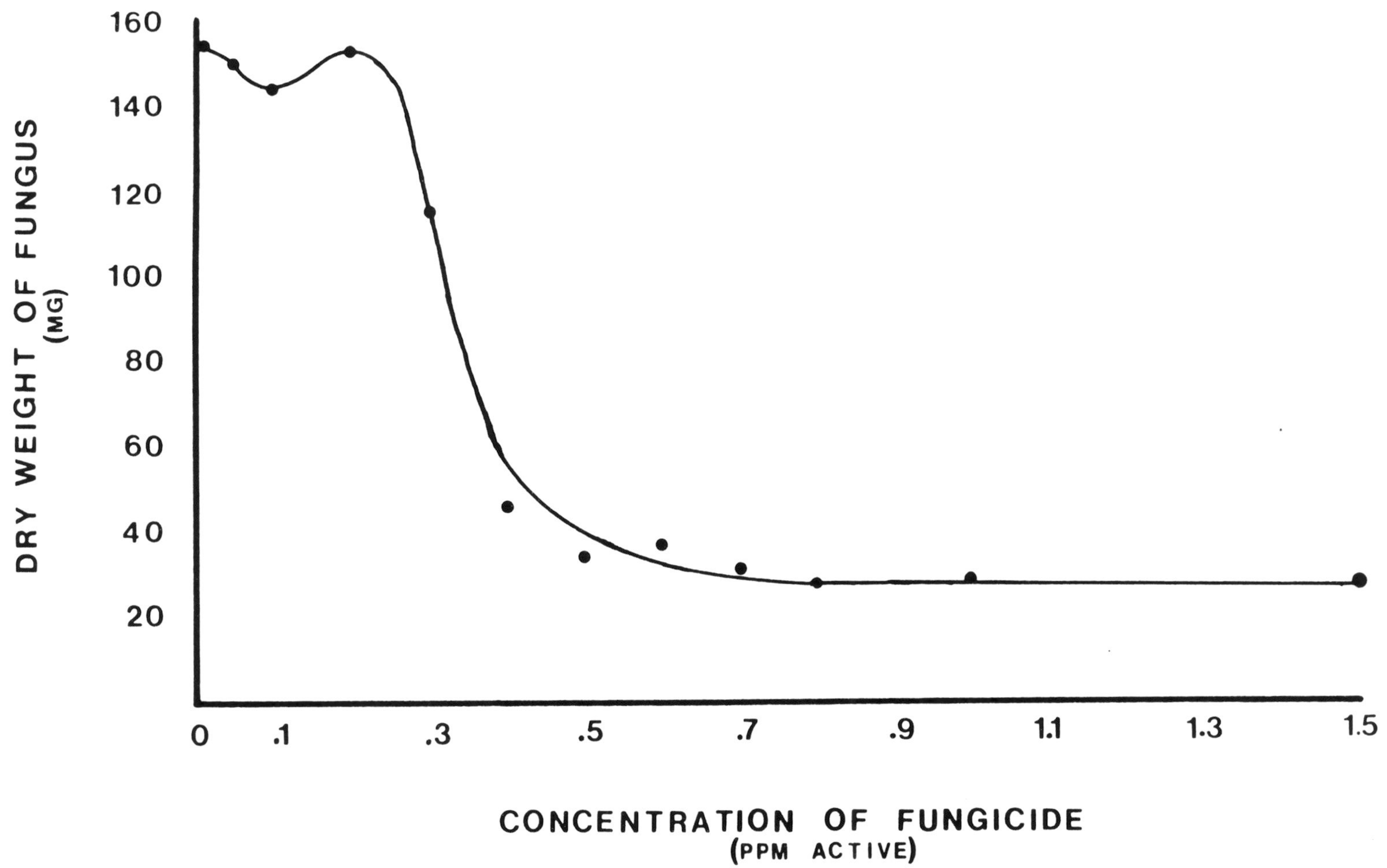
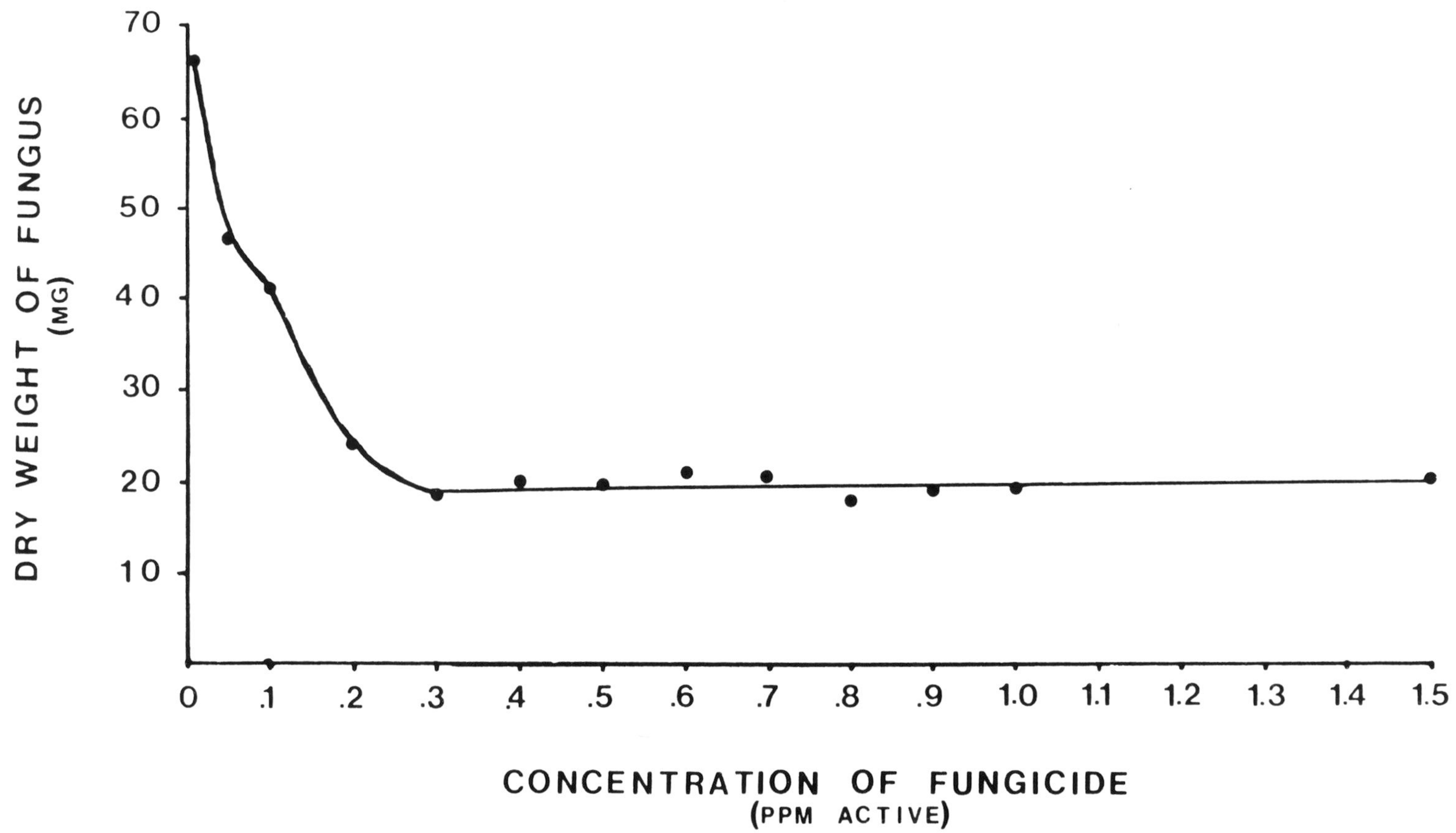
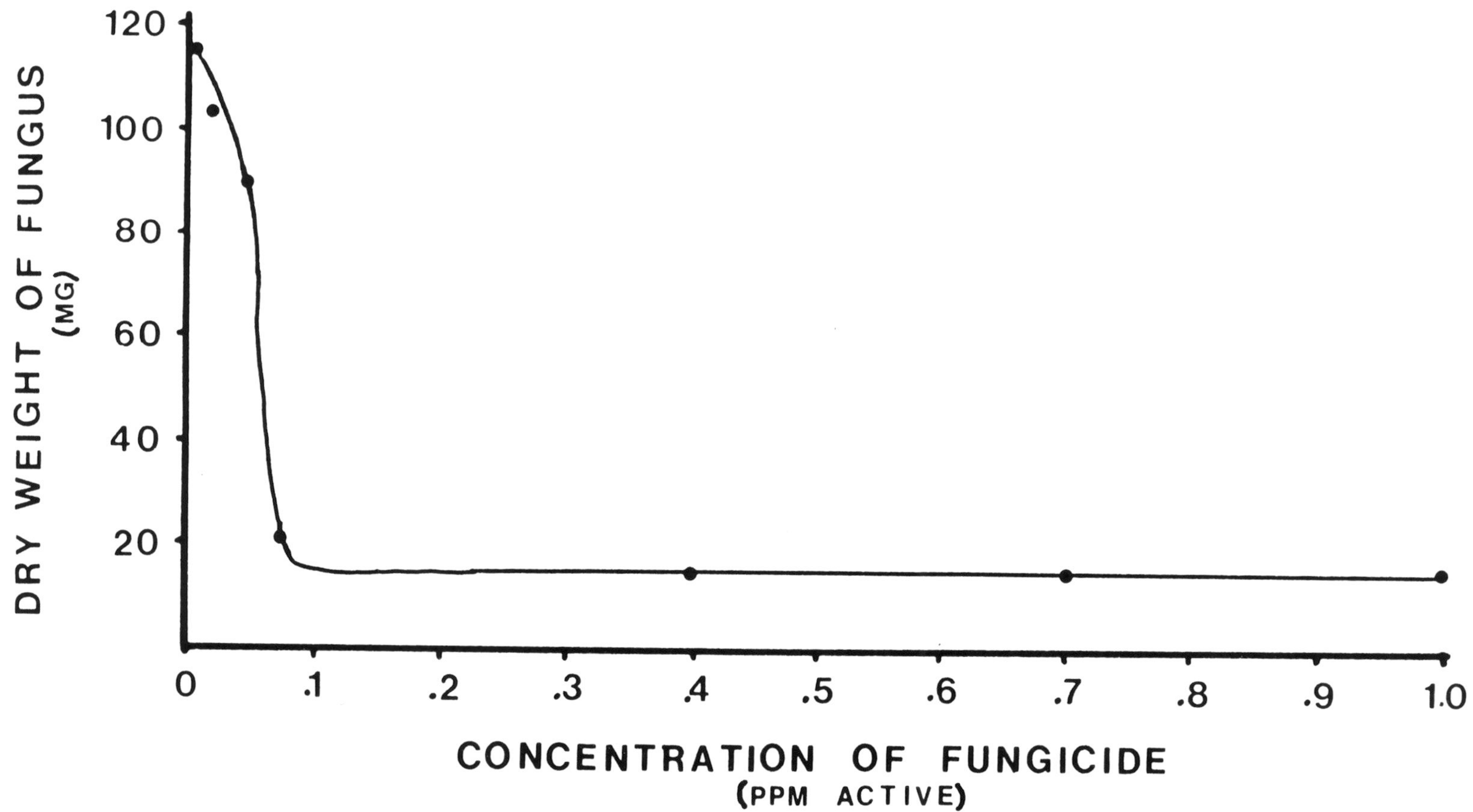

160
140
120
100
80
60
40
20
DRY WEIGHT OF FUNGUS (MG)
0
.1
.3
.5
.7
.9
1.1
1.3
1.5
CONCENTRATION OF FUNGICIDE (PPM ACTIVE)

Figure 7. Dosage-response of *Lagenidium callinectes*, L-16 isolate to EL-131



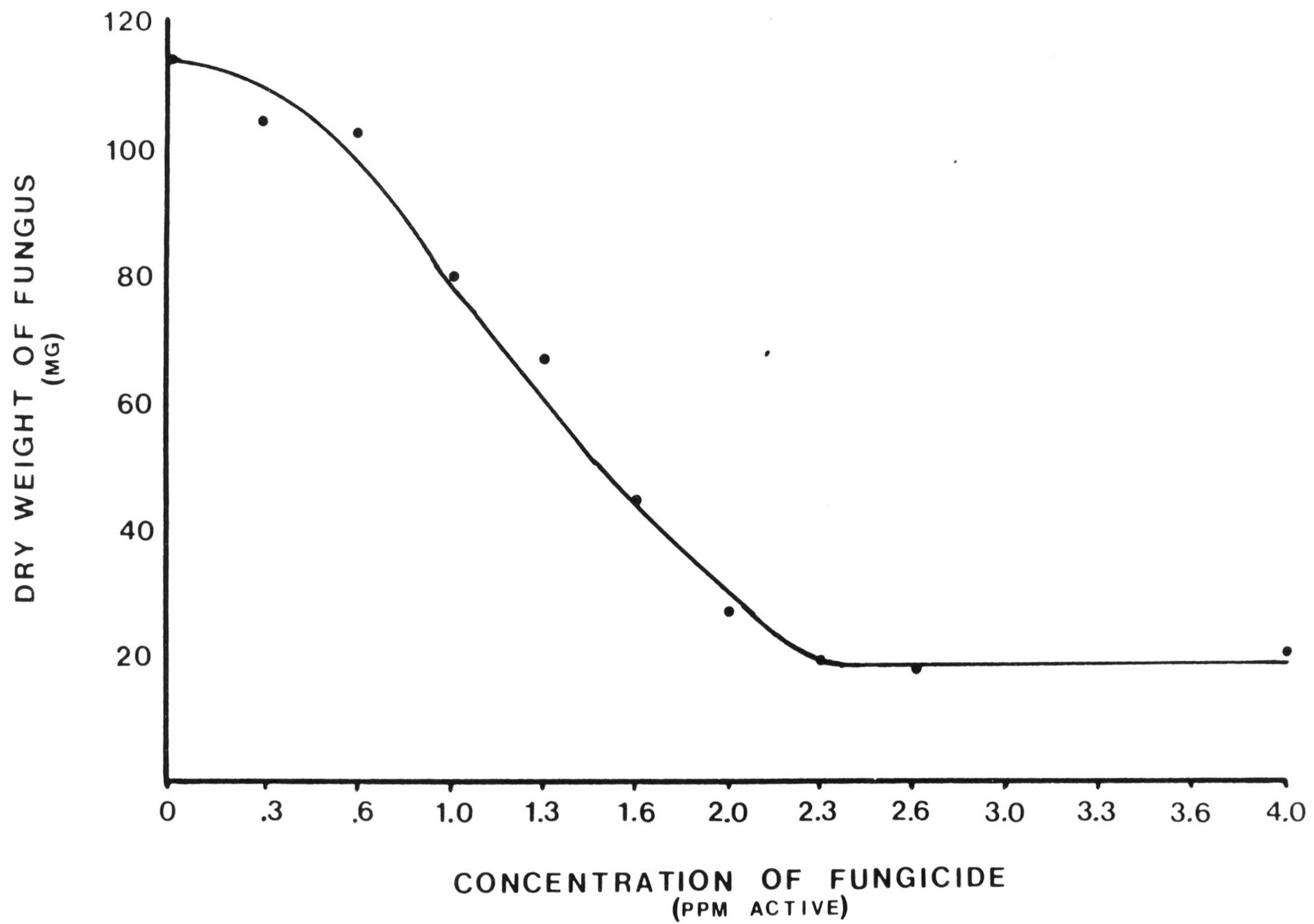
DRY WEIGHT OF FUNGUS (MG)
70
60
50
40
30
20
10
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
1.4
1.5
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 8. Dosage-response of *Lagenidium callinectes*, L-815 isolate to EL-131



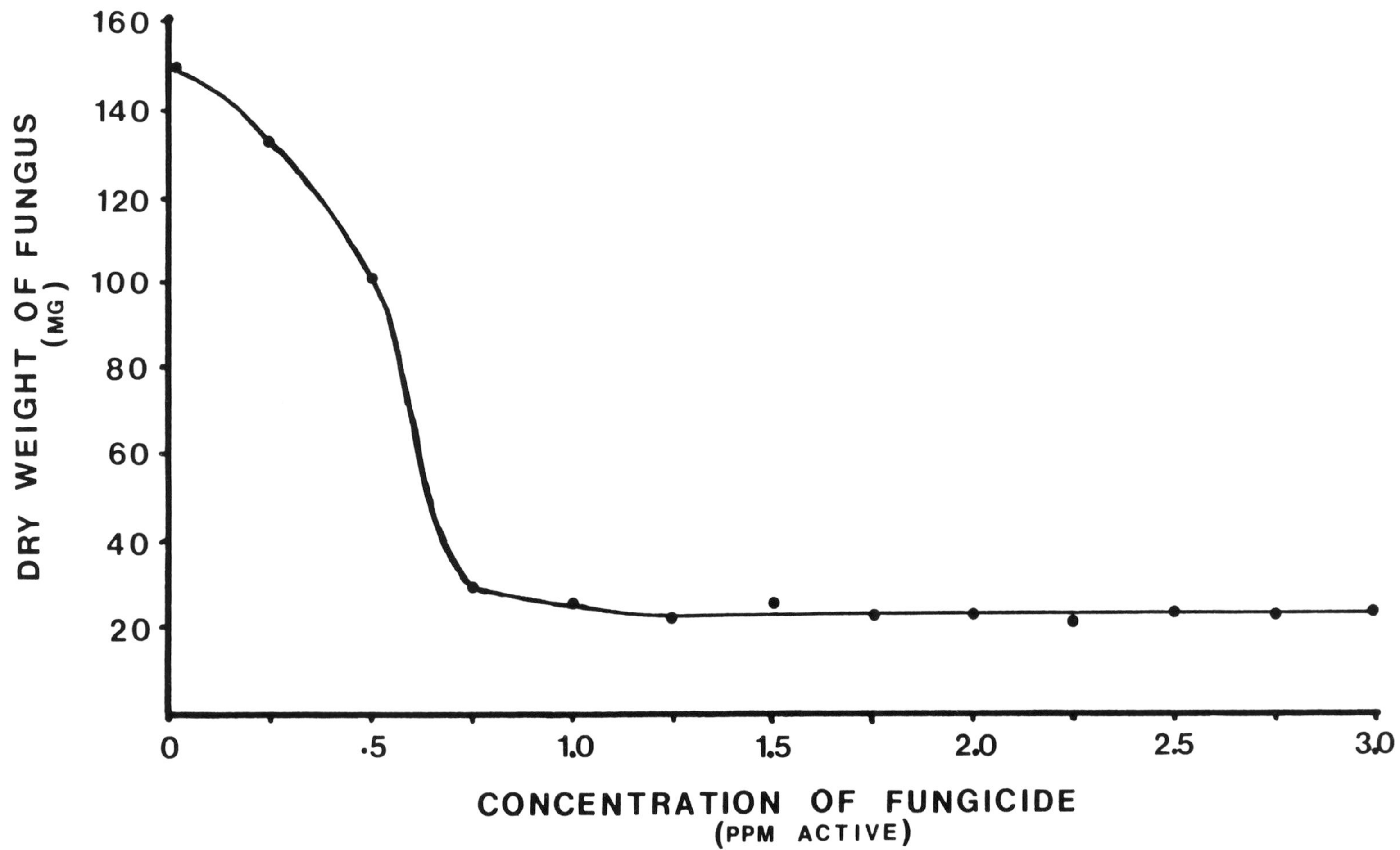
DRY WEIGHT OF FUNGUS
(MG)
120
100
80
60
40
20
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 9. Dosage-response of *Haliphthoros milfordensis*, H-222 isolate to EL-131



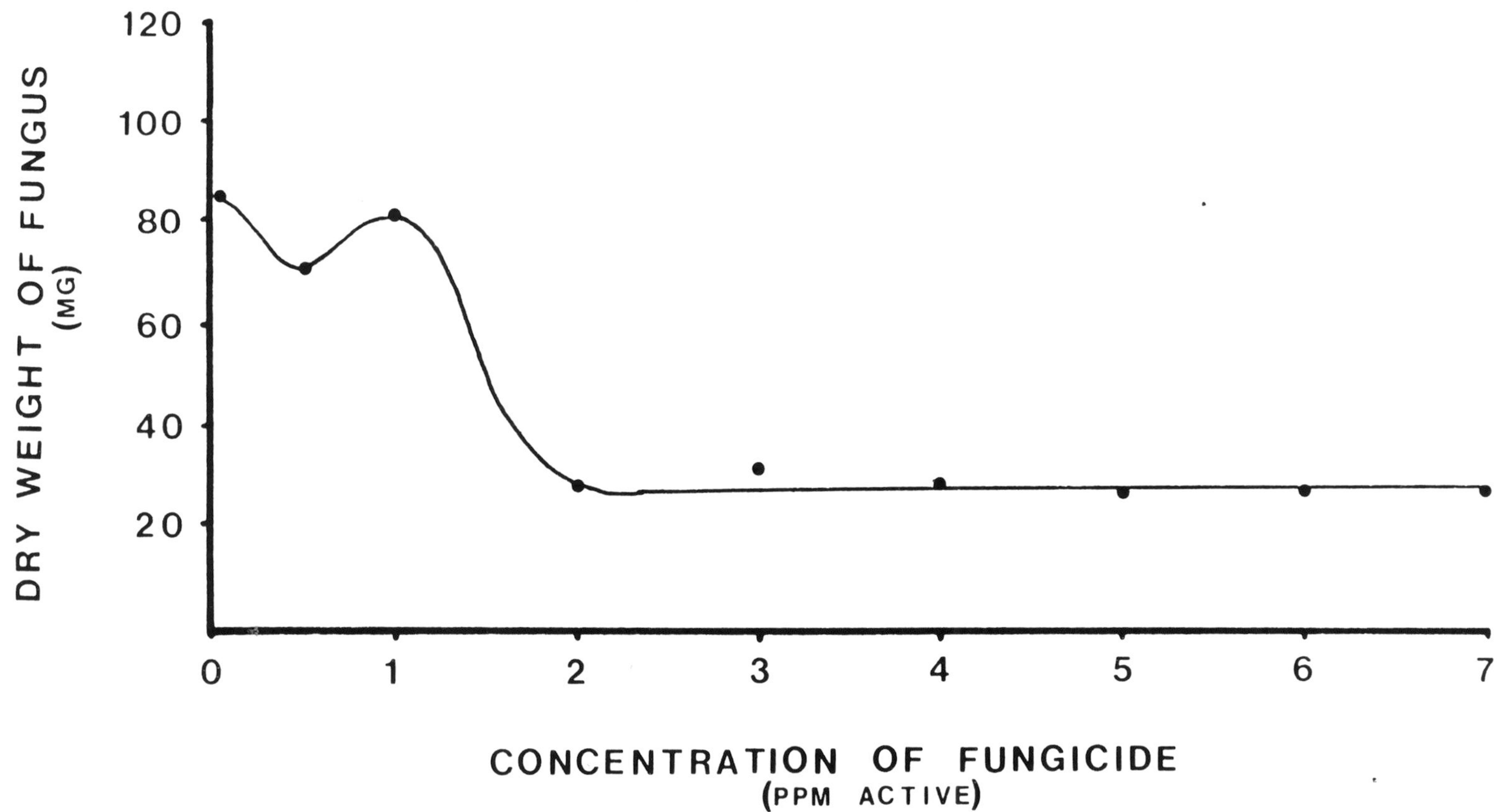
DRY WEIGHT OF FUNGUS
(MG)
120
100
80
60
40
20
0
.3
.6
1.0
1.3
1.6
2.0
2.3
2.6
3.0
3.3
3.6
4.0
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 10. Dosage-response of *Lagenidium callinectes*, L-3b isolate to IL-780



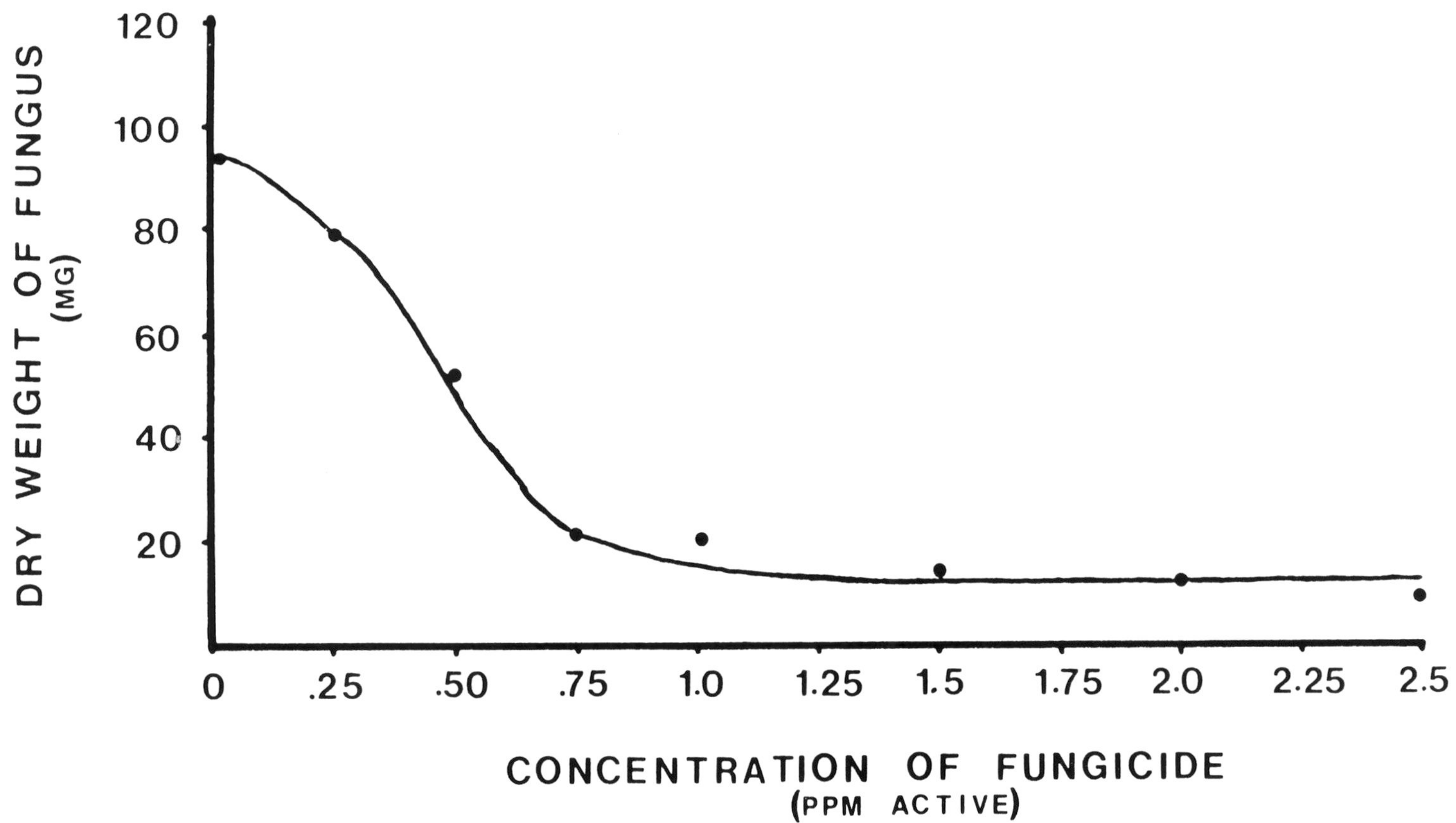
DRY WEIGHT OF FUNGUS (MG)
160
140
120
100
80
60
40
20
0
.5
1.0
1.5
2.0
2.5
3.0
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 11. Dosage-response of *Lagenidium callinectes*, L-16 isolate to IL-780



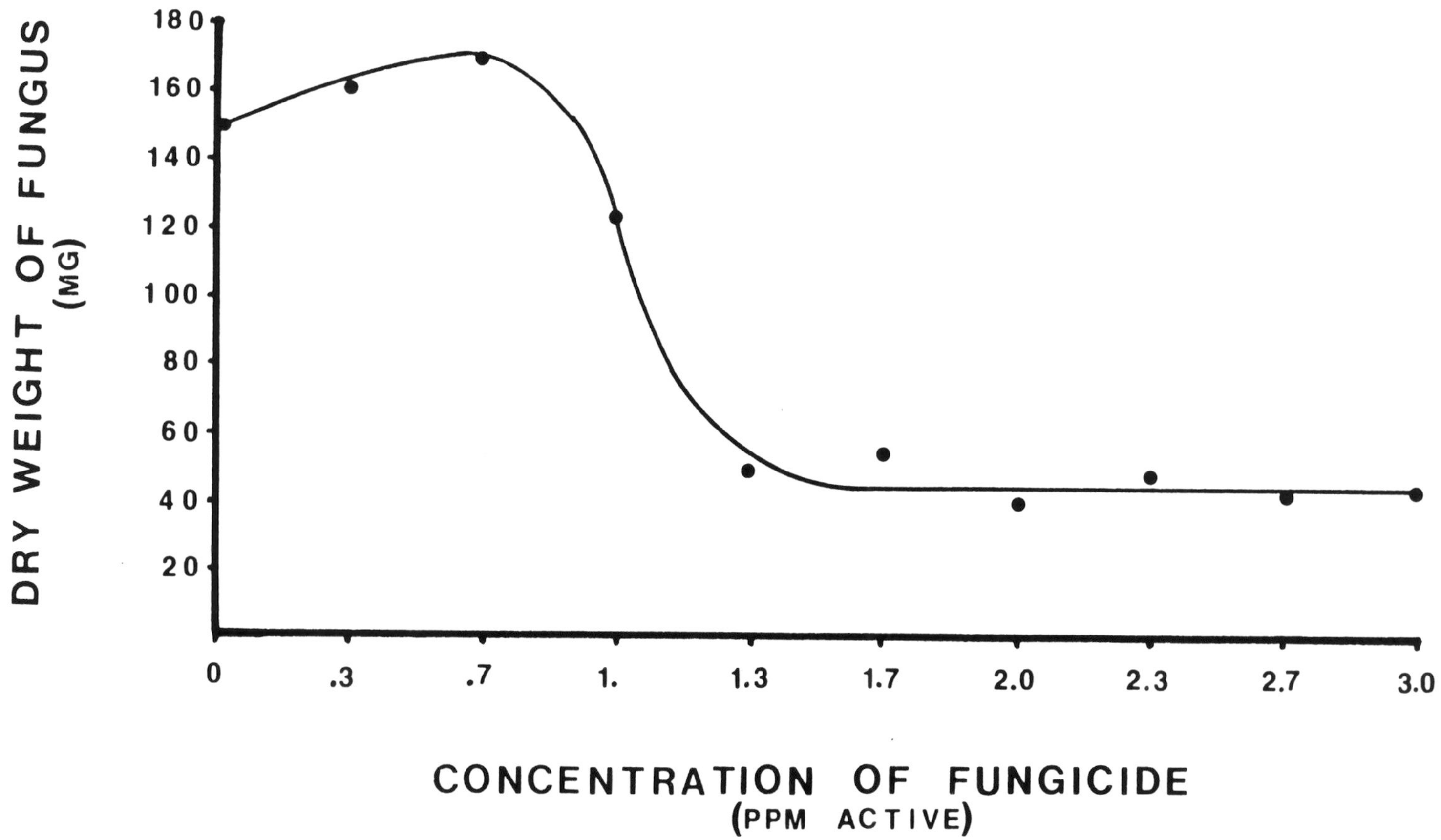
DRY WEIGHT OF FUNGUS
(MG)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 12. Dosage-response of *Lagenidium callinectes*, L-1 isolate to IL-780



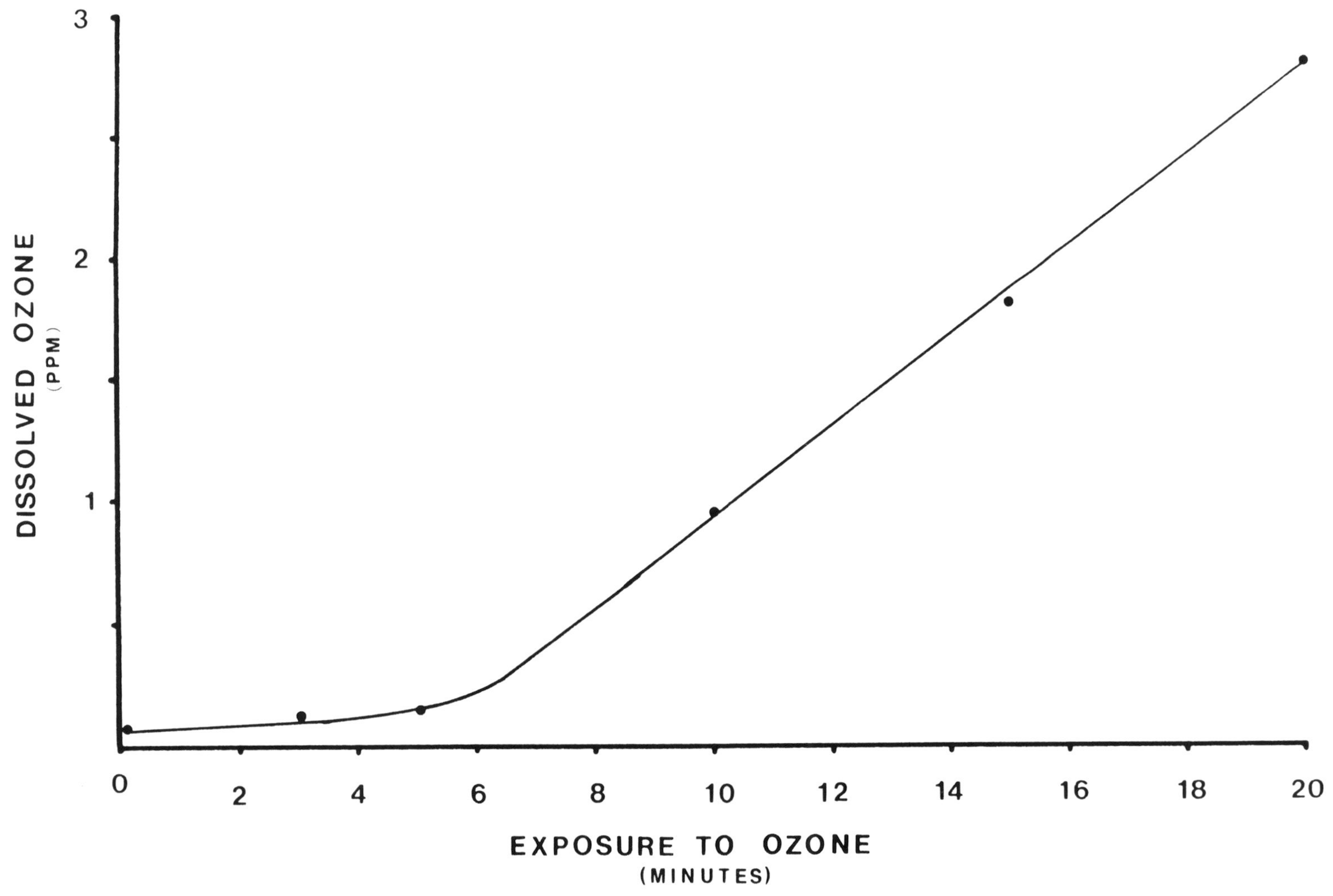
120
100
80
60
40
20
DRY WEIGHT OF FUNGUS
(MG)
0
.25
.50
.75
1.0
1.25
1.5
1.75
2.0
2.25
2.5
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 13. Dosage-response of *Haliphthoros milfordensis*, H-222 isolate to IL-780



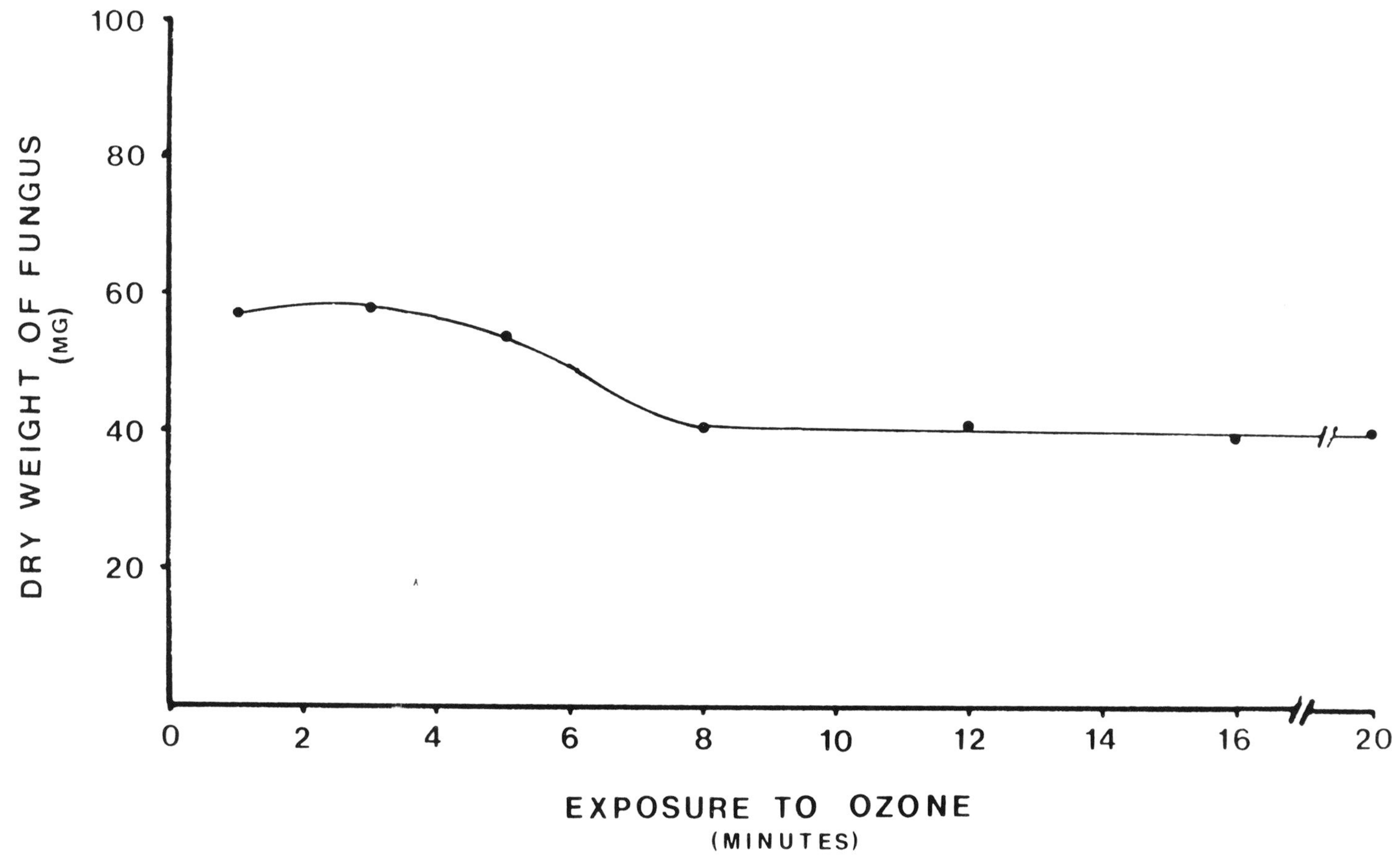
DRY WEIGHT OF FUNGUS
(MG)
180
160
140
120
100
80
60
40
20
0
.3
.7
1.
1.3
1.7
2.0
2.3
2.7
3.0
CONCENTRATION OF FUNGICIDE
(PPM ACTIVE)

Figure 14. Calibration curve for ozone apparatus



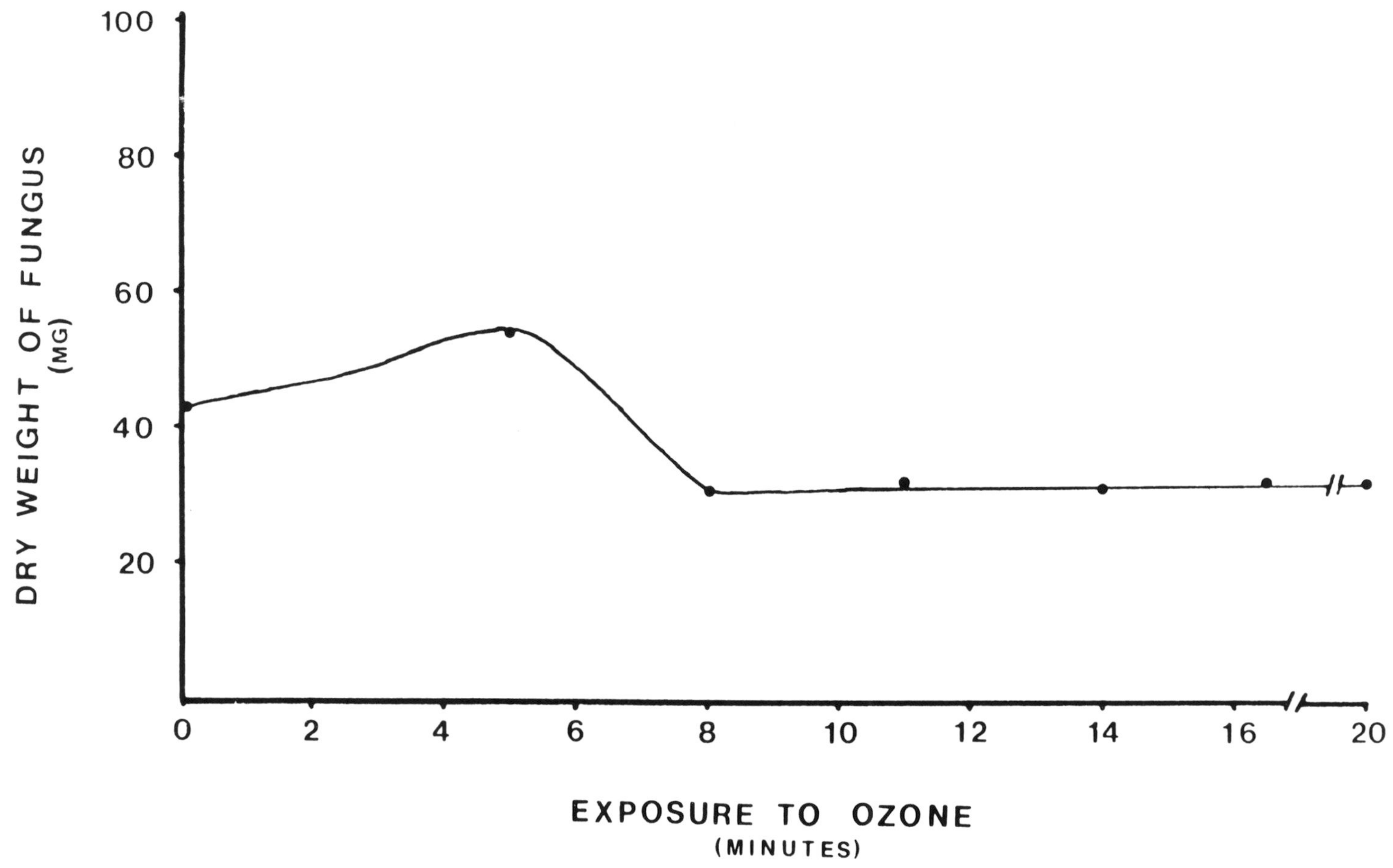
3
2
1
DISSOLVED OZONE
(PPM)
0
2
4
6
8
10
12
14
16
18
20
EXPOSURE TO OZONE
(MINUTES)

Figure 15. Dosage-response of *Lagenidium callinectes*, L-815 isolate to ozone



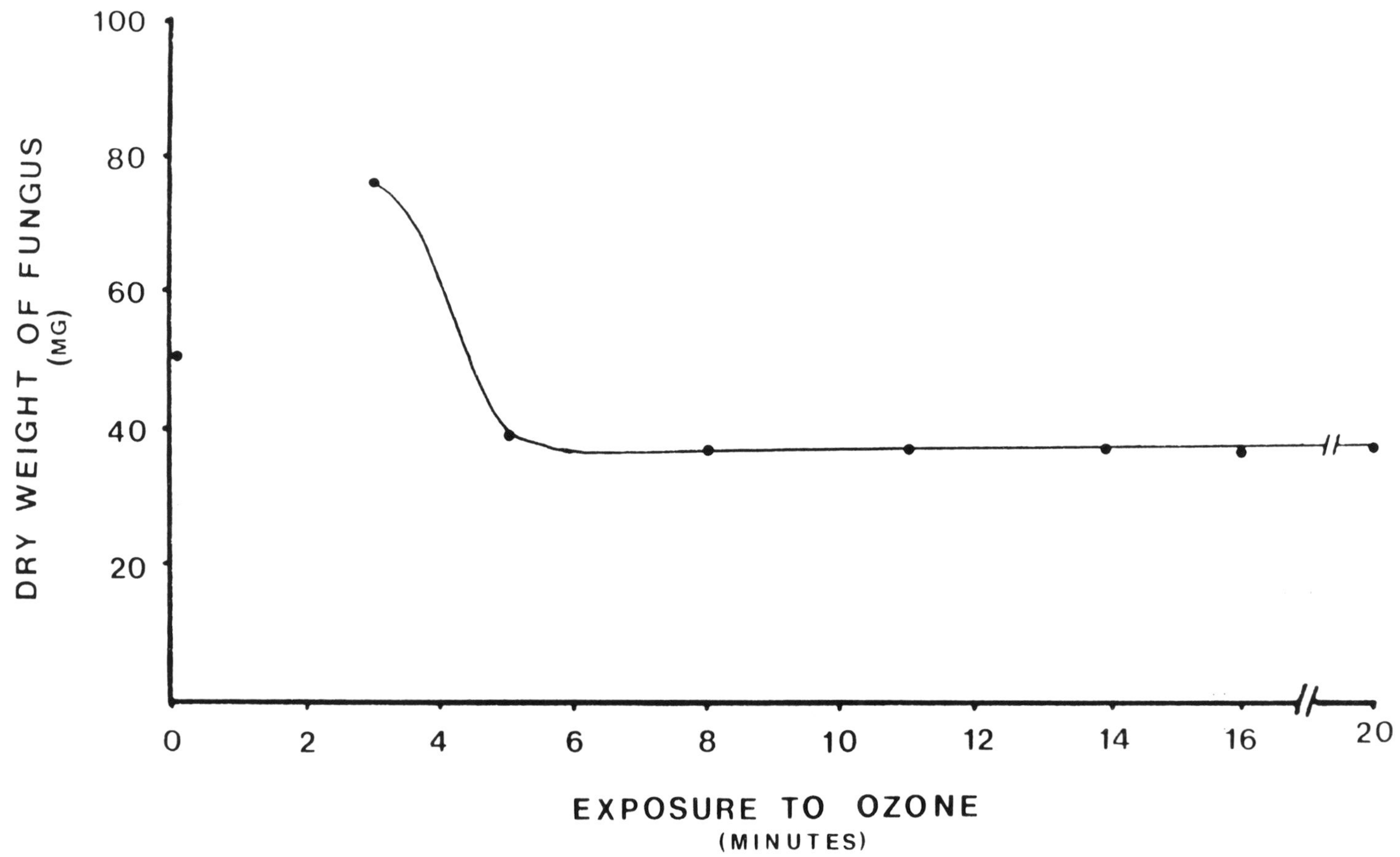
100
80
60
40
20
DRY WEIGHT OF FUNGUS
(MG)
0
2
4
6
8
10
12
14
16
20
EXPOSURE TO OZONE
(MINUTES)

Figure 16. Dosage-response of *Lagenidium callinectes*, L-1 isolate to ozone



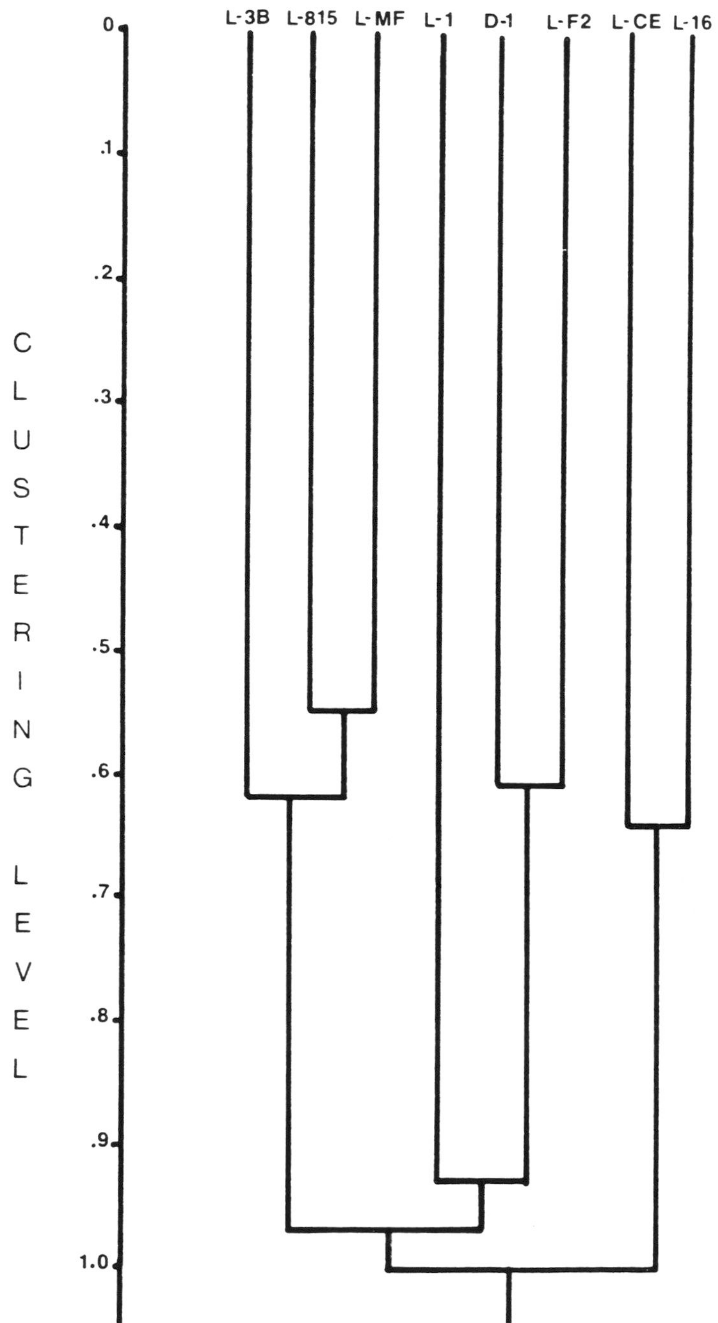
DRY WEIGHT OF FUNGUS
(MG)
100
80
60
40
20
0
2
4
6
8
10
12
14
16
20
EXPOSURE TO OZONE
(MINUTES)

Figure 17. Dosage-response of *Fusarium solani* to ozone



100
80
60
40
20
DRY WEIGHT OF FUNGUS
(MG)
0
2
4
6
8
10
12
14
16
20
EXPOSURE TO OZONE
(MINUTES)

Figure 18. Clustering analysis dendrogram for *Lagenidium callinectes* isolates



L-3B
L-815
L-MF
L-1
D-1
L-F2
L-CE
L-16
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
CLUSTERING LEVEL

Figure 19. Clustering analysis dendrogram for *Lagenidium callinectes* and *Haliphthoros milfordensis* isolates

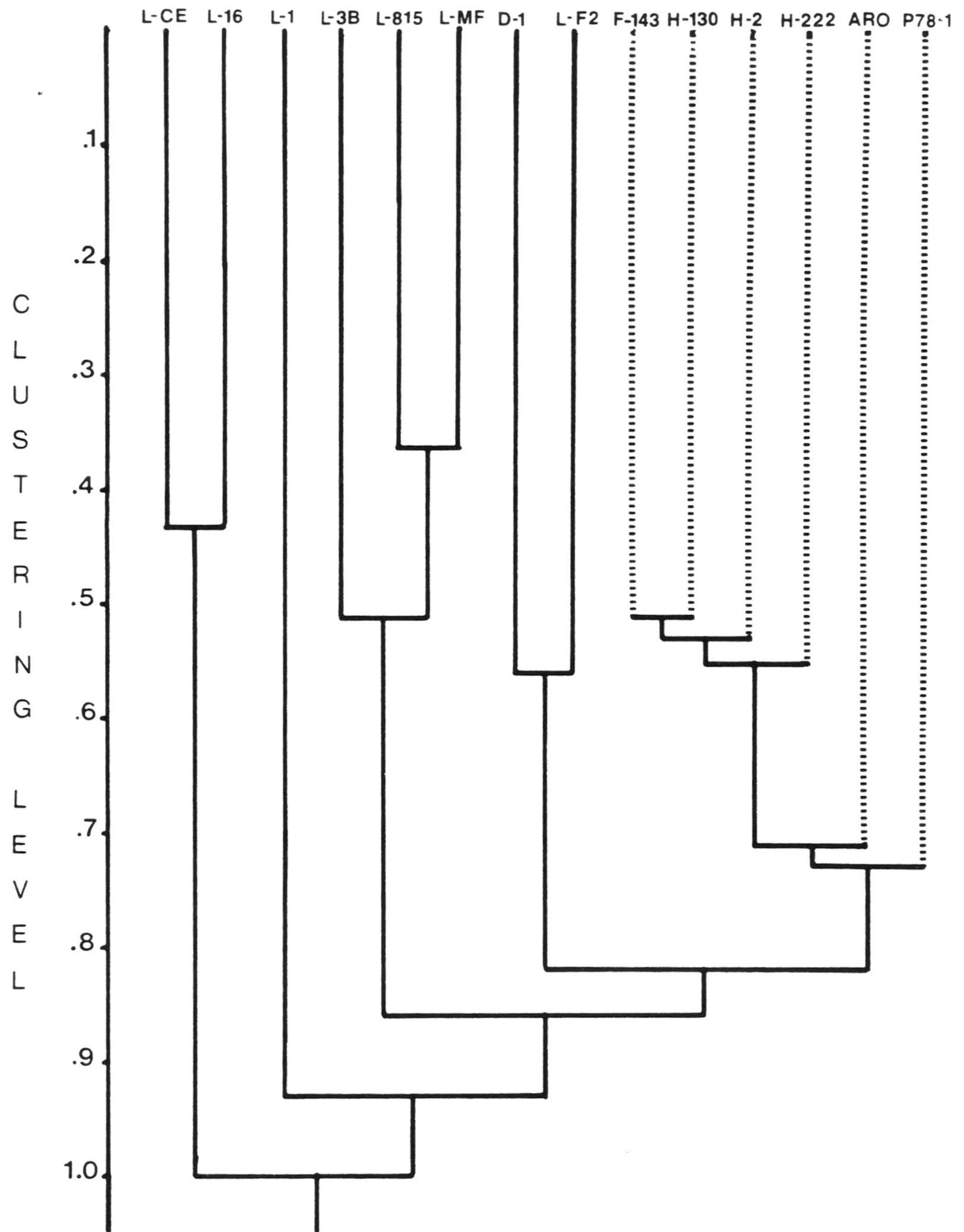

L-CE
L-16
L-1
L-3B
L-815
L-MF
D-1
L-F2
F-143
H-130
H-2
H-222
ARO
P78-1
CLUSTERING LEVEL
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0

Table 4. Tabulation of hyphal characterization results

HYPHAL CHARACTERISTICS

	L-1	L-3b	L-MF	L-815	L-16
Apical Segment Length	125.0µ(45.0)	159.9µ(91.3)	48.1µ(32.7)	48.9µ(30.2)	201.1µ(192.0)
Internodes	46.5µ(31.0)	50.4µ(36.0)	32.1µ(23.2)	30.0µ(29.0)	41.3µ(41.6)
Apical Segment Ratio	6.9 : 1	5.1 : 1	1.5 : 1	11.2 : 1	6.9 : 1
First Order Hyphae	26.0µ(21.7)	47.9µ(23.8)	17.5µ(10.1)	21.4µ(13.8)	24.8µ(22.8)
Second Order Hyphae	94.0µ(47.5)	99.0µ(0.0)	72.4µ(31.9)	20.0µ(0.0)	50.3µ(27.4)
Third Order Hyphae	N/A	N/A	79.0µ(0.0)	N/A	63.0µ(0.0)
Leading Hyphae Diameter	6.7µ(0.7)	10.7µ(1.8)	6.7µ(0.8)	4.5µ(0.5)	8.3µ(0.9)
Primary Hyphae Diameter	6.6µ(0.9)	10.0µ(2.1)	6.3µ(1.0)	4.9µ(0.8)	7.7µ(1.0)
Hyphal Segment Ratio	1.0 : 1	1.1 : 1	1.1 : 1	0.9 : 1	1.1 : 1
Average Angle	85.2 (21)	86.4 (16.6)	86.8 (12.8)	78.0 (16.2)	83.4 (11.3)
Hyphal Growth Unit	112.1µ/tp(41)	117.3µ/tp(29)	46.6µ/tp(11)	50.7µ/tp(18)	75.5µ/tp(31)

DISCUSSION

Fungicide Tests

The algal extracts 1, 2, 3, 8, 9, and 10 showed no apparent antifungal activity at concentrations up to 40 ppm. The combination of high concentrations required to establish an LC_{100}, as well as the high degree of impurity in the extracts of organic extraction solvents, precluded further investigation of these compounds.

The experimental compounds R-41-400, Imazalil nitrate, R-27-180 and R-41-782 are all derivatives of the compound miconazole, developed by Janssen Pharmaceutical of Belgium as an antimicrobial agent for both human and veterinary usage.[2] All derivatives were active against the L-3b isolate of *L. callinectes*, with lethal concentrations below 10 ppm. The R-41-782 derivative, or Enilconazole (trade name), which was selected for further investigation effected an LC_{100} of less than 2 ppm. for both the *L. callinectes* and *H. milfordensis* isolates, while having no effect on *F. solani*. Consistent with these results were Tavolek's finding which showed the R-41-782 derivatives to be most effective when tested against another phycomycete, *Saprolegnia*.[3] Their finding established an LC_{100} of less than 100 ppm. Because of the low

[2]Personal communcation of Dr. Donald Amend of Tavolek with author, 1981.

[3]Personal communication of Dr. Donald Amend with Dr. C. E. Bland, 1977.

LC_{100}'s of R-41-782 and the prospect of its becoming licensed for human use, research seems warranted for the establishment of LC_{100} for crustacean larvae.

IL-780 or polymeric biquanide was originally developed in England by ICI as a microbiocide for use in beverages, and is currently being produced commercially in England as Vantocil IB. An earlier form of IL-780 labelled DS-9073 was studied previously and found to effect an LC_{100} of 3.2 ppm. for the Lagenidium isolate L-3b and 1.3 ppm. for the L-1 isolate (Ruch, 1973). It was found also that lower concentrations ($\sim$ 0.1 ppm.) caused loss of spore motility which affected the pathogens ability for infection. The current study indicates that the latest formulation of Polymeric biquinide, IL-780, may be slightly more toxic than its earlier form DS-9073, since the LC_{100}'s for both L-3b and L-1 were lower (0.9 and 1.0 ppm. respectively). Research has shown IL-780 to have a very low mammalian toxicity (Anonymous, 1979). Acute oral LD_{50}'s for rats is at least 4.0 gms./Kg. of body weight -- with no effect when rats are fed 5,000 ppm for ninety days (Anonymous, 1979). Research has also shown IL-780 to be highly toxic to aquatic organisms. This is indicated in its LC_{50} for the bluegill, Lepomis microchirus, of 0.62 mg./l. (ppm.) during static test of ninety-six hours, and in its LC_{50} of 0.18 mg./l. for the water-flea, Daphnia magna.

Although concentrations lower than the LC_{100} for IL-780 against fungal pathogens may be helpful in keeping pathogens in check, it is doubtful that it can be used in view of its high toxicity to other aquatic organisms.

Prosulfalin or "Sward" (trade name) is an experimental preemergence herbicide developed by the Eli Lilly Company for use on turfgrass. Prosulfalin or EL-131 (original experimental designation) showed the lowest LC_{100} for L. callinectes of all compounds studied. In this regard an LC_{100} of less than 1.0 ppm. was shown for all isolates. Oddly, this compound was found to be the least effective against H. milfordensis in effecting an LC_{100} of 2.3 ppm.for the H-222 isolate. Research has shown acute oral LD_{50}'s for rats to be in excess of 2 gms./mg. of body weight (Anonymous, 1975). In addition to this, the bluegill, Lepomis microchirus, survived a concentration of 37 mg./1. with no effect during a ninety-six hour exposure (Anonymous, 1975). Because of the low LC_{100} required to remove Lagenidium and Haliphthoros as a contagion, and the relatively high dosages needed to affect both rats and fish, further research is warranted relative to EL-131's toxicity against cultured larval crustaceans.

The deuteromycete, F. solani, proved resistant to the compounds R-41-782 and IL-780, as well as EL-131. In all cases, no lethal dosage was reached at concentrations up to 40 ppm. The nature of Fusarium resistance is unknown, however it may be a reflection of its cell wall composition. The cell wall may be linked to a resistance to fungicide action. Recently, the problem of F. solani as a contagion has been significantly reduced by filtration of fresh

air as it enter the culture center grow-out area.[4]

Ozonation Test

The ozone concentration determination showed no build-up of ozone within the first five minutes of input of ozone to the contact tube. Succeeding this point, ozone concentration increased linearly at approximately 0.2 mg./l./min., with a total concentration of 2.8 ppm. after twenty minutes (Fig. 14). The lack of build-up of ozone during the early period of testing is probably due to ozone utilization during oxidation of compounds within the sea water.

For _L. callinectes_ isolates L-1 and L-815, and for _F. solani_, there was no apparent effect of ozonation during the first five minutes. Yet in each case 100% mortality occurred by the eight minute period. This suggests that LC_{100}'s for these organisms can be placed about 0.8 ppm. Motile zoospores of _L. callinectes_ were observed up to the point of death (8 minutes), with only cellular debris remaining after this point. This suggests that cellular lysis occurs during the first eight minutes of ozonation, after oxidation of other compounds in the sea water. The idea of ozone induced cellular lysis is in accordance with the finding of Rosen, Lowther and Clark (1975) who postulated that disinfection by ozone occurs by lysis of the pathogen when it comes in contact with a bubble of ozone. They also postulated that lysis is independent of ozone concentration. From this, I conclude that the contact time with ozone, rather than its concentration, is of prime importance when

[4]Personal communication of Dr. D. V. Lightner with Dr. C. E. Bland, 1979.

using ozone as a means of disinfection.

Ozonation as a means of bacterial and fungal pathogen control has been used by other investigators and with similar results. (Straub, V. M., 1975; Blogoslawski, and Alleman, 1979; Danald, Ure, and Lightner, 1979; Wedemeyer, Nelson, and Yasutake, 1979). Ozone has been shown also to be effective in improvement of water quality in aquaculture by increasing dissolved oxygen and decreasing ammonia and nitrite levels, thus allowing higher stocking densities (Honn, 1979; Rosenthal and Otte, 1979).

However, Danald et. al. (1979) noted high mortalities of shrimp placed in ozonized water for periods in excess of ten minutes, even twenty-four hours after ozonation. Since ozone's half-life in sea water is approximately fifteen minutes (Honn, 1979), it is probable that the post ozonation mortality is due to other long lived oxidative compounds produced by the initial ozonation (Danald et. al., 1979; Honn, 1979). It has been suggested that these compounds may be removed from the system by charcoal filtration (Danald, 1979).

The possibility of using ozone as means of pathogen control and water quality improvement appears to be quite feasible as long as the proper precautions have been made to ensure protection of cultured organisms from the long lived oxidative compounds. This use of ozone would necessitate placing a charcoal filtering system just behind the contact chamber in the filtration system prior to reentry to the aquarium.

Clustering Analysis

Bahnweg's (1980) nutritional and physiological study supported

the belief of other investigators that when comparisons are made between different isolates, Lagenidium callinectes is quite heterogeneous. He concluded further there are at least two groups within the species, one heterogeneous assemblage composed of the isolates L-MF, L-3b, L-815 and L-1, and another more homogeneous grouping composed of the isolates L-F2 and D-1. Members of the first group were characterized as having a narrow range of substrate utilization and being obligately marine, while members of the second group possessed similar growth rates, similar temperature optima, a wide range of substrate utilization, and the ability to grow in media with no NaCl.

When data from this study was originally subjected to Clustering Analysis, three groups (Clusters), rather than Bahnweg's two, were observed. One cluster was formed by the isolates L-MF, L-3b and L-815, a second was formed by the isolates L-F2 and D-1 and a third was composed of the single isolate L-1. Closer examination of Bahnweg's original paper revealed that these same three groups are identifiable in his charting of growth versus pH, as well as growth versus temperature. In each case the isolate L-1 was found intermediate to the extremes formed by the other two groups.

In 1981, Dr. Bahnweg made available new data concerning the most recent isolates, L-16 and L-CE. When subjected to Clustering Analysis these isolates formed a fourth and separate grouping (Fig. 18).

Closer examination of these results showed that these clusters were not only physiological groupings, but also geographical

groupings. (Fig. 20) The first cluster composed of the isolates L-MF, L-3b and L-815 were isolated from the warm waters of the Gulf of Mexico (L-MF & L-3b) and the Gulf of California (L-815). The second cluster composed of L-F2 and D-1 were isolated from the cooler waters of the north Pacific. The third cluster composed of the single isolate L-1 was isolated from the more temperate waters of the mid-Atlantic. The fourth and final cluster composed of the isolates L-16 and L-CE were both isolated from the Philippines.

When _Lagenidium callinectes_ and _Haliphthoros milfordensis_ were subjected to Clustering Analysis together, a single cluster of all _Haliphthoros_ isolates was formed at the 0.73 clustering level, while at the same level the four _Lagenidium_ clusters retained their identity. This further supports the idea that _Lagenidium callinectes_ is quite heterogeneous and that the four clusters represent true biological subgroups within the species.

Conclusion

The original description of _L. callinectes_ as a new species was made by John N. Couch (1942) from living and formalin preserved material on ova of the blue crab, _Callinectes sapidus_. An abbreviated form of this description follows:

Lagenidium callinectes -- Couch (1942)

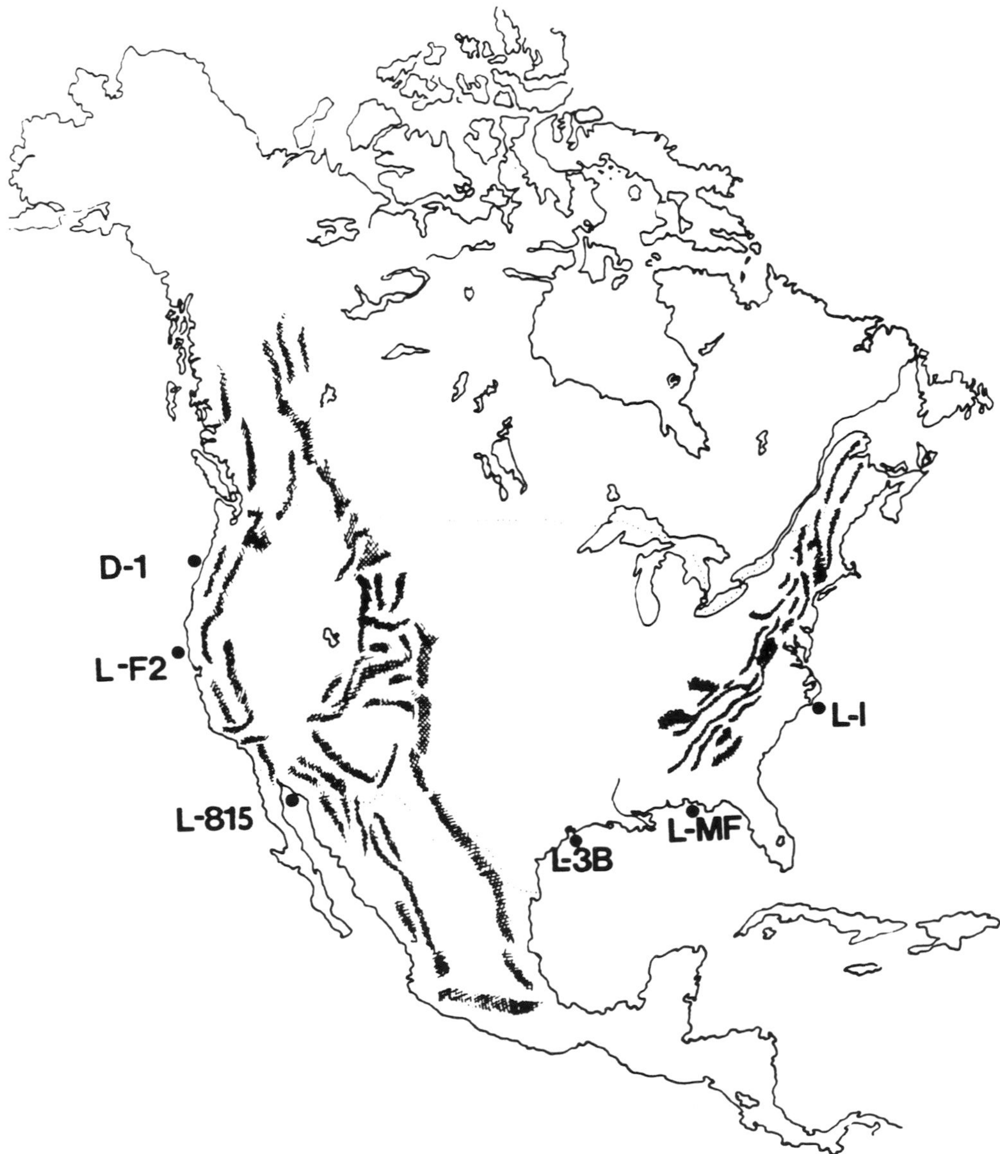
-Parasitic on the ova of the blue crab, _Callinectes sapidus_

-Irregularly branched, sparingly septate hypha, 5.4μ to 12.6μ

-Asexual reproductive vesicle (containing zoospores) variable to 100μ

-Vesicle persistent after release of zoospores

Figure 20. Geographical distribution of U. S. *Lagenidium callinectes* isolates


D-1
L-F2
L-815
L-3B
L-MF
L-I

-Reniform, biflagellate zoospores, 9.6μ X 12.6μ

-Encysted zoospores, oblong or subglobose, monoplanetic, 10.0μ X 11.3μ

-Resting bodies, 18μ X 30 μ

Since then, L. callinectes has been collected by a number of investigators, from different hosts and usually from aquaculture situations. Differences in morphology and physiology have been observed, with the most noted of these being: degree of extramatrical hyphae, length of discharge tubes, formation of vesicles, salinity tolerance, growth rates, as well as susceptibility to various fungicides (Couch, 1942; Johnson and Bonner, 1960; Ruch, 1974; Armstrong et. al., 1976; Daniels, 1977; Bahnweg, 1980). Other observed differences include: vesicle size, spore size, hyphal diameter, method of encystment, temperature tolerances and pH tolerances (Couch, 1942; Johnson, 1957; Johnson et. al., 1960; Scott, 1962; Fuller, 1964; Lightner et. al., 1973; Bland and Amerson, 1973; Ruch, 1974; Armstrong et. al., 1976; Daniels, 1977; Bahnweg, 1980). It should be noted, however, that these observations were not made under standardized conditions; i.e., the isolates were on different hosts, in water of different salinity, and were cultured in different types of media.

When isolates of L. callinectes were tested under standardized conditions it was found that there was no significant difference among the isolates with respect to zoospore size. These standardized observations showed zoospores of L. callinectes to be highly variable, with a mean width of 9.9μ (with a range from 7.1μ to

13.6μ) and a mean length of 12.6μ (with a range from 10.1μ to 18.5μ). Similarly, standardized observations of the vesicles from several isolates yielded no significant difference with respect to maximal diameter. In this case the vesicle was also shown to be highly variable, with a mean diameter of 47.9μ (and range from 33.0μ to 88.1μ).

The hyphal characterization was also proved to be of little use in discerning one isolate from another. The only hyphal characters which were statistically different, and could be used for grouping, were the length of first order hyphal and the hyphal growth unit. In each case the characters placed the isolates L-1 and L-3b into one group, and L-MF, L-815, and L-16 into a second. It is my belief that little weight should be placed on these groupings since sampling of the L-1 and L-3b isolates was mostly from second order hyphal systems (no third and fourth order hyphal systems were observed). It should be noted, however, that these morphological characters may be helpful in describing the species as a whole. Most significant in this regard is the mean Apical Segment Lengths of 95μ and mean Internode Length of 40μ. These figures give an average Apical Segment Ratio of 2.4 : 1, which suggests a strong degree of Apical Hyphal Dominance. Apical Hyphal Dominance is believed to suggest that branching may be controlled by a maximum rate of tip extension, or by changes in the volume of protoplasm within the apical tip (Trinci, 1973; Gull, 1975). It should also be noted that _L. callinectes_ branched somewhat consistently at 83° (Std. dev. 16.5°).

This investigation not only reinforces the belief of Bahnweg

(1980) that the species L. callinectes is heterogeneous, but suggests that L. callinectes is composed of at least four races which can only be recognized by a number of comparative physiological and biochemical tests. In this case, the four groups (L-MF, L-3b, L-815 / L-F2, D-1 / L-1 / L-16, L-CE) were elucidated by nutritional and physiological test results that were analyzed by the "Clustering Analysis" computer program. This study indicates that the previously noted differences among the various isolates are a net result of intraspecific variation, as well as variations arising from differences in host and culturing conditions.

In a strict comparison with the original description of L. callinectes by Couch (1942), differences in length of discharge tubes, amount of protoplasm filling the vesicles, rate of sporogenesis, as well as differences in host, caused Bian, Hatai, Po and Egusa (1979) to describe Lagenidium scyllae as a new and separate species from the Philippines. In this regard, the present research shows members of the species Lagenidium callinectes to be heterogeneous in both morphology and physiology. Therefore strict comparison to the original description as in L. scyllae may prove invalid.

Since indentification of phycomyceteous fungi relies almost entirely on morphology (Coker, 1923; Sparrow, 1960; Talbot, 1971; Ainsworth, Sparrow and Sussman, 1973), any changes in the taxonomic status of strains of L. callinectes must be dependent upon the

existence of clearly identifiable morphological differences. In conclusion, it appears from this study that there are no such morphological characters which may be used in discerning one group from another and that all of these isolates, and possibly the newly described species *Lagenidium scyllae* (Bian, Hatai, Po, Egusa, 1979), are in fact representatives of the single species *Lagenidium callinectes*. For this reason, a redescription of *Lagenidium callinectes* is warranted and should encompass the heterogeneity observed in the present study.

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