COMPARATIVE PHYSIOLOGY AND CHEMICAL CONTROL OF SELECTED STRAINS OF THE MARINE FUNGUS, *Lagenidium callinectes* COUCH

by

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ABSTRACT

William Henry Daniels. COMPARATIVE PHYSIOLOGY AND CHEMICAL CONTROL OF SELECTED STRAINS OF THE MARINE FUNGUS, Lagenidium callinectes COUCH. (Under the direction of Dr. Charles E. Bland) Department of Biology, November, 1977.

The fungus Lagenidium callinectes Couch, is a marine, holocarpic, biflagellate, phycomycete. First described by Couch (1942) as a parasite of the ova and early larval stages of the blue crab Callinectes sapidus, other strains of Lagenidium have recently been isolated and identified as parasites (pathogens) of other marine crustacea. To facilitate control of Lagenidium in the mariculture of crustacea and as an aid to taxonomic studies, in vitro physiological and fungicidal control tests were conducted. First performed were physiological tests including salinity, temperature, and cation replacement responses. Differing growth rates and sporulation levels were noted for some strains. Secondly, various chemicals reported to have fungitoxic potential were tested for the control of L. callinectes. Those showing possible value in fungal control and warranting further testing were Surflan, Terrazole, Formalin, and Furanace.

ACKNOWLEDGEMENTS

I would sincerely like to acknowledge Dr. Charles E. Bland for his constant supervision throughout the completion of this thesis. Also, I wish to thank Drs. Graham Davis, Charles R. Coble, and Donald Jeffreys for their proofreading and comments. I would also like to thank Mrs. Carol Lunney for her technical assistance. This work was made possible by grants from the NOAA Office of Sea Grant, Department of Commerce, and the State of North Carolina, Department of Administration.

DEDICATION

This thesis is dedicated to my mother, whose guidance and support made its completion possible.

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INTRODUCTION

The fungus *Lagenidium callinectes* Couch, class Oomycetes, family Lagenidiaceae, is a marine, holocarpic, biflagellate, phycomycete. First described by Couch (1942) as a parasite of the ova and young larvae of the blue crab, *Callinectes sapidus*, additional strains of *L. callinectes* have recently been isolated and identified as parasites (pathogens) of other marine invertebrates. The organisms from which these isolates were obtained include the blue crab, *Callinectes sapidus* (Bland and Amerson, 1973), the Dungeness crab, *Cancer magister* (Armstrong, Buchanan, and Caldwell, 1976), the white shrimp *Penaeus setiferus* (Lightner and Fontaine, 1972), and the American lobster, *Homarus americanus* (Nilson, Fisher, and Schleser, 1975). An additional strain identified as *L. callinectes* was isolated from drift algae (Gotelli, 1974). Cultures of these strains were sent to Bland (Department of Biology, East Carolina University) for confirmation of identification and study.

Observations indicate that several subtle but consistent morphological differences exist between the strain of *L. callinectes* first described by Couch and these recent isolates (Bland, personal communication). There is at present, however, no taxonomic basis for distinguishing the new isolates of *L. callinectes*. Since basic morphological differences have been observed between the strains, a series of comparative physiological tests was indicated. It was hoped that such tests might facilitate future taxonomic study and offer some insight into control measures for these parasites. Concerning the latter, in addition to basic physiological tests, various chemicals with fungitoxic

potential were tested as possible agents for the control of fungal diseases affecting crustacean mariculture. A need for such control measures currently exists as diseases of fungal etiology are seriously hampering efforts to culture marine crustacea (Bland, 1975).

MATERIALS AND METHODS

Strains Used

Five strains, tentatively identified as L. callinectes, were used in the various comparative studies. They are: L-1, isolated by Bland and Amerson (1973) from ova of the blue crab Callinectes sapidus collected in the Newport Estuary, N. C.; 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; LAG-F2, isolated by Nilson et al., (1975) from larvae of the American lobster, Homarus americanus, raised at the Bodega Marine Laboratory, Bodega Bay, California; D-1, isolated by Armstrong, et al., (1976) from the larvae of the Dungeness crab, Cancer magister, under study at Oregon State University Marine Science Center; and LAG-G, isolated by Gotelli (1974) from drift algae, Friday Harbour, Washington.

Culture Maintenance and Inoculation Technique

Stock cultures of the fungal strains in which sporulation is easily induced (L-1, L-3b, LAG-G) were maintained in PYGS broth medium (dextrose - 3 grams, Bacto peptone - 1.25 grams, yeast extract - 1.25 grams per liter of sea water at approximately 30 ppt salinity) which was sterilized for 15 minutes at 121° C under 15 pounds of pressure.

The procedure for stock culture continuation and spore collection was as follows: The PYGS broth was removed from a 72-hour old culture of L. callinectes by placing a sterile nylon filter over the mouth of the flask and decanting the broth. The same volume of sterile sea water was returned to the flask which was placed on a horizontal rotary shaker (109 revolutions per minute) for 18-24 hours at approximately 25° C.

The spores were then filtered from the flask into an empty sterile 125 ml Erlenmeyer flask. The intensity of sporulation was measured by counting the number of spores per unit volume (spores per cubic centimeter) using a hemacytometer. Sterile spinal needles and disposable syringes were used to extract the spore suspension from the flask to inoculate PYGS growth medium for continuation cultures and to inoculate various test media. Stock cultures, inoculated with 60,000 spores, were placed on a horizontal rotary shaker for 72-78 hours*. After a three day growth period, the procedure for inducing sporulation was repeated.

In the strains resistant to spore induction (D-1 and LAC-F2), inoculation was by mycelial fragmentation. Scrapings of the strain grown on PYGS agar in Petri dishes were placed in 50 ml of sterile PYGS broth in a 125 ml Erlenmeyer flask which was placed on a horizontal rotary shaker for 72-78 hours. The resulting mycelia were collected, washed twice in sterile deionized water and fragmented for 30 seconds using a Waring blender microcup. The amount of fragmented mycelia used for inoculating was determined turbidometrically with a Klett-Summerson colorimeter. The turbidity of the fragmented mycelia was measured in Klett units and could be varied by the addition or removal of sterile deionized water.

Using strain L-3b, comparable experimental results were obtained with either of the prescribed inoculation procedures.

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*60,000 spores per cubic centimeter was determined previously to be the optimum inoculation count for sporulation with respect to continuation cultures (Ruch, 1974).

IN VITRO Tests

All In vitro studies were designed to test comparable growth parameters of individual strains of L. callinectes using 125 ml Erlenmeyer flasks containing 50 ml of a particular broth medium. The organic components of all test media were peptone, yeast extract, and dextrose. Unless otherwise noted, there were five replications of each test medium at each individual concentration.

The flasks containing the test media were sterilized in the same manner as the stock culture medium, allowed to cool, and inoculated with 60,000 spores or 1 cc of fragmented mycelia at a turbidity of 300 Klett units. After inoculation, the flasks were placed on a horizontal rotary shaker for 72 hours. At the end of this period, the flasks were removed and the mycelial growth was collected with a Buchner funnel on tared Whatman #3 qualitative 5.5 cm filter paper. The mycelia were then oven-dried for 48 hours at 70° C, placed in a desiccator and allowed to cool to room temperature, and weighed to within one ten-thousandth of a gram on an H16 Mettler balance.

Defined Saline Medium

In those physiological tests requiring the selective substitution or elimination of various components of the saline medium, a defined saline medium (artificial sea water of Vishniac, 1960) was utilized. Preparation of the medium is as follows:

NaCl	20.0 grams/liter
MgCl	2.5 " "
KCl	1.0 " "

KH_2PO_4	0.1 grams/liter
$CaCl_2$	0.4 " "
$(NH_4)_2SO_4$	0.4 " "
TRIS buffer	0.25 " "

Trace metal mix (100 microliters/liter of defined saline medium)

NaEDTA	5.0 grams/liter
$FeSO_4 \cdot 7H_2O$	0.05 " "
$ZnSO_4 \cdot 7H_2O$	0.02 " "
$MnSO_4 \cdot H_2O$	0.01 " "
$CoSO_4 \cdot 7H_2O$	2.0 milligrams/liter
$CuSO_4 \cdot 5H_2O$	0.2 " "
H_3BO_3	2.0 " "
$Na_2MoO_4 \cdot 2H_2O$	0.2 " "

Salinity

Two types of salinity tests were conducted with each strain of L. callinectes. First, a salinity tolerance test was performed in which the concentration of a raw sea water and marine salts mixture was varied between 0 and 60 ppt at 5 ppt increments. Salinity was determined with a Goldberg refractometer (AO Instruments). A second salinity test utilized the defined saline medium. In this test the concentration of NaCl (in an otherwise complete defined medium) was varied between 0 and 4.0% at 0.5% increments.

Monovalent Cation Replacement

Utilizing the defined saline medium, equimolar cation substitutions

were made for KCl and NaCl. In this procedure, six media were used for each strain, with each including three controls and three experimental solutions. They were as follows:

1. Control. - Containing both KCl and NaCl in the concentrations that promote optimum growth in marine organisms.
2. Control 1. - NaCl was omitted from an otherwise complete defined saline medium.
3. Control 2. - NaCl and KCl were omitted from an otherwise complete defined saline medium.
4. Experimental LiCl - NaCl and KCl were substituted for by LiCl in equimolar concentrations.
5. Experimental NaCl - KCl was substituted for by NaCl in equimolar concentrations.
6. Experimental KCl - NaCl was substituted for by KCl in equimolar concentrations.

Growth Diameters on Agar Plates

Petri plates, filled to a depth of approximately 5 millimeters with PYGS agar (1.5% agar), were inoculated with each of the selected strains (one strain per plate and three plates per stain). To insure uniformity of inoculum size when transferring from continuation plate cultures to test plates, a sterile number 3 cork bore was used to obtain inocula. After being inoculated, the test plates were incubated at 25° C for 5 days, after which diameters were measured and averaged for each strain.

Temperature

Determination of optimum growth temperature for each strain was carried out on a rotary shaker which was housed in a growth chamber in which the temperature could be precisely controlled. Employing PYGS as the growth medium, the temperatures were varied between 5 and 45° C at 5° increments.

At each temperature in which actual growth was observed, an attempt was made to induce sporulation. If sporulation was observed, the intensity was measured with a hemacytometer.

Fungicide Tests

To determine their fungitoxic potential, several chemicals were tested against strains L-1 and L-3b. All tests were conducted in PYGS broth containing varying concentrations of the selected chemicals. Preparation of the test media was as follows: One ml of various concentrations of stock fungicide solutions were added to 49 ml of PYGS media in 125 ml Erlenmeyer flasks. The stock solution concentration was determined by multiplying the concentration desired for testing by the dilution factor in the Erlenmeyer flasks (50X final concentration needed). When the stock solution concentration was too small for direct measurement and dilution, a double stock dilution was necessary. The chemicals tested were as follows:

1. Surflan (75% active) - active constituent oryzalin (3,5-dinitro-N^4,N^4-dipropyl sulfanilamide); a commercial herbicide marketed by Elanco Products Company, a division of Eli Lilly and Co.

2. Terrazole (35% active) - active constituent 5-ethoxy-3-trichloromethyl-1,2,4-thiadiazole; a commercial fungicide marketed by Olin Chemicals.

3. Formalin (formaldehyde, 37.3% pure) - a chemotherapeutant to eradicate fungus on fish eggs and external parasites on fish.

4. Furanace (P-7138, nifurpirinol, nitrofuran) - a broad spectrum chemotherapeutant developed in Japan by Dainippon Pharmaceutical Company and distributed by Abbott Laboratories; used to eradicate fungus on fish eggs and external parasites on fish in aquaria.

5. Potassium permanganate - used as a disinfectant and to control fungi and algae in aquaria.

6. Betadine (povidone iodine aqueous solution, 10% w/v) - a bacteriostatic agent believed to have potential as a fungicidal agent; distributed by the Purdue Frederick Co.

RESULTS

Correlation of Inoculation Procedures

PYGS broth media were inoculated with 1 cc inocula of varying concentrations of fragmented L-3b hyphae in an effort to determine an inoculum concentration standard which would elicit a growth response comparable to that obtained with media inoculated with 60,000 spores. The desired growth response occurred at a turbidity of around 300 Kletts (Fig. 1).

Effect of Salinity on Growth

These experiments were designed to study growth responses at varying "natural" salinities and NaCl levels. Strains L-1 and LAG-G grew poorly at low levels of sea water and did not without NaCl in the defined saline medium (Fig. 2).

Strains D-1 and LAG-F2 showed appreciably different responses to the same testing parameters. In contrast to L-1 and LAG-G, these strains showed optimum growth in the absence of significant amounts of salts (Fig. 2) and with the absence of NaCl in the percent NaCl tests (Fig. 2). They not only grew well without significant amount of NaCl and KCl, but grew at an optimum rate in deionized water with only PYG as a nutrient source.

Strain L-3b had a growth response intermediate (as pertains to media salinity) to that observed for the other two groups of strains (D-1, LAG-F2; L-1, LAG-G). In the salinity tolerance tests, L-3b failed to grow in deionized water containing PYG but did exhibit optimum growth at lower salinities than did L-1 and LAG-G (Fig. 2). When tested

by the percent NaCl procedure (Fig. 2) L-3b grew without NaCl, but growth was not optimum without significant amounts of NaCl.

Monovalent Cation Replacement

Based upon growth responses to specific cation deletions or substitutions (Fig. 3), the five strains of L. callinectes tested can be divided into three groups.

Strains L-1 and LAG-G did not grow without both KCl and NaCl and neither cation could be substituted for by the other or by LiCl. In contrast, strains D-1 and LAG-F2 grew at or near optimum levels when either NaCl or KCl or both were omitted from the defined media. Strains D-1 and LAG-F2 failed to grow well only when NaCl was substituted for in equimolar amounts by KCl (Fig. 3) and when NaCl and KCl were substituted for in equimolar amounts by LiCl (Fig. 3). Thus both LiCl and KCl inhibited growth when they were present in amounts (equimolar) approximating that of NaCl in sea water.

For strain L-3b, unlike L-1 and LAG-G, moderate growth was noted when NaCl was omitted from the defined medium and when KCl was substituted for by NaCl in equimolar amounts in the defined medium (Fig. 3). Contrary to the results with D-1 and LAG-F2, strain L-3b failed to grow when both NaCl and KCl omitted from the medium.

Based upon the growth data, relative to salinity and cation replacement responses, it is possible to place these five strains of L. callinectes into specific groupings (Table 1).

Growth Rate on Agar Plates

A significant difference in growth rates on agar plates was observed

among the strains after a five-day incubation period. Within a growth range of approximately 5 cm, extremes of growth were with strains D-1 and LAG-F2 having diameters of about 6 cm and L-1 having a diameter just above 1 cm. Strains L-3b and LAG-G exhibited a growth rate intermediate to the others with both reaching nearly 3 cm (Fig. 4).

While growth rates of LAG-G on agar plates were moderate, its growth in broth media has been shown to be slower, comparable to that of strain L-1.

Effect of Temperature Upon Growth and Sporulation

Four of the five strains exhibited optimum growth at approximately 20° C, although the upper and lower temperature limits for growth varied slightly among the strains. One strain, L-3b, exhibited optimum growth at 35° C and also had a wider near optimum temperature growth range than the other four strains tested (Fig. 5).

Regarding sporulation at various temperatures, the temperature for optimum sporulation of a strain was approximately $\pm$ 5° C that at which optimum growth occurred (Table 2).

Fungicide Tests

An essential criterion for establishing the efficacy of a particular chemical as a fungitoxic agent is its fungicidal activity; expressed as the minimal lethal concentration (MLC), (The lowest concentration at which no growth is recorded). Generally, the lower the MLC of a particular chemical, the more desirable it may be as a fungicide. The chemicals tested which demonstrated the best potential for use in the control of fungal growth were Surflan, Terrazole,

Formalin, and Furanace. The MLC of potassium permanganate and Betadine were never ascertained as they failed to inhibit fungal growth at concentrations approaching 40 ppm, at which point testing was discontinued. The MLC of the effective chemicals for strains L-1 and L-3b are given in Table 3.

Although the MLC for several compounds was relatively high, the effective concentration for each may, in fact, be much lower. For example, the MLC of Terrazole for strain L-1 was 2.0 ppm, but growth was negligible at the much lower concentration of 0.2 ppm (Fig. 7). Therefore, although the MLC was 2.0 ppm, Terrazole had a fungistatic effect upon strain L-1 at the much lower concentration of 0.2 ppm. This same fungistatic effect was noted for Terrazole with strain L-3b (Fig. 7), but was much less pronounced than with strain L-1. Furanace exhibited also a fungistatic effect on strain L-3b at a concentration lower than the MLC as did strain L-1 with Surflan (Table 4).

TABLE 1. Grouping of strains of *Lagenidium callinectes* based upon salinity and cation replacement test data

Strains	Marine Classification	Remarks
L-1 & LAG-G	Obligately Marine	Requires both NaCl and KCl in concentrations approximating those found in sea water for optimum growth to occur
L-3b	Obligately Marine	Grows without significant amounts of NaCl when KCl is present in the medium, but needs it in the amounts found in sea water for optimum growth to occur
D-1 & LAG-F2	Facultative Marine	Grows at optimal levels without significant amounts of NaCl and KCl in the medium, but is halotolerant, growing well in media having an NaCl concentration of up to 4.0%

TABLE 2. Sporulation at varying temperatures

Strains	Temperature (°C)								
	5	10	15	20	25	30	35	40	45
L-1	-	-	+	+++	++	0	-	-	-
LAG-G	-	0	+++	++	+	-	-	-	-
L-3b	-	-	-	0	+++	++++	+	0	-
D-1	-	-	0	++	+++	-	-	-	-
LAG-F2*	-	-	-	-	-	-	-	-	-

++++ 100,000+ spores/cc
+++ 50,000-100,000 spores/cc
++ 25,000-50,000 spores/cc
+ less than 25,000 spores/cc
0 unmeasurable
- sporulation not observed

*LAG-F2 sporulation not inducable

TABLE 3. The minimum lethal concentrations of the chemicals exhibiting significant fungicidal activity

Chemical Tested	Minimum Lethal Concentration Strain L-1	Strain L-3b
Surflan[+]	0.2 ppm*	0.06 ppm
Terrazole[++]	2.0 ppm	1.0 ppm
Formalin[o]	1.5 ppm	2.5 ppm
Furanace[oo]	0.3 ppm	4.0 ppm

*Parts per million
+Figure 6
++Figure 7
oFigure 8
ooFigure 9

TABLE 4. Comparison of MLC* and chemical concentration eliciting fungistatic effect

Chemical Tested	Strain	Fungistatic	MLC
Surflan	L-1	0.06 ppm	0.2 ppm
Terrazole	L-1	0.2 ppm	2.0 ppm
Terrazole	L-3b	0.5 ppm	1.0 ppm
Furanace	L-3b	2.0 ppm	4.0 ppm

*Minimum lethal concentration

TABLE 5. Tentative taxonomic grouping of strains of *Lagenidium callinectes* based upon physiological studies

Strains	Group Characteristics
L-1 & LAG-G	1. Obligately marine 2. Slow growth rates 3. Sporulation easily inducible 4. Optimal growth and sporulation occurs at comparable temperatures of 15-20° C
L-3b	1. Obligately marine* 2. Moderate growth rate 3. Sporulation easily inducible 4. Optimal growth temperature at 35°C 5. Optimal sporulation temperature at 30°C
D-1 & LAG-F2	1. Facultative marine 2. Rapid growth rates 3. Optimal growth temperature at 20° C 4. Sporulation not easily inducible for D-1 and not observed for LAG-F2

*Unlike strains L-1 and LAG-G, some growth occurs when NaCl is not present in the medium.

TABLE 6. Comparison of toxicity of selected chemicals to Penaeus stylirostris and Lagenidium callinectes

Chemical Tested	P. stylirostris*	L. callinectes
Surflan	Negative toxicity up to 0.5 ppm	L-1 - 0.2 ppm L-3b - 0.06 ppm
Terrazole	Negative toxicity up to 5.0 ppm	L-1 - 2.0 ppm L-3b - 1.0 ppm

*Nauplius and protozoea were tested by Lightner, University of Arizona.

Figure 1. Growth response of Lagenidium callinectes, strain L-3b, in relation to fragmented hyphal inocula (turbidity).

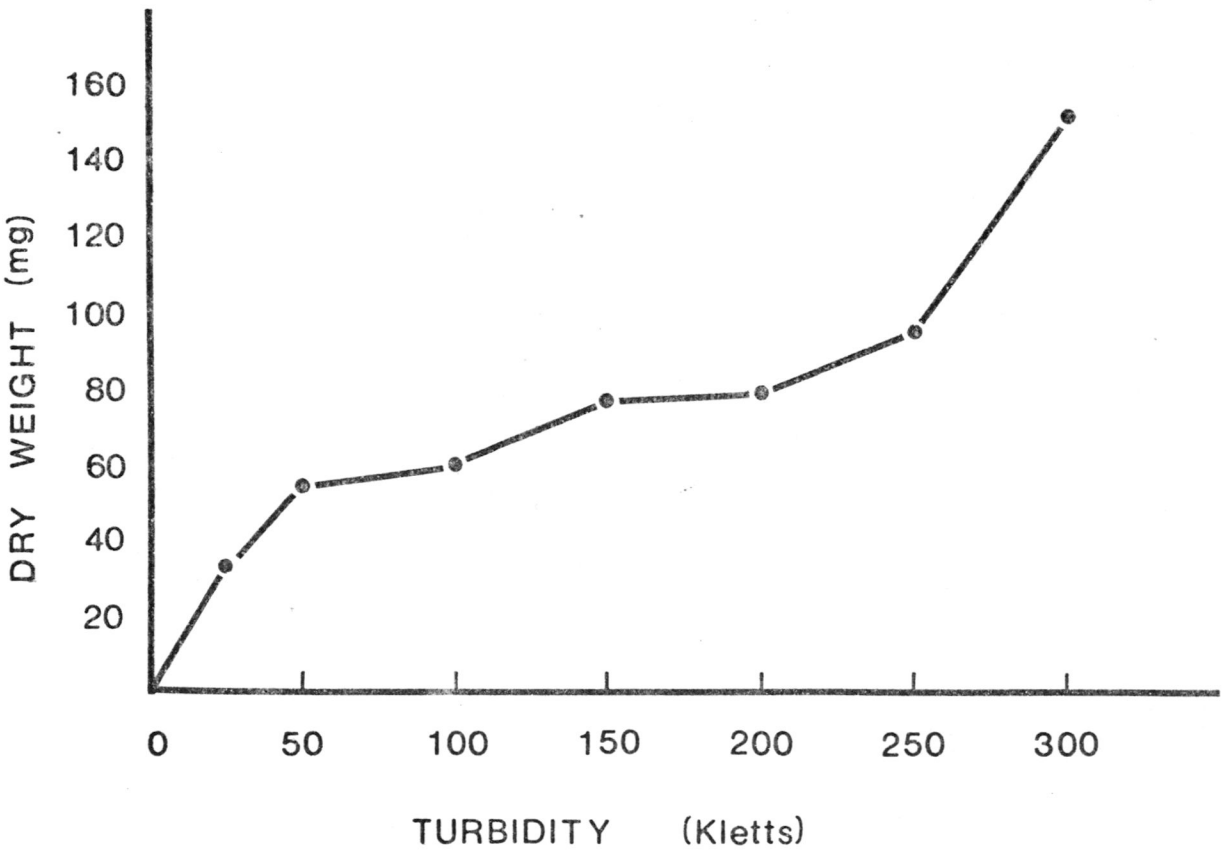

160
140
120
100
80
60
40
20
DRY WEIGHT (mg)
0
50
100
150
200
250
300
TURBIDITY (Kletts)

Figure 2. Effect of salinity upon growth of Lagenidium callinectes.

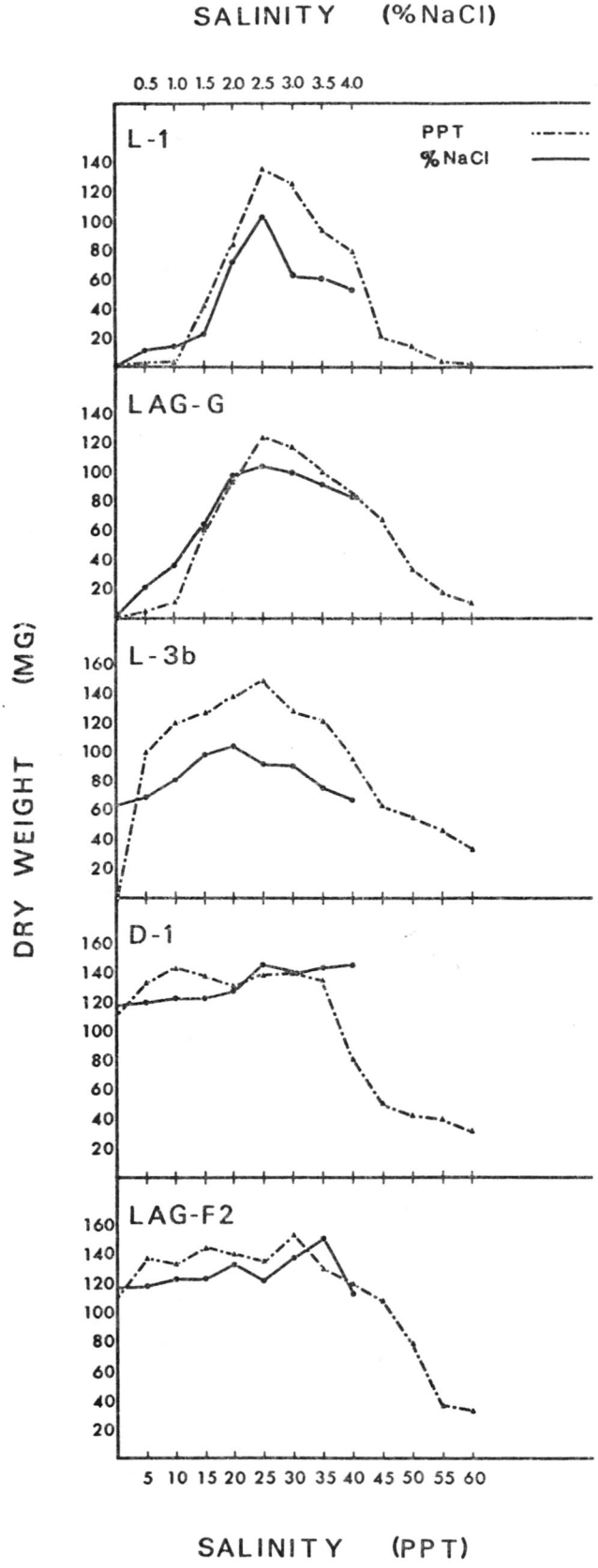

SALINITY (%NaCl)
0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
L-1
PPT
%NaCl
LAG-G
L-3b
D-1
LAG-F2
DRY WEIGHT (MG)
5 10 15 20 25 30 35 40 45 50 55 60
SALINITY (PPT)

Figure 3. Growth of *Lagenidium callinectes* in relation to monovalent cation replacement.

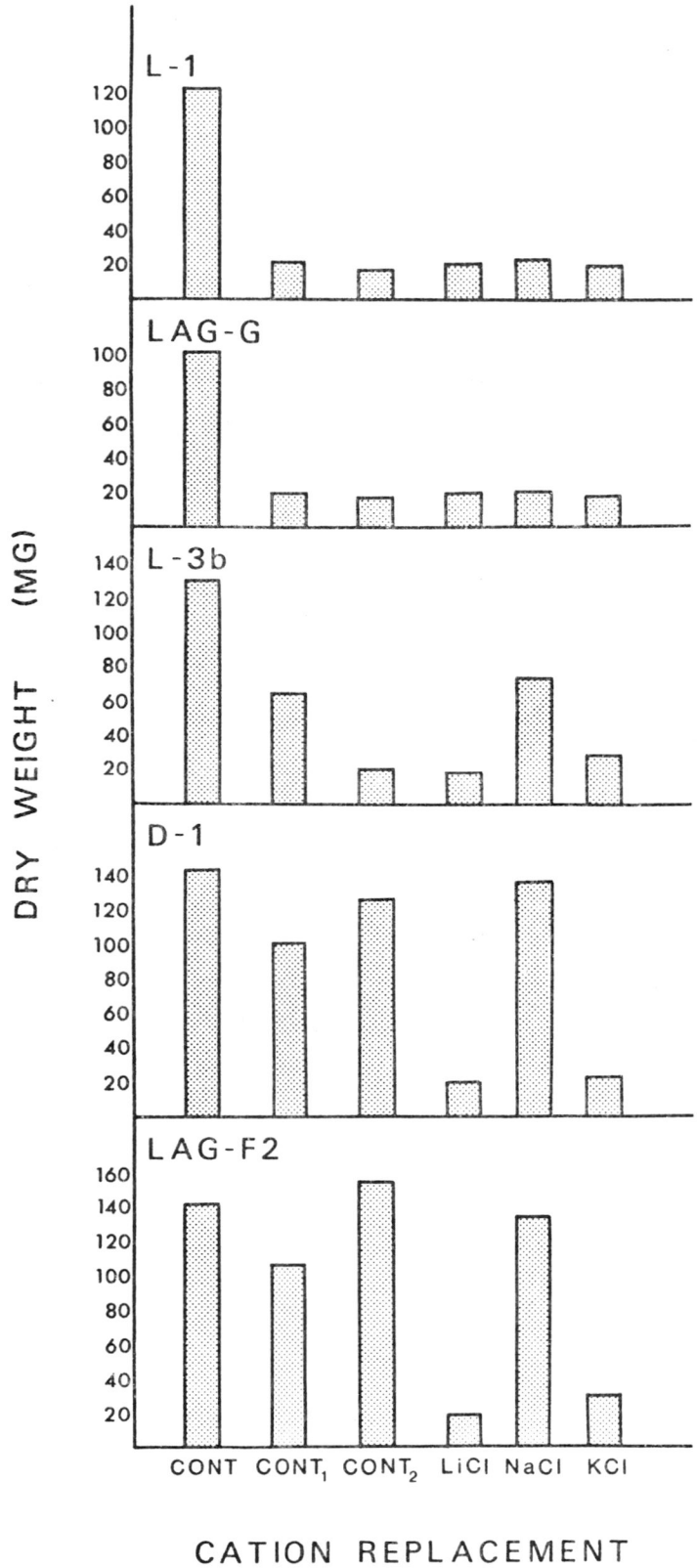
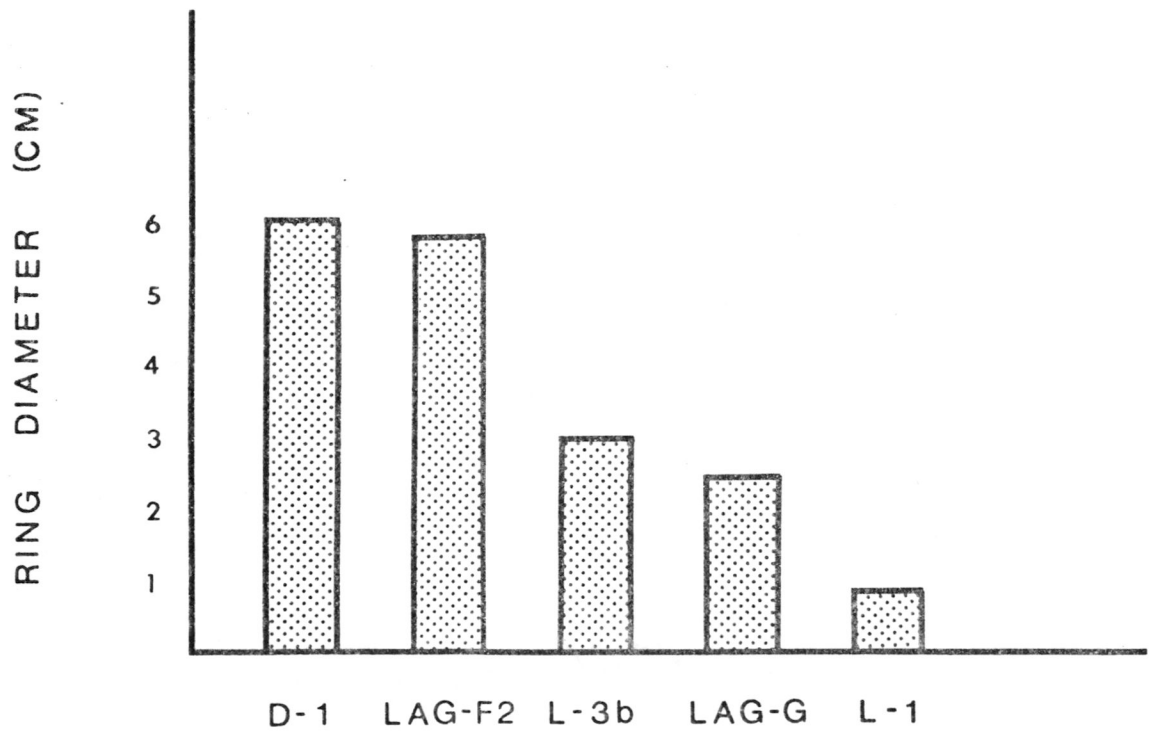

L-1
120
100
80
60
40
20
LAG-G
100
80
60
40
20
L-3b
140
120
100
80
60
40
20
D-1
140
120
100
80
60
40
20
LAG-F2
160
140
120
100
80
60
40
20
DRY WEIGHT (MG)
CONT
CONT1
CONT2
LiCl
NaCl
KCl
CATION REPLACEMENT

Figure 4. Comparison of growth rates of the five strains of *Lagenidium callinectes* on agar.



GROWTH PATTERNS ON AGAR

Figure 5. Growth of Lagenidium callinectes at varying temperatures.

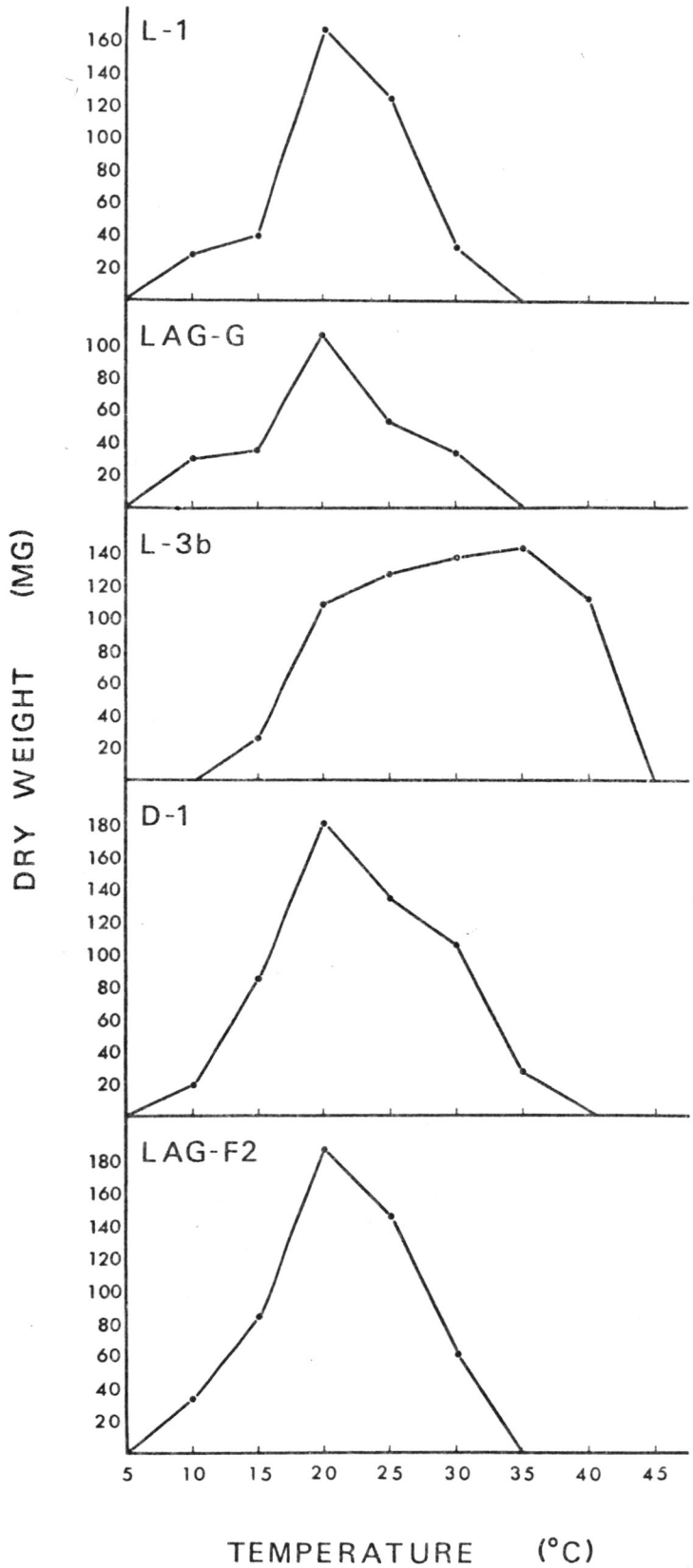
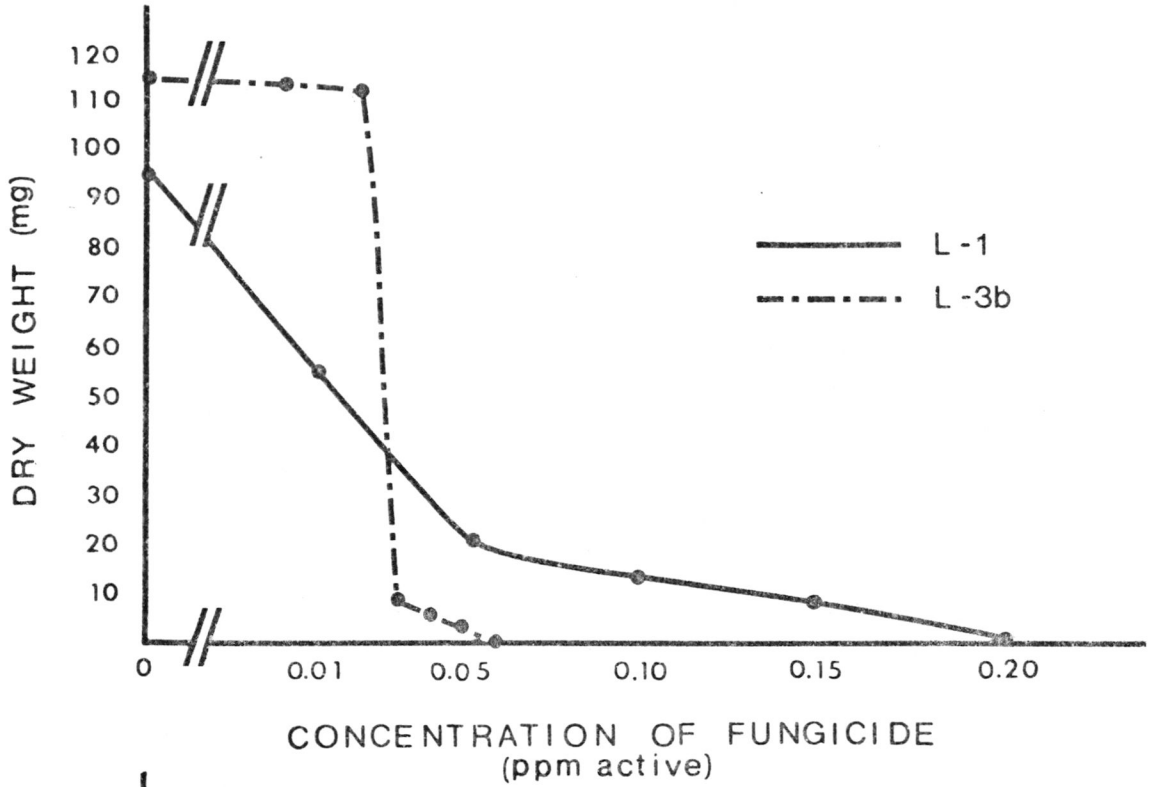

L-1
LAG-G
L-3b
D-1
LAG-F2
DRY WEIGHT (MG)
TEMPERATURE (°C)
5 10 15 20 25 30 35 40 45

Figure 6. Growth responses of Lagenidium callinectes, strains L-1 and L-3b, to different concentrations of the chemical Surflan.

Figure 7. Growth responses of Lagenidium callinectes, strains L-1 and L-3b, to different concentrations of the chemical Terrazole.


DRY WEIGHT (mg)
120
110
100
90
80
70
60
50
40
30
20
10
0
0.01
0.05
0.10
0.15
0.20
L-1
L-3b
CONCENTRATION OF FUNGICIDE
(ppm active)

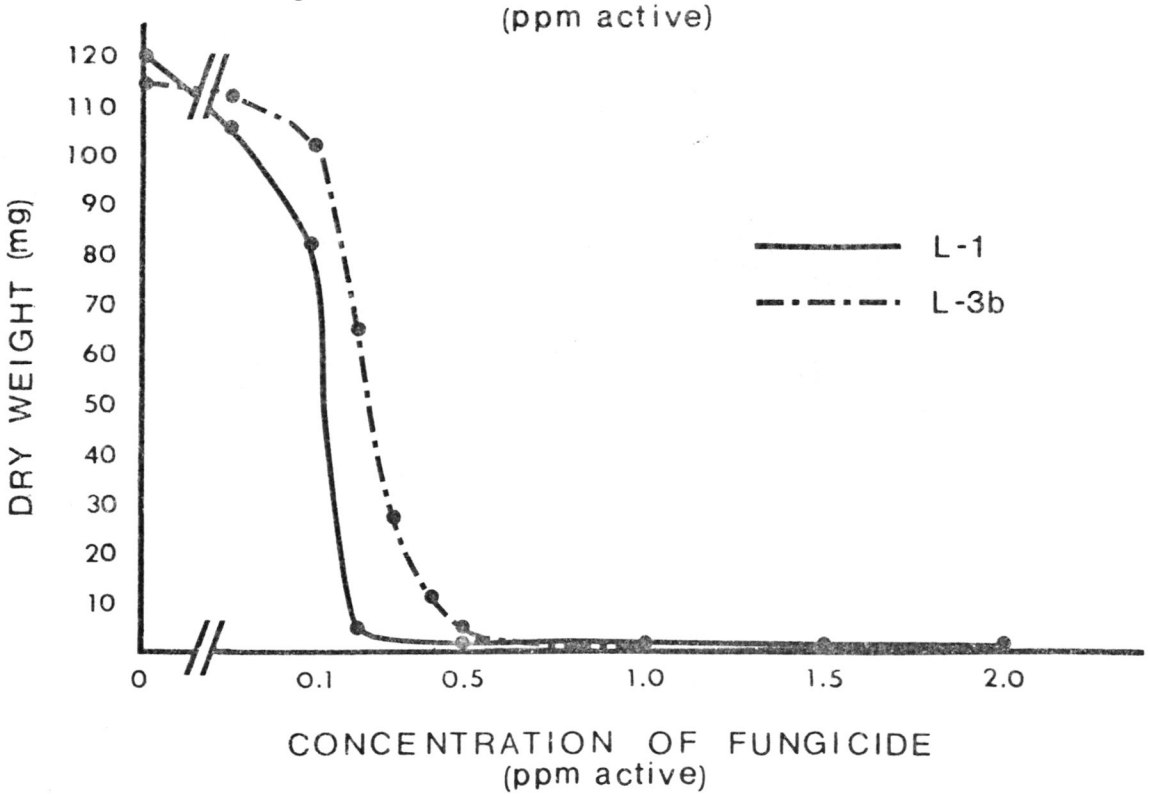
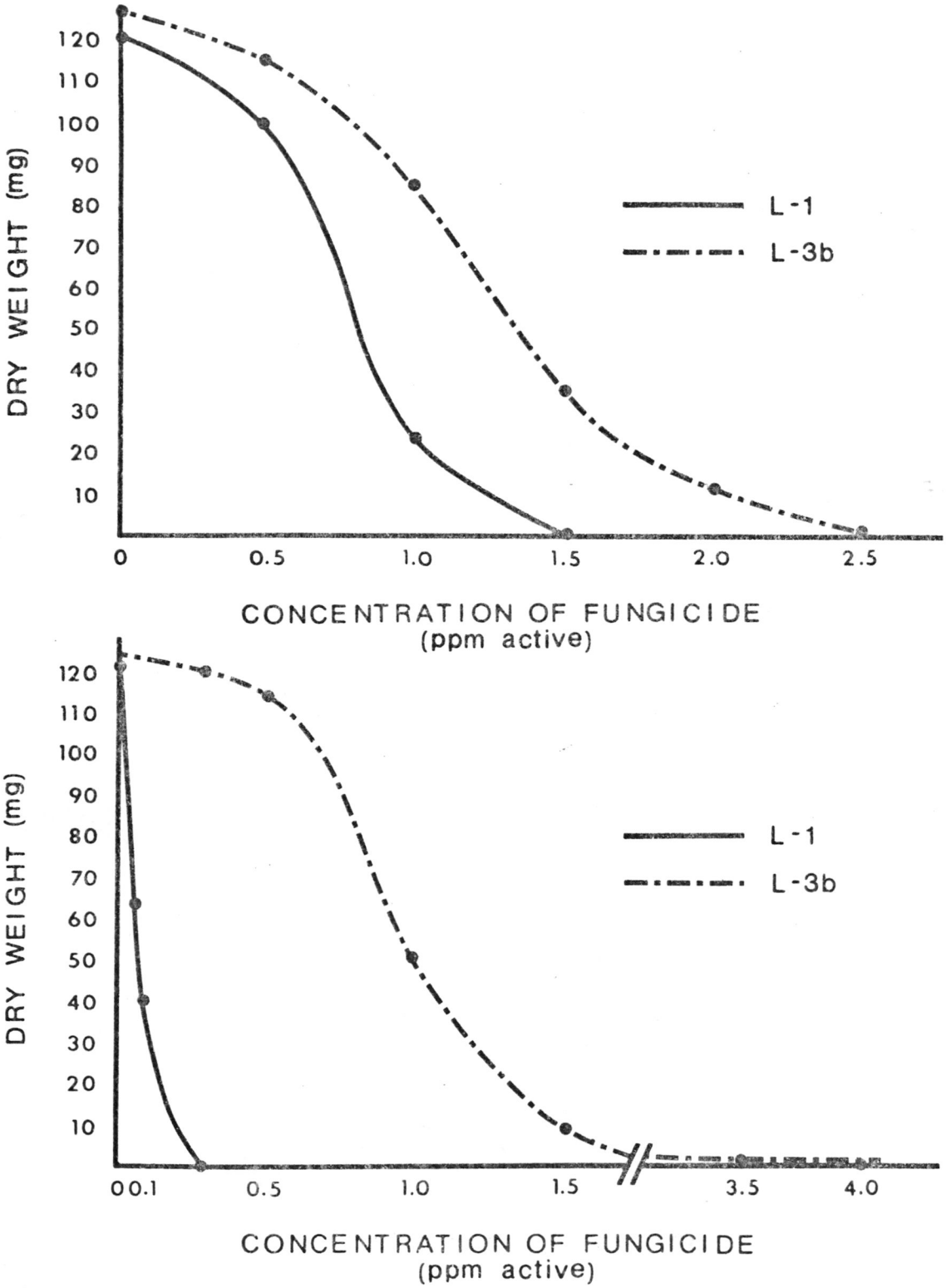

DRY WEIGHT (mg)
120
110
100
90
80
70
60
50
40
30
20
10
0
0.1
0.5
1.0
1.5
2.0
L-1
L-3b
CONCENTRATION OF FUNGICIDE
(ppm active)

Figure 8. Growth responses of Lagenidium callinectes, strains L-1 and L-3b, to different concentrations of the chemical Formalin.

Figure 9. Growth responses of Lagenidium callinectes, strains L-1 and L-3b, to different concentrations of the chemical Furanace.


DRY WEIGHT (mg)
120
110
100
90
80
70
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
L-1
L-3b
CONCENTRATION OF FUNGICIDE
(ppm active)
DRY WEIGHT (mg)
120
110
100
90
80
70
60
50
40
30
20
10
00.1
0.5
1.0
1.5
3.5
4.0
L-1
L-3b
CONCENTRATION OF FUNGICIDE
(ppm active)

DISCUSSION

The attempt to establish criteria for discerning truly marine fungi from those considered to have a transient association with the marine environment has met with two basic problems. First, mechanisms by which these fungi are able to reside in environments of high salt content and the fate of these salts with respect to nutrition is not clearly understood. Secondly, some fungi do not recognize discrete boundaries between freshwater and marine environments, growing equally well in either.

With these problems in mind, Johnson and Sparrow (1961), when writing about fungi in oceans and estuaries, defined marine fungi in two different ways. It was first proposed that if a fungus, during any part of its life cycle, resided in the marine environment, it could be considered a marine fungus. It was also theorized that, if a salt or group of salts common to the marine environment are required by an organism in the concentrations approximating those occurring in sea water, then the fungus is a marine organism.

As an extension of Johnson and Sparrow's attempt to categorize marine fungi with respect to habitat, Kohlmeyer defined a marine fungus as any fungus which "lives and reproduces in the sea" (Kohlmeyer, personal communication).

Vishniac (1960) considered a marine fungus to be any fungus requiring NaCl for growth. Vishniac's definition of a marine fungus raises an important question. Is the marine status of a microorganism to be based upon habitat or on nutrition or both? Whether or not the consideration is based upon nutrition alone, additional terminology is

needed to distinguish between those microorganisms which are able to live in the sea and those microorganisms which must live in the sea. Thus the terms "obligate" for those which must live in the sea, requiring NaCl in concentrations consistent with those of the marine environment, and "facultative" for those which live in marine and freshwater environments. The reliance upon habitat as a singular method for defining marine fungi seems much less desirable since it would be impossible to distinguish between those microorganisms which require high saline environments for growth, and those which simply tolerate them.

Regarding the present study, the most desirable system for classifying marine fungi is one which includes both nutrition and habitat and which specifies whether the fungi are obligate or facultative. Thus, if the fungus is isolated from the marine environment or from a host found in the marine environment, but does not require a salt or salts at levels present in this environment, then it may be considered facultatively marine. Alternatively, if the fungus is isolated from the marine environment or a host from the sea, and requires a salt or salts present in the marine environment for growth, then the fungus may be considered obligately marine. The marine environment or sea, as pertains to this study, may be defined as that part of the aquatic environment having a salinity of approximately 30 $\pm$ 5 ppt.

Strains L-1 and LAG-G, having exhibited a specific need for both NaCl and KCl, can be classified as obligately marine. In considering the obligately marine nature of these two strains of Lagenidium, one must also contemplate the role which NaCl and KCl play in the life of these fungi. A need for NaCl in concentrations comparable to those

of sea water has been demonstrated in other obligately marine fungi. Siegenthaler, Belsky, and Goldstein (1967) utilizing the obligately marine fungus, Thraustochytrium roseum, found NaCl to function in ion transport, specifically phosphate uptake. Sykes and Porter (1973) obtained similar results with the marine fungus Labyrinthula sp. Additionally, Kazama and Fuller (1973) state that for Pythium marinum NaCl has a function other than that of an osmoticum, specifically, ion transport. Thus, it appears that NaCl in obligately marine fungi functions other than as an osmoticum. This fact may be, in part, substantiated by studies of Lagenidium callinectes (Gotelli, 1974) and Labyrinthula sp. (Sykes and Porter, 1973) in which the osmotic potential of the growth medium was stabilized by osmotica such as mannitol and KCl. Hence, they demonstrated that certain marine fungi have a specific growth requirement for NaCl, rather than utilizing it simply in maintenance of osmotic pressure. Consequently, these studies seem to complement the observations of Siegenthaler, Belsky, and Goldstein (1967) described above. The mechanism of ion transport described for these other marine fungi is quite likely functional also in the obligately marine strains of L. callinectes (L-1 and LAG-G) considered in the present study. However, research to substantiate or refute this claim remains to be done.

Strain L-3b appears to have both obligate and facultative marine properties. Although growth was observed in the absence of significant amounts of NaCl, optimum growth was not realized until the growth medium contained NaCl in the concentration approximating sea water. Thus, this strain has specific nutritional requirements which make it

difficult to classify as either obligately or facultatively marine.

Strains D-1 and LAG-F2, although isolated from marine hosts as were strains L-3b, L-1, and LAG-G, seem to be least typically marine fungi. These strains not only grow well in the absence of significant amounts of NaCl and other marine salts, but also grow well under conditions in which the salt content is equal to or higher than that found in sea water. The ability of these strains to grow optimally under these variable conditions makes them highly facultative with respect to the marine environment. A possible explanation, relative to the high saline tolerances of strains D-1 and LAG-F2, comes from research by Norkrans and Kylin (1969) concerning salt tolerances in yeasts. Their research indicates that fungi may achieve high salt tolerances by maintaining a favorable potassium to sodium ratio inside the cell though the external environment may exhibit an unfavorable sodium to potassium ratio. This favorable potassium to sodium ratio may be achieved through the ability of these fungi to utilize energy for the extrusion of sodium from the cell while retaining or incorporating potassium.

Temperature tests conducted during the present study failed to demonstrate significant differences among most of the strains studied. Only strain L-3b, isolated from the warmer waters of the Gulf of Mexico (Lightner and Fontaine, 1973) had a significantly different growth response to variable temperatures. The remaining strains, isolated from locales of annually or seasonally cold waters, had much lower optimum growth temperatures than strain L-3b. Thus, temperature data has proven to be relatively ineffective in separating the strains

taxonomically.

The effect of temperature upon sporulation, correlating well with the effect of temperature on growth of the strains, contributed little to the taxonomic evaluation (grouping) of the strains. However, general observations regarding sporulation do indicate characteristic differences between the individual strains. These differences include variable sporulation intensities between strains at their optimum temperature for sporulation (Table 2); variation in techniques necessary to induce sporulation (specifically D-1); and failute of one strain, LAG-F2, to sporulate under conditions favorable to sporulation for the other strains.

In comparing the various strains considered in the present study, several morphological differences among the strains are obvious. These differences are manifested in such morphological features as length of discharge tube, number of spores cleaved within the discharge vesicle, hyphal diameter, and frequency and angle of hyphal branching (Bland, personal communication). In addition to the morphological differences, the mechanism of infection of eggs or larvae of marine crustacea by zoospores varies from one strain to another (Bland, personal communication).

Based on observations made in the present study, a tentative grouping of the five strains of L. callinectes is possible (Table 5). This data, along with the obvious morphological difference which exist between the strains may facilitate a taxonomic re-evaluation of the strains of Lagenidium which must now be classified as L. callinectes.

In the testing of certain chemical compounds for control of the

fungus Lagenidium, four compounds, Surflan, Terrazole, Formalin, and Furanace have exhibited fungicidal activity similar to the activity demonstrated previously by the fungicide malachite green in a chemical control study by Ruch (1974). Two of the four compounds, Terrazole and Surflan, have undergone animal toxicity studies at the University of Arizona (Lightner, personal communication). The crustacean used in the toxicity studies was Penaeus stylirostris. These studies show the fungicides to be much less toxic to the larval crustacea than to L. callinectes (Table 6). The concentration margin between animal and fungal toxicity provides a significant working range for the use of these chemicals in the conrol of fungal diseases affecting the mariculture of marine crustacea.

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