

STUDIES ON THE HOST RANGE AND CHEMICAL CONTROL OF FUNGI
ASSOCIATED WITH DISEASED TROPICAL FISH

by

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INTRODUCTION

The ability of certain saprolegniaceous fungi to attack fish and other aquatic or amphibious animals has been reported frequently by various investigators during the last century. In many cases the death of the host has been attributed to the fungal invader. To a large extent, previous investigations concerning mycoses of fish have been of a taxonomic nature dealing with host range studies and devoted mainly to the description and biology of the fungus involved (Tiffney, 1939b). Investigations conducted in this laboratory have been concerned primarily with fungus infections of fresh-water hatchery species (Scott and O'Bier, 1962). Few investigations of this nature have been devoted to tropical fish as a group. Frequent reports from tropical fish growers implicating certain aquatic fungi with the death of tropical fish suggested the need for additional information. The term tropical fish, as used here, refers to those species considered as such by Axelrod and Vorderwinkler (1957). This includes both tropical and sub-tropical popular aquarium species.

Two main approaches were taken in this research. The first phase was concerned primarily with host range studies and associated investigations. Fungi were isolated from infected fish and specific identifications, when possible, were made. Infection studies using parasitic isolates and selected fish species were carried out under controlled laboratory conditions in order to determine degrees of pathogenicity and host specificity. The second phase of this study

involved determinations made to establish the fungicidal effect of certain chemicals in vitro. Also of importance were studies to determine the tolerance of selected species of fish to the lowest effective concentrations (minimum lethal dose) of the chemicals tested.

LITERATURE REVIEW

Host Range Studies and Associated Investigations

The first significant studies of fish mycoses were carried out by Tiffney (1939a) who demonstrated the ability of Saprolegnia parasitica Coker to parasitize a wide range of fish and amphibious species. His experiments also revealed varying degrees of resistance to fungal infection among the different hosts. Experimentally wounded fish appeared to be more susceptible to infection than those not wounded. In addition, Tiffney (1939b) isolated members of Achlya and Dictyuchus from various living fish, amphibians, and reptiles in nature. This suggested that members of the Saprolegniaceae other than Saprolegnia are capable of attacking aquatic animals.

More recently Vishniac and Nigrelli (1957) conducted laboratory experiments which demonstrated the ability of known saprophytic species of the Saprolegniaceae to infect the Mexican platyfish, Xiphophorus maculatus. The results of these investigations suggest strongly that any member of the Saprolegniaceae may function as a wound parasite of fish. In addition, they contributed significant histological studies which showed physical disruption of the host tissue due to hyphal penetration.

Other noteworthy work is that of Scott and O'Bier (1962). These investigators collected sixty-four isolates of aquatic fungi from fish and fish eggs from fourteen states. Five fungal species not previously reported as naturally occurring pathogens were collected, while six

saprolegniaceous species were isolated from fish eggs for the first time. In this same work, infection studies similar to those conducted by Vishniac and Nigrelli established five species as wound parasites on Xiphophorous maculatus. Also of importance was the pertinent discussion by these authors of the confusing and uncertain taxonomy of certain species of Saprolegnia mentioned throughout the literature. It was pointed out that a comprehensive systematic treatment of the genus Saprolegnia is needed.

Chemical Control Studies

For the last 30 years fish hatchery operators, aquaria enthusiasts, and interested biologists have been greatly concerned with finding an effective control for mycotic infections of fish. Numerous chemicals have been tested as possible fungicides with varying degrees of success.

Foster and Woodbury in 1936 reported that dipping fungus-infected species of trout in a 1:10,000 solution of malachite green for 3 to 5 minutes was effective in ridding the host of the infection.

In a more extensive investigation, O'Donnell (1941) tested malachite green on 18 species of popular sport fish. He reported that a dip treatment in a 1:15,000 malachite green solution for 10-30 seconds had very definite fungicidal and therapeutic effects. He also conducted toxicity tests from which he derived the previously mentioned recommended dipping time. However, it should be pointed out that very little taxonomic information concerning the fungi involved was given in this report. He briefly states that the fungi incurred were

members of the family Saprolegniaceae; the most common species encountered being Saprolegnia parasitica Coker. Also, it is unclear as to whether or not all of the "infected" fish treated had macro- or microscopically observable fungal infections.

Potassium permanganate, another much tested compound, was found to be ineffective on diseased trout in a 1:10,000 solution by Foster and Woodbury (1936). However, Estes (1957) conducted long (24 hours) exposure treatments on infected goldfish and reported that potassium permanganate was a very effective fungicide at concentrations of 2.0 ppm. and 3.0 ppm. He also pointed out the effectiveness of this compound in short (60 minutes) exposure experiments at a concentration of 8.0 ppm. Again, the taxonomy of the fungi and the extent of the infection was somewhat vague. In addition, to the above mentioned works, Hoshina and Ookubo (1956) reported that a 1:10,000 solution of potassium permanganate was lethal to cultured Saprolegnia parasitica Coker.

Copper sulfate is also mentioned in the literature as a compound tested for its fungicidal abilities. Foster and Woodbury (1936) reported that it was toxic to the fish at a 1:2,000 concentration and ineffective against the fungus at a 1:1,000 concentration. O'Donnell (1947) stated that copper sulfate was an effective fungicide, but the effective concentration changed with the varying calcium content of different waters. This was verified by Burrows in 1949.

Rankin (1952, 1953) introduced a new group of compounds as possible fungicides. He reported that Phenoxetol (B-phenoxyethylalcohol) was effective in curing fungus infections on varieties of goldfish at a

concentration of 0.01%. Propylene-phenoxetol was also effective at 0.01%, while Para-chloro-phenoxetol was reported effective at 0.005%. Rankin also conducted toxicity tests using his recommended concentrations; but as he suggested, his investigations were not extensive enough to yield conclusive results. Moreover, the pathogenic organism was referred to only as white fungus with taxonomic descriptions completely lacking.

Various other compounds such as table salt, methylene blue, etc., have been used in attempts to control fungus diseases of fish. However, these compounds are too numerous and their methods of application too complicated to mention here.

MATERIALS AND METHODS

Host Range Studies

Fungus-infected fish were obtained from two main sources:

(1) tropical fish hatcheries located in Florida and California; and
(2) local aquaria. The method for receiving collections from the above mentioned hatcheries was the one employed by Scott and O'Bier (1962). Fungi were isolated from both living and dead hosts. Small bits of mycelium were rinsed thoroughly in distilled water and placed in Petri dishes containing 40-50 ml. of chemically defined SPS medium, (Table 1) Scott, et al. (1963). The glass ring technique, as reported by Raper (1936), was used to obtain unifungal, bacteria-free cultures. Stock cultures were maintained on 3.0% potato dextrose agar slants refrigerated at approximately 8°C.

The identifications of the fungi incurred were made according to Sparrow (1960) and Johnson (1956a). The species of fish received were previously identified by the sender. Authorities for verification of fish identifications were Axelrod and Vorderwinkler (1957) and Frey (1961).

Infection Studies

In order to demonstrate the pathogenicity of the isolates obtained, controlled infection investigations were conducted in the laboratory. A representative strain of each of the three fungal genera isolated (Saprolegnia sp., Achlya americana, Pythium sp.; Table 2) was tested

for its pathogenic effects on the five selected species of tropical fish (Table 2). The techniques utilized in these investigations were generally similar to those used by Tiffney (1939a), Vishniac and Nigrelli (1957), and Scott and O'Bier (1962). All infection studies were conducted at room temperature (25-30°C.). The method employed is listed below.

Preparation of infection dishes

Covered Pyrex baking dishes, 3" x 9 1/2" x 5 1/2", were partially wrapped in aluminum foil and sterilized in a hot-air oven at 175°C. for two hours. Filtered, sterile stream water (1,250 ml. per dish) was added aseptically to each of two dishes per experiment. A vibrator pump was used for aeration of the water throughout each experiment. Four fungus-inoculated blocks and four uninoculated blocks of SPS medium (1 sq. cm. each) were placed at opposite ends of each infection dish. After two days fish food was added and the experimental fish were introduced to the dishes.

Preparation of experimental fish

Two to three days prior to exposure to the fungus, the test fish were dipped in a solution of malachite green (1:15,000 dilution) for 15 seconds (O'Donnell, 1941) and then placed in a clean aquarium. Boiled hemp seed halves (Cannabis sativa) were placed in the aquarium to test for possible fungal contamination. After the period of segregation, four or five experimentally injured fish, each a different species, and two uninjured species were placed in each dish.

The following step-wise procedure was used for experimentally wounding the fish:

1. The fish was anesthetized in a solution of Tricaine-methanesulfonate (MS-222). The concentration used was 8 ml. of 0.5% MS-222 per 300 ml. of sterile water. The required time for anesthetization ranged from 3 to 8 minutes depending on the species and individuals involved.
2. The unconscious specimen was placed on a paraffin block and observed under a dissecting microscope. Injury was inflicted by scraping scales from the left side of the front area of the caudal peduncle and also by clipping the caudal fin.
3. The fish was then revived by placing it in fresh, sterile water before being introduced into the infection dish.

Observations

Developments in the infection dishes were observed and recorded periodically each day for a total of 18 days. If death resulted, the fish was immediately removed from the dish and examined. If infection occurred, the fungus growing on the experimental fish was identified and compared with the original inoculum.

In Vitro Chemical Control Studies

Certain chemicals, potentially valuable as fungicides, were tested in vitro for their inhibitory effects on pathogenic species of aquatic fungi. Pertinent information concerning the chemicals tested is given in Table 4.

Sterile, deep Petri dishes (9 cm. in diameter and 2 cm. in depth) were filled with 25-30 ml. of SPS medium. Fungus-inoculated blocks of (0.5 sq. cm. each) were cut from pure cultures growing on SPS medium and centrally placed in each dish (one block per dish). Various concentrations of the chemical being tested were pipetted aseptically in 10 ml. volumes per dish. Three replicates of each concentration were tested. In order to establish a control measure for this type of experimentation, 10 ml. of filtered, sterile stream water was added to each of three fungus-inoculated dishes (per concentration of chemical tested). The three fungal strains used in the infection studies were also used in these tests. Growth was determined by placing a millimeter scale on the bottom of the Petri dish and measuring increases in colony diameter. Measurements were made in the horizontal plane along two perpendicular axes (ex. 40 mm. x 39 mm.). Measurements were recorded at 24, 48, and 72 hour intervals.

Tolerance Tests

In order to test the toxicity of the chemicals found to have a definite fungicidal effect, fish were placed in battery jars containing serated solutions of various concentrations. The concentration was considered lethal when the fish ceased to be active and appeared unconscious. The time of this occurrence was recorded, and the fish was placed in fresh water.

The test fish for these experiments were Lebistes reticulatus and Helostoma temminski, one each per concentration. These species were chosen because of their availability, size difference, and taxonomic differences.

RESULTS

Host Range Studies

A total of 19 isolates representing three fungal genera (Saprolegnia, Fig. 1; Achlya, Fig. 2; Pythium, Fig. 3) were obtained from various infected tropical fish (Table 2). Due to difficulty in inducing sexual fruiting in the fungal isolates, it was possible to identify only two isolates to species. Both of these infectious agents were found to be Achlya americana. Eleven different host species of fish were identified.

Infection studies

Infection studies conducted under controlled laboratory conditions demonstrated the pathogenic and parasitic abilities of the three isolates. Saprolegnia and Achlya appear to be the most lethal of the fungi tested, while Pythium is the least vigorous of the three.

Achlya americana showed no specificity in attacking members of the five species of fish used (Table 5). Hyphae of the parasite could be observed macroscopically (Fig. 4) growing from the injured areas of the experimentally wounded specimens within 7-10 hours after placing the fish in the infection dishes, while death occurred in all injured hosts within 24 hours. Two of the eight unwounded fish developed mycoses and death followed.

The test isolate of Saprolegnia also showed no host specificity in causing infections in each of the five different fish species (Table 6). Again, mycelia were observed at the injured areas of the

previously wounded fish within 7-10 hours after inoculation. All wounded fish died within 29 hours after being exposed to the pathogen. Infection and death occurred in two control fish, while a third died of unknown causes. The isolate of Pythium, less vigorous than the two genera previously discussed, infected only 13 of the 18 wounded fish supplied (Table 7). Pythium infected at least two members of each of the five different species, thus discounting a specific preference for any particular species. It should be pointed out that Pythium mycelia could not always be observed macroscopically before death as with Achlya and Saprolegnia. Death occurred no earlier than three days in the specimens infected with Pythium. All control fish employed in the Pythium tests remained uninfected and living throughout the test periods.

As derived by observing the times of infection and death, differences in resistance to infection between the five test species of fish were not appreciable.

Chemical Control Studies and Tolerance Tests

Phenoxetol

As shown in Table 8, the effective fungicidal concentration of Phenoxetol is between 0.25% and 0.50% for all three organisms tested. The lack of growth increase of Saprolegnia sp. after the first day was due to heavy bacterial contamination. However, it is felt that the growth pattern was developed at the 24 hour measurement and therefore this data is still valid. As compared with the control, the

concentrations of 0.10% and 0.01% had little or no inhibitory effect; while 0.25% Phenoxetol retarded growth.

Tolerance tests with Phenoxetol (Table 14) showed that all concentrations greater than 0.25% were highly toxic to both test species of fish in under two minutes. The toxic effects then decreased gradually in relation to time with a decrease in concentration. At 0.02% both fish survived although appearing very sluggish, while at 0.01% no toxic effects were evident.

Propylene-phenoxetol

Propylene-phenoxetol effectively stopped growth of Saprolegnia sp. at concentrations greater than 0.50% and less than 0.75% (Table 9). Growth of A. americana and Pythium were completely inhibited at concentrations between 0.25% and 0.50% (Table 9). As with most of the other chemicals tested, Saprolegnia appeared to be the most vigorous of the three fungal genera tested.

Concentrations of Propylene-phenoxetol of 0.25% and above proved to be highly toxic to all test fish within one minute (Table 15). Tolerance in the fish increased gradually with a decrease in concentration with 0.01% being the highest possible concentration at which survival appeared certain.

Chloro-phenoxetol

Chloro-phenoxetol, in concentrations between 0.04% and 0.20%, prohibited growth in each of the three fungi tested (Table 10). Toxicity tests showed (Table 16) that a 0.04% concentration was highly

toxic to the test fish within 50 seconds and therefore additional experiments to establish narrower limits of effective fungicidal concentrations were felt unnecessary. Also, tests using concentrations as low as 0.005% proved toxic to the fish within 40 minutes.

Nipagin M

No growth occurred in dishes containing either Saprolegnia, A. americana, or Pythium which were flooded with Nipagin M concentrations of 0.10% (Table 11). Saprolegnia grew more vigorously in 0.06% Nipagin M than either A. americana or Pythium (very scanty growth).

In testing for tolerance, it was found that the guppies were rendered unconscious in less than one minute by 0.1% and 0.06% concentrations of Nipagin M (Table 17). The kissing gouramis, however, withstood the effects of these concentrations eight to ten times as long as the guppies. Further tests showed no ill effects suffered by either fish in a 0.009% solution.

Potassium permanganate

Growth of both Saprolegnia sp. and A. americana occurred in cultures containing 0.25% potassium permanganate, while no growth was observed in those dishes containing 0.50% solutions. In Pythium sp., however, growth was suppressed completely at a concentration of 0.10% (Table 12).

As shown in Table 18, all concentrations of potassium permanganate tested were found to be toxic to the test fish.

Malachite green

Malachite green proved highly effective as a fungicide in concentrations ranging from 0.0066% (66 ppm.) to 0.0002% (2 ppm.) (Table 13). Again, however, no concentration of solution tested was found to be suitable for sustained fish culturing (Table 19). However, it is possible that a concentration lower than those tested would be suitable.

DISCUSSION AND CONCLUSIONS

Host range studies in this work differed from those conducted by other workers in that isolations of fungal pathogens were made only from infected tropical fish species. Tiffney (1939a) conducted a more general study in isolating fungi from three main groups of vertebrates (fish, reptiles, and amphibians). Also, the work done by Scott and O'Bier (1962) was of wider scope in that these investigators made collections from fish eggs and fish of various groups (including several species of tropical fish). As stated previously, reports of fungal epidemics in tropical fish hatcheries supported the need of a more specific type of investigation.

The three genera of fungi (Saprolegnia, Achlya and Pythium) isolated by this worker are among those commonly reported in the literature as fish pathogens. The difficulty experienced here of inducing sexual fruiting in order to make specific identifications is a common problem shared by mycologists working with various groups of fungi. Little is known about the physiology of the formation of reproductive structures in fungi, and it is certainly an area where additional work is needed.

In addition to the isolation and identification of naturally occurring pathogens, it was considered desirable and necessary to test relative degrees of pathogenicity and host specificity of representative fungal isolates under controlled laboratory conditions. Experiments of this type have been carried out by Vishniac and Nigrelli (1957) and by Scott and O'Bier (1962).

The results of this type of test (Tables 5-7) concur generally with those of the above workers in that the fungal pathogens appear to function primarily as wound parasites. In this work 91% of the experimentally wounded fish tested became infected and died, while only 17% of the unwounded test species developed mycoses. Moreover, in two of the four unwounded fish which became infected, the fungus was first observed growing from a particular area on the body of the fish. This suggests that these two fish sustained an accidental injury during the experiment, thus becoming more vulnerable to fungus infection. It is of further interest that the strains of Saprolegnia sp. and Achlya americana employed were more vigorous pathogens than the Pythium tested. This is supported by Scott and O'Bier who reported negative results in laboratory inoculation tests using isolates of Pythium. Although infection with Pythium was accomplished in these experiments, its status as a relatively highly infectious fish parasite is doubtful. However, Saprolegnia and Achlya proved to be highly pathogenic agents under laboratory conditions.

Vishniac and Nigrelli as well as Scott and O'Bier used the Mexican platyfish, Xiphophorus maculatus, as the test fish for inoculation studies. This investigation, however, compared and contrasted the pathogenic effects of three fungal strains on five selected species of tropical fish (Table 3). No patterns of host specificity could be established, thus demonstrating the versatility of the fungi tested. Also, the differences in resistance to fungal invasion among the different fish species was not constant.

It appears conclusive, in regard to the work of Tiffney, Vishniac and Nigrelli, Scott and O'Bier, this author and others, that certain saprolegniaceous fungi are primary wound invaders and lethal pathogens of various fish species. However, little is known about the actual host-parasite relationships once the fungus infects the fish.

Histological investigations and other studies which may reveal the intimate details of the fungus infection are of essence.

The problem of controlling and preventing fungus infection has been of paramount interest to fish culturists for many years. Various workers have demonstrated the fungicidal ability of a number of diversified chemicals. Most of these remedies have been applied in the form of dips for various lengths of time. Rankin (1952, 1953) using Phenoxetol compounds reported the ability of goldfish and swordtails to survive indefinitely in fungicidal concentrations of these compounds. This would be a more desirable and effective preventive method. General criticisms which can be made regarding the work of Rankin (1952), O'Donnell (1941), Estes (1957) and others are that information concerning taxonomic descriptions of the fungi involved, type of infection and extensiveness of infection is vague, if not completely lacking. Without this pertinent information, it is difficult to conclude the actual fungicidal ability of the chemicals tested. The writer was primarily concerned with the effect of certain chemicals (Table 4) on pathogenic isolates grown in vitro; that is, a test of the fungicidal abilities of the test chemicals on a fungus growing on a substrate (SPS agar) suitable for abundant and healthy growth.

Although these results cannot be compared directly with those derived from dip treatments, etc., they are valuable in establishing the value of a potential fungicide.

All chemicals tested showed positive fungicidal effects in varying concentrations (Tables 8-13). Malachite green, Nipagin M, and Chloro-phenoxetol were effective in comparatively lower concentrations. Concentrations of Phenoxetol, Propylene-phenoxetol, and Nipagin M (Tables 14, 15, 17) below the minimum lethal dose were determined in which the test fish could survive. Experiments using two injured and one uninjured species revealed, however, that the fungus (Saprolegnia in this case) was lethal to the fish at these concentrations. This disagrees with Rankin (1953) who reported that Phenoxetol and Propylene-phenoxetol cured fungus infections of goldfish at 0.01% concentrations.

The most effective of the chemicals tested was malachite green. O'Donnell (1941) reported positive curing and therapeutic results by dipping infected trout in 1:15,000 solution (0.0066%) of malachite green for 10-30 seconds. These tests showed that this chemical was an effective fungicide in vitro at concentrations as low as 2 ppm. (Table 13). Also, the test fish were able to retain consciousness for as long as two to three hours in the lowest effective concentration. It should be noted that the solutions of malachite green lose their effectiveness in 2-3 days after mixture.

Although no effective concentration which would allow sustained fish culturing was found, the chemicals tested proved to have definite fungicidal abilities and should not be discounted for possible use as

fish fungicides in the future. Possible methods of application (too extensive for this work) are: (1) the usage of sustained dip treatments (in relation to the known limit of tolerance) at various concentrations (especially malachite green), (2) the development of resistance in the fish by first placing it in a suitable concentration and then increasing the concentration slowly until the minimum lethal concentration is reached.

It is believed by the writer that tests similar to these using the above mentioned and other chemical compounds will possibly be of value in the control and prevention of fish mycoses.

SUMMARY

Collections of Saprolegniaceous fungi were made from diseased tropical fish. Nineteen strains, representing three fungal genera (Saprolegnia, Achlya and Pythium), were isolated from 11 fish species. Due to difficulty in inducing sexual fruiting, only two strains were identified to species; both of these being A. americana.

Laboratory infection studies revealed the ability of strains of each different fungus to infect five selected tropical fish species. Saprolegnia sp. and A. americana proved to be more vigorous and lethal pathogens than Pythium sp. No indications of host specificity were evident.

All chemicals tested (Table 4) showed definite fungicidal abilities. No effective fungicidal concentration of the chemicals tested would permit sustained fish culturing. Malachite green was the most effective fungicide tested, being functional in concentrations as low as 2 ppm.

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FIGURE 1

Loach infected by Saprolegnia sp. (body mycosis).

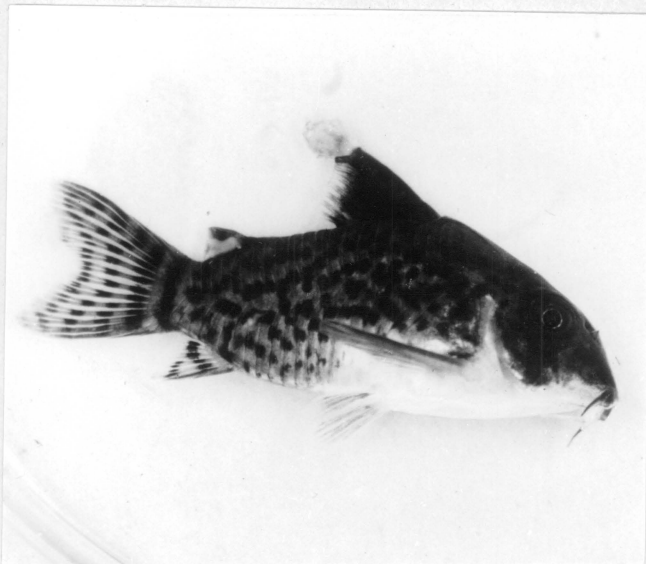


FIGURE 2

Achlya americana growing from the dorsal fin of
a living tropical catfish.

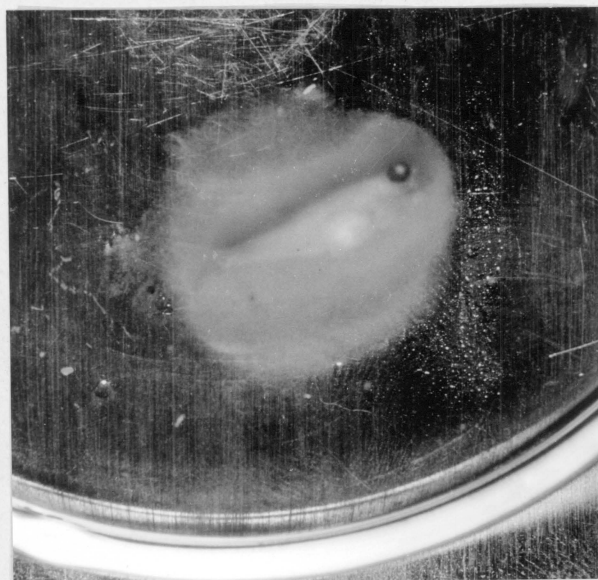


FIGURE 3

Pythium sp. growing as a body fungus from a neon tetra.



FIGURE 4

Fungus (A. americana) growing from injured areas of experimental guppy 24 hours after inoculation.

TABLE 1^a

The Ingredients of SPS Agar

1. Chelation agent:		
Ethylenediaminetetraacetic acid	0.5	grams/liter
2. Buffer for pH 7.0:		
K_2HPO_4	0.17	"
KH_2PO_4	0.14	"
3. Inorganic nutrients:		
$MgCl_2 \cdot 6H_2O$	1.0	"
$CaCl_2 \cdot 6H_2O$	0.02	"
$MnCl_2 \cdot 4H_2O$	0.06	"
$ZnCl_2$	0.04	"
$FeCl_3 \cdot 6H_2O$	0.0013	"
4. Organic Nutrients:		
D L Methionine	0.05	"
Glucose	5.0	"
Sodium glutamate (mono)	2.0	"
5. Dissolve the ingredients of steps 1, 2, 3 and 4 in 971 ml. pre-distilled ion-exchanged water and adjust the pH of the solution to 7.0 with KOH pellets.		
6. Agar (Difco Bacto-agar). Add to the above solution.	20.0	grams/liter
7. Autoclave the medium at 15 lbs. for 30 minutes.		

^aNote: taken from Scott et al. (1963).

TABLE 2

Host Range List

Organism	Common Host Name	Scientific Name	Locality	Date
<u>Pythium</u> sp.	Guppy	<u>Lebistes reticulatus</u>	City of Industry, California	12-19-62
"	Kissing Gourami	<u>Helostoma temminckii</u>	" "	1-22-63
"	Neon Tetra	<u>Hypessobrycon innesi</u>	" "	1-22-63
"	Swordtail	<u>Xiphophorus helleri</u>	Blacksburg, Virginia	2- 2-63
"	Black Molly	<u>Mollienisia</u>	" "	5-14-63
"	Black Platyfish	<u>Xiphophorus maculatus</u>	" "	5-20-63
<u>Achlya americana</u>	Swordtail	<u>Xiphophorus helleri</u>	Blacksburg, Virginia	3- 9-63
"	Cat Fish (Tropical)	<u>Corydoras</u> sp.	" "	2-18-63
<u>Achlya</u> sp.	Black Molly	<u>Mollienisia latipinna</u>	" "	3- 9-63
<u>Saprolegnia</u> sp.	Neon Tetra	<u>Hypessobrycon innesi</u>	City of Industry, California	1-17-63
"	Dwarf Gourami	<u>Colisa lalia</u>	" "	"
"	Loach	<u>Acan Thopthalmus semicinctus</u>	" "	"
"	Black Platyfish	<u>Xiphophorus maculatus</u>	" "	4- 5-63
"	Kissing Gourami	<u>Helostoma temminckii</u>	" "	4- 5-63
"	Black Platyfish	<u>Xiphophorus maculatus</u>	Micco, Florida	6- 1-63
"	Black Molly	<u>Mollienisia latipinna</u>	Blacksburg, Virginia	7-15-63
"	Swordtail	<u>Xiphophorus helleri</u>	" "	7-18-63
"	Guppy	<u>Lebistes reticulatus</u>	" "	7-20-63
"			" "	7-22-63

TABLE 3

Nomenclature and Classification
of Experimental Species of Fish

Family	Common Name	Scientific Name
Cyprinodontidae (Sub-family Poeciliinae)	Mexican Platyfish	<u>Xiphophorus maculatus</u> Guenther
Cyprinodontidae (Sub-family Poeciliinae)	Swordtail	<u>Xiphophorus helleri</u> (Heckel)
Cyprinodontidae (Sub-family Poeciliinae)	Guppy	<u>Lebistes reticulatus</u> Peters
Cyprinodontidae (Sub-family Poeciliinae)	Black Molly	<u>Mollienisia latipinna</u> Cuvier and Valenciennes
Anabantidae	Kissing Gourami	<u>Helostoma temminckii</u> Cuvier and Valenciennes

TABLE 4

Information Concerning Test Chemicals

Name Commonly Used	Other Name(s)	Manufacturer	Chemical Formula	Solubility in Water	Stock Solution Concentrations (room temp.)
Phenoxetol	Ethyleneglycolphenylether or -phenoxyethyl alcohol	NIPA Labs, LTD	$(C_6H_5OCH_2CH_2OH)$	2.4% (200 C.)	1.0% (1 ml. per 99 ml. H ₂ O)
Propylene- phenoxetol	Phenylether of propylene glycol	NIPA Labs, LTD	$(C_6H_5OCH_2CH_2CH_2OH)$	1.0% (25-26° C.)	1.0% (1 ml. per 99 ml. H ₂ O)
P-chloro- phenoxetol	P-chloro-phenylether of ethylene glycol	NIPA Labs, LTD	$(ClC_6H_4OCH_2CH_2OH)$	0.4% (25-26° C.)	0.4% (1 gm. per 100 ml. H ₂ O)
Mipagin M	Methyl ester of p-hydroxybenzoic acid	NIPA Labs, LTD	$(p-HOC_6H_4CO_2CH_3)$	0.3% (25° C.)	0.1% (.1 gm. per 100 ml. H ₂ O)
Malachite green	Aniline green; China green; etc.	Merck & Co., Inc.	$(C_{25}H_{25}ClN_2)$	Very soluble	0.0066% (.066 gms. per 1000 ml. H ₂ O)
Potassium permanganate	Chameleon mineral	Merck & Co., Inc.	$(KMnO_4)$	Very soluble	0.5% (.5 gm. per 100 ml. H ₂ O)

TABLE 5

Controlled Laboratory Studies Demonstrating the Infectious Ability of
Achlya americana on Wounded and Unwounded Selected Fish Species

Name and Number of Fish Wounded	Unwounded	Mycosis evident; death occurred	No Mycosis; death occurred	Dead within 24 hours	Dead within; after 18 days
<u>M. latipinna</u> - 2 (Black Molly)		2		2	
<u>L. reticulatus</u> - 4 (Guppy)		4		4	
<u>H. temminckii</u> - 4 (Kissing Gourami)		4		4	
<u>X. maculatus</u> - 4 (Platyfish)		4		4	
<u>X. helleri</u> - 4 (Swordtail)		4		4	
	<u>L. reticulatus</u> - 2 (Guppy)	1			12 days 1
	<u>H. temminckii</u> - 4 (Kissing Gourami)				4
	<u>X. helleri</u> - 2 (Swordtail)	1			5 days 1

TABLE 6

Controlled Laboratory Studies Demonstrating the Infectious Ability of
Saprolegnia sp. on Wounded and Unwounded Selected Fish Species

Name and Number of Fish Wounded	Unwounded	Mycosis evident; death occurred	No Mycosis; death occurred	Dead within 24 hours	Dead within 29 hours	Living and Uninfected after 18 days
<u>X. helleri</u> (Swordtail) - 4		4		4		
<u>X. maculatus</u> (Platyfish) - 4		4		4		
<u>H. temminckii</u> (Kissing Gourami) - 4		4		3	1 29 hours	1
<u>L. reticulatus</u> (Guppy) - 4		4		4		
<u>M. latipinna</u> (Black Molly) - 2		2		2		
<u>H. temminckii</u> (Kissing Gourami) - 2						2
<u>L. reticulatus</u> (Guppy) - 2		1			5 days	1
<u>M. latipinna</u> (Black Molly) - 2		1		1		1
<u>X. helleri</u> (Swordtail) - 2			1	1		1

TABLE 7

Controlled Laboratory Studies Demonstrating the Infectious Ability of
Pythium sp. on Wounded and Unwounded Selected Fish Species

Name and Number of Fish Wounded	Unwounded	Mycosis evident; death occurred	No Mycosis; death occurred	Dead within 24 hours	Dead within; after 18 days	Living and Uninfected after 18 days
<u>M. latipinna</u> - 2 (Black Molly)		2			3;12 days	
<u>L. reticulatus</u> - 4 (Guppy)		3			3;12;16 days	1
<u>H. temminckii</u> - 4 (Kissing Gourami)		2			3;13 days	2
<u>X. maculatus</u> - 4 (Platyfish)		2			11;14 days	2
<u>X. helleri</u> - 4 (Swordtail)		4			4;7;10;15 days	2
	<u>L. reticulatus</u> - 2 (Guppy)					2
	<u>H. temminckii</u> - 4 (Kissing Gourami)					4
	<u>X. helleri</u> - 2 (Swordtail)					2

TABLE 8

In vitro Studies on the Effects of Phenoxetol on the Growth
of Representative Strains of Three Fungal Genera

Concentration (%)	<u>(Saprolegnia sp.)</u>			<u>(Achlya americana)</u>			<u>(Pythium sp.)</u>		
	24 hrs.	48 hrs.	72 hrs. ^a	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
1.0	0	0	0	0	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0
0.50	0	0	0	0	0	0	0	0	0
0.25	16.6x17.0	20.5x20.6	24.5x24.0	12.6x13.6	15.0x15.6	21.0x20.6	16.0x14.3	15.6x16.0	17.0x16.6
0.10	20.0x20.0	21.0x20.0	21.0x20.3	18.0x16.6	26.6x24.6	51.5x51.3	15.6x14.0	35.0x32.0	46.0x44.0
0.01	22.0x22.3	22.6x23.0	23.3x23.3	31.0x21.0	35.0x33.3	40.0x40.0	19.0x17.0	39.0x39.3	54.6x55.0
Control	26.0x25.0	26.6x25.3	26.6x26.0	20.6x21.6	36.5x33.3	42.0x44.6	17.0x18.0	38.6x38.3	55.3x56.0

^a Each measurement is an average of three replicates.

TABLE 9

In vitro Studies on the Effects of Propylene-Phenoxetol on the Growth
of Representative Strains of Three Fungal Genera

Concen- tration (%)	(Saprolegnia sp.)			(Achlya americana)			(Pythium sp.)		
	Growth in mm. ^a			Growth in mm.			Growth in mm.		
	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
1.0	0	0	0	0	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0
0.50	b	b	b	0	0	0	0	0	0
0.25	21.0x19.6	31.3x28.3	45.6x44.3	b	b	b	0	b	10.3x10.6
0.10	40.3x41.0	64.0x65.0	87.0x86.0	28.0x20.0	32.6x30.0	43.2x42.3	21.6x20.0	37.3x35.0	50.6x49.6
0.01	45.3x44.0	68.3x69.0	89.0x89.0	28.6x19.3	32.3x29.6	45.3x43.6	11.3x12.3	22.6x22.3	32.3x31.3
Control	44.0x47.6	68.6x70.0	39.0x89.0	28.6x19.0	40.6x37.0	47.0x43.6	22.6x21.6	40.0x39.0	60.3x53.0

^aEach measurement is an average of three replicates.

^bIndicates that only a few unmeasurable hyphal strands were present.

TABLE 10

In vitro Studies on the Effects of Chloro-Phenoxetol on the Growth of Representative Strains of Three Fungal Genera

Concentration (%)	<u>(Saprolegnia sp.)</u>		<u>(Achlya americana)</u>		<u>(Pythium sp.)</u>				
	Growth in mm. 48 hrs.	Growth in mm. 72 hrs. ^a	Growth in mm. 24 hrs.	Growth in mm. 48 hrs.	Growth in mm. 24 hrs.	Growth in mm. 48 hrs.			
0.4	0	0	0	0	0	0			
0.2	0	0	0	0	0	0			
0.04	20.3x22.6	29.0x29.3	45.0x45.6	12.5x13.3	14.0x16.0	16.5x19.6	13.5x15.3	18.6x18.0	18.6x18.3
Control	27.0x27.3	46.6x49.6	89.0x89.0	22.0x22.6	32.6x32.6	50.5x52.3	22.0x23.3	37.6x38.6	67.0x68.6

^a Each measurement is an average of three replicates.

TABLE 11

In vitro Studies on the Effects of Nipagin M on the Growth of Representative Strains of Three Fungal Genera

Concentration (%)	(Saprolegnia sp.)			(Achlya americana)			(Pythium sp.)		
	24 hrs.	48 hrs.	72 hrs. ^a	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
0.1	0	0	0	0	0	0	0	0	0
0.06	10.6x11.6	14.3x15.6	26.6x27.0	b	9.0x9.3	9.0x9.3	0	b	b
0.01	32.0x33.3	59.3x61.6	86.6x87.0	19.0x19.3	35.0x36.6	50.3x51.3	15.6x14.6	28.3x28.0	42.3x42.6
Control	34.6x35.6	66.3x66.6	89.0x89.0	22.0x21.6	40.0x41.3	54.6x52.0	20.0x20.3	34.3x33.0	52.0x54.3

^a Each measurement is an average of three replicates.

^b Indicates that only a few unmeasurable hyphae were present.

TABLE 12

In vitro Studies on the Effects of Potassium Permanganate on the Growth of Representative Strains of Three Fungal Genera

Concen- tration (%)	(Saprolegnia sp.)			(Achlya americana)			(Pythium sp.)		
	Growth in mm. ^a			Growth in mm.			Growth in mm.		
	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
0.50	0	0	0	0	0	0	0	0	0
0.25	16.0x16.3	39.5x40.0	51.3x52.0	b	b	b	0	0	0
0.10	19.6x21.0	51.0x52.0	62.3x63.0	16.0x17.0	37.0x38.0	53.3x55.0	0	0	0
0.04	21.6x24.0	48.3x50.6	71.3x73.0	17.6x17.3	55.6x58.0	53.0x54.6	9.6x9.6	21.3x22.3	35.3x36.0
Control	31.0x31.6	56.0x55.6	89.0x89.0	21.3x20.3	40.6x40.6	59.6x61.0	17.0x17.3	39.3x40.6	58.6x61.0

^aEach measurement is an average of three replicates.

^bIndicates that only a few unmeasurable hyphal strands were present.

TABLE 13

In vitro Studies on the Effects of Malachite Green on the Growth of Representative Strains of Three Fungal Genera

Concentration (%)	(Saprolegnia sp.)		(Achlya americana)		(Pythium sp.)				
	Growth in mm. 24 hrs.	Growth in mm. 48 hrs.	Growth in mm. 24 hrs.	Growth in mm. 48 hrs.	Growth in mm. 24 hrs.	Growth in mm. 48 hrs.			
.0066	0	0	0	0	0	0			
.0040	0	0	0	0	0	0			
.0020	0	0	0	0	0	0			
.0010	0	0	0	0	0	0			
.0005	0	0	0	0	0	0			
.0002	0	0	0	0	0	0			
Control	32.0x34.0	60.0x63.0	89.0x89.0	18.6x19.6	29.0x29.6	51.0x51.3	20.6x19.0	34.0x32.0	60.0x58.6

^a Each measurement is an average of three replicates.

TABLE 14

Toxicity Effects of Phenoxetol on Two Selected
Fish Species in Relation to Time

Concentration (%)	Guppy	Kissing Gourami
1.00	8 sec.	25 sec.
.75	13 sec.	45 sec.
.50	19 sec.	1 min. 25 sec.
.25	27 sec.	1 min. 50 sec.
.10	1 min. 15 sec.	3 min. 10 sec.
.06	4 min. 45 sec.	6 min. 20 sec.
.03	4 hrs. 20 min.	2 hrs. 25 min.
.02	Sluggish, but lives	Sluggish, but lives
.01	Non-toxic	Non-toxic

TABLE 15

Toxicity Effects of Propylene-Phenoxetol on Two Selected
Fish Species in Relation to Time

Concentration (%)	Guppy	Kissing Gourami
1.00	15 sec.	22 sec.
.75	25 sec.	39 sec.
.50	30 sec.	46 sec.
.25	35 sec.	52 sec.
.08	5 min. 27 sec.	7 min. 20 sec.
.04	15 hrs. 20 min.	11 hrs. 50 min.
.02	Sluggish, but lives	Sluggish, but lives
.01	Non-toxic	Non-toxic

TABLE 16

Toxicity Effects of Chloro-Phenoxetol on Two Selected
Fish Species in Relation to Time

Concentration (%)	Guppy	Kissing Gourami
.04	20 sec.	50 sec.
.02	1 min. 30 sec.	1 min. 30 sec.
.008	9 min. 16 sec.	10 min. 26 sec.
.005	36 min.	25 min. 45 sec.

^aNo non-toxic concentration found.

TABLE 17

Toxicity Effects of Nipagin M on Two Selected
Fish Species in Relation to Time

Concentration (%)	Guppy	Kissing Gourami
.1	35 sec.	4 min. 50 sec.
.06	50 sec.	9 min. 40 sec.
.01	1 hr. 15 min.	1 hr. 39 min.
.009	Non-toxic	Non-toxic

TABLE 18

Toxicity Effects of Potassium Permanganate on Two Selected
Fish Species in Relation to Time

Concentration (%)	Guppy	Kissing Gourami
.5	2 min. 5 sec.	1 min. 40 sec.
.25	11 min. 15 sec.	10 min. 25 sec.
.002	10 hours	9 hrs. 30 min.

^aNo non-toxic concentration found.

TABLE 19

Toxicity Effects of Malachite Green on Two Selected
Fish Species in Relation to Time

Concentration (%)	Guppy	Kissing Gourami
.0066	10 min.	16 min.
.0040	22 min.	33 min.
.0020	24 min.	38 min.
.0010	32 min.	48 min.
.0005	2 hrs. 10 min.	1 hr. 3 min.
.0002	3 hrs. 30 min.	2 hrs. 14 min.

^aNo non-toxic concentration found.

ABSTRACT OF
STUDIES ON THE HOST RANGE AND CHEMICAL CONTROL OF FUNGI
ASSOCIATED WITH DISEASED TROPICAL FISH

by
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Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute
in candidacy for the degree of
MASTER OF SCIENCE
in
Biology

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Blacksburg, Virginia

This investigation was concerned with the aquatic fungi infecting tropical fish. The research was divided into two main phases: (1) host range studies and associated investigations; and (2) studies to determine the fungicidal effects of certain chemicals in vitro.

Nineteen strains, representing three fungal genera (Saprolegnia, Achlya, and Pythium), were isolated from 11 fish species. Two strains were identified specifically as Achlya americana.

Laboratory infection studies revealed the ability of each isolate to infect the following selected fish species:

Mexican Platyfish	- <u>Xiphophorus maculatus</u>
Swordtail	- <u>Xiphophorus helleri</u>
Guppy	- <u>Lebistes reticulatus</u>
Black Molly	- <u>Mollienisia latipinna</u>
Kissing Gourami	- <u>Heleostoma temminchi</u>

Saprolegnia sp. and Achlya americana proved to be more vigorous and lethal pathogens than Pythium sp. No indications of host specificity were evident.

Six chemicals (Phenoxetal, Propylene-phenoxetol, P-chloro-phenoxetol, Nipagin M, Malachite green, and Potassium permanganate) were tested. Each compound exhibited fungicidal activity. Malachite green was found to be the most effective fungicide tested, being functional in concentrations as low as 2 ppm. Sustained fish culturing could not be carried out in the minimum fungicidal concentrations of the various test chemicals.