Bulletin 68

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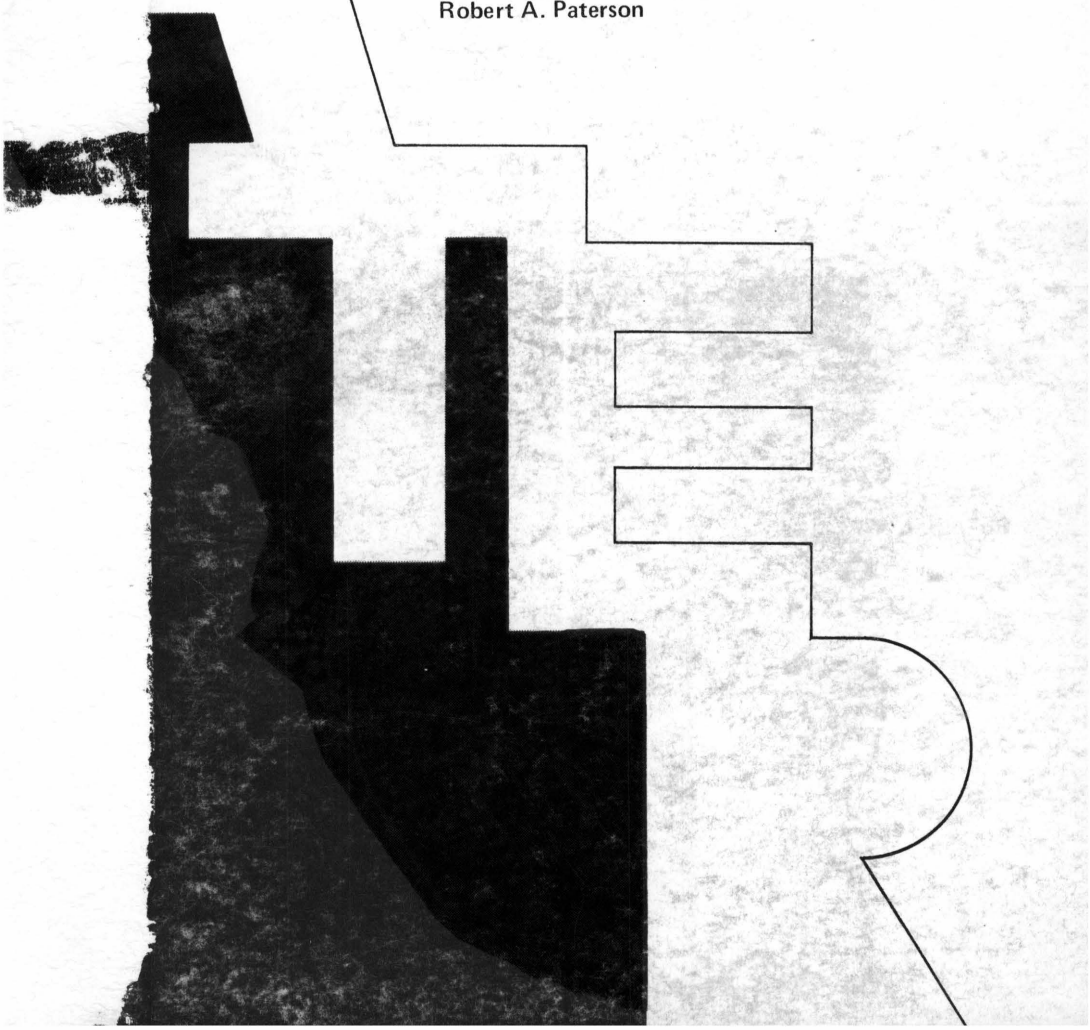

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# PREFACE

Two main groups of fungi are found in aquatic habitats, the aquatic imperfect fungi (aquatic Hyphomycetes) and the aquatic Phycomycetes. The aquatic imperfect fungi have been the subject of a number of studies. However, of the Phycomycetes, which are found in a variety of habitats, little is known concerning their ecological role, especially in rivers and streams. This paper deals with this group of organisms in the lotic environment.

The first section details the kinds of fungi found in the New River and Little Stony Creek, Virginia, a tributary of the New River. Special consideration is given to the effects of the effluents from an ammunition plant on the population of fungi. Selected fungi from these environments were isolated and grown under controlled laboratory conditions. The second phase of this investigation involved a study of the effect of some common toxic chemicals on the growth and reproduction of the fungi grown under controlled conditions.

These investigations should help to expand our knowledge of these little-studied organisms as well as point the way toward additional studies which might further define the ecological significance of these fungi in rivers.

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# ABSTRACT

Two lotic habitats in the vicinity of Blacksburg, Virginia were selected for field investigations. The New River represented a river with a high nutrient load. Little Stony Creek, a tributary of the New River, has no sources of man-made effluent which might contribute nutrients to the stream. Collecting sites on the New River were located above, just below, and some distance farther downstream from the effluent outfall of a munitions plant. The effect of this effluent on the number of taxa was not conspicuous. However, there was a reduction in the number of taxa per collection at the station near the effluent when compared with the other stations.

Filamentous aquatic Phycomycetes such as *Achlya, Saprolegnia,* and *Pythium* were commonly found in both habitats. However, a greater diversity of the chytrid type of aquatic Phycomycete was found in Little Stony Creek as compared to the New River. Twelve chytrid taxa were found in Little Stony Creek and two in the New River.

Two fungi, *Achlya caroliniana* from the New River and *Rhizidium* sp. from Little Stony Creek, were studied in pure culture in terms of the effect of common pollutants on their growth and reproduction. The *A. caroliniana* had higher tolerances to zinc, cyanide, and mannitol as compared to the *Rhizidium* sp. The *Rhizidium* was more tolerant to higher concentrations of detergents than the *Achlya*.

# INTRODUCTION

Two main groups of fungi are found in aquatic habitats, the aquatic imperfect fungi (aquatic Hyphomycetes) and the aquatic Phycomycetes. Aquatic Hyphomycetes are characterized by the presence of nonmotile asexual spores and appear to be important in the decay of leaves in streams and rivers (Cummins et al., 1973). The aquatic Phycomycetes possess motile asexual spores. These fungi are found in a variety of habitats, but little is known concerning their ecological role (Sparrow, 1960). This paper deals with the aquatic Phycomycetes of the lotic environment (streams and rivers).

Few studies have dealt in any detail with this group of fungi in the lotic environment (Sparrow, 1960). Some investigations have been conducted on the occurrence of aquatic fungi with special reference to the populations found in polluted streams (Cooke and Bartsch, 1959, 1960; Harvey, 1952). These studies dealt primarily with the filamentous aquatic Phycomycetes such as *Saprolegnia* and *Achlya* and little attempt was made to observe other types of Phycomycetes. Some of the species commonly encountered were *Achlya americana* Humphry, *Dictyuchus monosporus* Leitgeb; *Leptomitus lacteus* (Roth) Agardh, and *Saprolegnia ferax* (Gruith.) Thuret. The work of Cooke and Bartsch (1960) on streams in Ohio indicated little if any correlation between the degree of pollution and the occurrence of the fungi. No particular influence on water mold population was noted in the East Fork of the Little Miami River either by the effluent from the sewage treatment plant at Batavia or the raw sewage at Williamsburg. In the Potomac River, the stations in the cleaner water did not have as many aquatic fungi. In Mill Creek, there was a downstream decline in occurrence of aquatic fungi thought to be related to increasingly heavy loads of industrial wastes which may contain substances toxic to aquatic fungi. No aquatic fungi were found with distribution restricted to polluted reaches of streams, and similarly no aquatic fungi have been found which were restricted to so-called clean water portions of streams.

Willoughby and Collins (1966) collected river water samples from above and below the introduction of effluent from a sewage treatment plant. Samples were placed on nutrient agar medium and from this an estimate of the number of propagules was made. *Saprolgenia ferax*, a species often encountered in polluted waters, was present in large numbers in the sewage treatment plant effluent. The occurrence of *S. ferax* persisted downstream for a considerable distance. This species was not found in the stream water above

the site of effluent addition. Conversely, the genus *Achlya* was not present in the sewage effluent and its distribution downstream was fairly uniform. Cooke and Matsaura (1969), using similar plating techniques, found an increase in the number of propagules below the addition of effluents from a wastewater stabilization pond. Waterhouse (1942) studied a small stream over a year's period, finding members of several genera.

Previous studies have thus demonstrated that aquatic Phycomycetes are present in rivers under a variety of conditions. But specific information concerning the community structure or responses to toxic chemicals is not available.

With the current interest in the development of a sound understanding of the functioning of ecosystems, it appears that this group of organisms needs to be more fully investigated. This is especially true in the lotic environment where it is now generally believed that most of the primary production comes from outside the stream in the form of leaves and other plant parts (Cummins et al., 1973). Aquatic Phycomycetes may be active in the decomposition of this material. These fungi, having also been reported from polluted areas, may have some role in the decomposition of pollutants thus contributing to stream purification.

This investigation has sought to:

1. Determine the Phycomycete flora of a river,

2. Compare the species composition of streams of different quality,

3. Ascertain the effect of the introduction of an industrial effluent on population structure under natural conditions, and

4. Analyze the response of representative species to selected toxicants under laboratory conditions.

# FIELD STUDIES

Two lotic habitats in the vicinity of Blacksburg, Virginia were selected for the field investigation. One was the New River, which was chosen because it represents a river with a high nutrient load. Indeed, numerous towns and factories are located along its shores. The other habitat was Little Stony Creek, which flows from the Appalachian Mountains northwest through the Jefferson National Forest, and is a tributary of the New River. Except for occasional dwellings and small farms along its banks, there are no sources of man-made effluents which might contribute nutrients to the stream (Table 1).

## I. Materials and Methods

Samples were collected from various locations within each stream in an attempt to obtain the greatest diversity of fungi. These included the bottom sediments, areas where vegetation was present, riffle areas, etc. Samples were also collected from the soil immediately at the edge of each stream where water level fluctuations are most notable. This area covers a transition zone between the stream and the upland vegetation. Soil samples were also collected from the upland vegetation area. The latter were collected in an attempt to determine the extent to which the flora of each stream may be enriched by the passive movement of fungi from the upland soils into the water. Runoff during rains and wind action that blows soil into the water are two possible ways in which fungi of the upland soil could become deposited in the stream. It is possible that many of the upland forms found in the water represent transient species not a part of the stream community.

In the New River, collections were made from river sediments and upland and water edge soils in the vicinity of the Radford Army Ammunition Plant near Radford, Virginia. In addition, three specific sampling sites were established in the bottom sediments of the river. As a result of the munitions manufacturing operation, large amounts of effluent were introduced into the river in this area. Station 1 was located above the entrance of the effluent from the plant. Station 2 was located just below the effluent outfall, and Station 3 was located downstream at a point where surveys of other organisms have indicated a general recovery from the effects of the effluent (Cairns and Dickson, 1973). Samples were collected from each of the stations on July 25, August 29, October 10, and December 10, 1972. A total of 82 samples were collected. Table 2 lists the species found at each station.

Table 1

Chemical Characteristics of the Collecting Sites*

| | New River Number 1 | New River Number 2 | New River Number 3 | Little Stony Creek |
|---|---|---|---|---|
| pH | 7.5 | 7.6 | 7.6 | 7.0 |
| Total Hardness, ppm | 68.4 | 136.8 | 68.4 | 17.1 |
| Oxygen, ppm | 7.0 | 7.0 | 7.0 | 10.0 |
| Total Phosphate, ppm | 0.215 | 0.410 | 0.180 | — |
| Nitrate, ppm | 1.29 | 13.90 | 2.23 | — |

*The values for Little Stony Creek were obtained at the time of collection. The values for the New River were obtained from the Center for Environmental Studies at Virginia Polytechnic Institute and State University and are representative of the conditions during the collecting periods.

Table 2

Aquatic Phycomycetes Found in the New River, Virginia

| | Station 1 | | | Station 2 | | | Station 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Upland | Edge | Main Channel | Upland | Edge | Main Channel | Upland | Edge | Main Channel |
| *Achlya* sp. | + | + | + | + | – | + | + | – | + |
| *A. americana* | – | – | – | – | – | – | + | – | – |
| *A. caroliniana* | – | + | + | – | – | – | + | – | – |
| *A. diffusa* | – | – | – | + | – | – | – | – | – |
| *A. flagellata* | – | – | + | – | – | – | – | + | + |
| *A. prolifera* | + | – | – | – | – | – | – | – | – |
| *Aphanomyces* sp. | + | + | + | + | + | + | + | + | + |
| *Catenaria* sp. | – | – | – | + | – | – | – | – | – |
| *Dictyuchus* sp. | + | – | + | + | + | + | + | – | – |
| *Leptolegnia* sp. | – | – | – | – | – | – | – | + | – |
| *Leptomitus* sp. | – | – | – | – | – | – | – | – | + |
| *Nowakowskiella hemisphaerospora* | – | – | + | – | – | + | – | – | + |

Table 2
(Continued)

| | Station 1 | | | Station 2 | | | Station 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Upland | Edge | Main Channel | Upland | Edge | Main Channel | Upland | Edge | Main Channel |
| *Phlyctorhiza variabilis* | + | + | + | + | + | + | + | + | + |
| *Pythium* #1 | + | + | + | + | + | + | + | + | + |
| *Pythium* #2 | + | + | + | + | + | + | + | + | + |
| *Pythium* #3 | – | – | – | + | – | – | – | – | – |
| *Rhizophlyctis* #1 | + | + | – | – | – | – | – | – | – |
| *Rhizophlyctis* #2 | + | – | – | + | – | – | + | – | – |
| *Rhizophlyctis* #3 | – | – | – | + | – | – | + | – | – |
| *Rhizophlyctis rosea* | + | – | – | + | + | + | + | + | – |
| *Rhizophydium* #1 | + | – | + | + | – | – | + | – | – |
| *Rhizophydium* #2 | – | + | – | – | – | – | + | + | + |
| *Saprolegnia* sp. | + | – | + | + | + | + | + | + | + |
| *S. ferax* | – | – | – | + | – | + | – | – | – |
| *S. megasperma* | – | – | – | – | – | – | + | – | – |

At Little Stony Creek, 11 samples were collected on October 15, 1971, and again on September 12, 1972. Samples were taken from various habitats within the creek as well as from the upland areas. The species list from these collections and general location of each is given in Table 3.

All soil samples were collected using two opposing hemispherical scoops attached to tongs. The scoops were driven into the soil so that only the upper portion of the soil was collected. The tongs were closed by a scissors movement in such a way that the same amount of soil was collected each time. The samples were placed in sterile Whirl-Pak bags. The collecting apparatus was sterilized between samples with 75% ethanol.

Field samples were returned immediately to the laboratory and placed in 100 x 20 mm Petri dishes according to methods commonly used by investigators who study aquatic fungi. Various organic materials, or "baits," were added to the dishes for the fungi to colonize. The "baits" used were pine pollen, cellophane, chitin, hemp seeds, and snake skin. Because of the high nutrient level in the samples from the New River, it was necessary to aerate the dishes by bubbling filtered air through the cultures. After three or more days, the baits were removed and examined under the microscope for fungal growth. If fungi were present on the baits, they were then isolated into unifungal or pure cultures, where possible, for future identification.

**II. Results**

The fungi found in the cultures of soil which were collected from the New River and Little Stony Creek belong to three orders of the aquatic Phycomycetes. One, the Chytridiales, is characterized by a simple, non-filamentous plant body of determinate growth (Figure 1). The other two, the Saprolegniales and Peronosporales, have members that are filamentous with extensive indeterminate growth (Figure 2). Fungi belonging to all of the groups produce motile flagellated spores.

Many of the fungi that were isolated have not as yet been identified as to their species designation. Members of the Chytridiales are especially difficult to isolate in pure culture. Therefore, work is still in progress on this aspect of the study. Most of the members of the Saprolegniales and one genus of the Peronosporales that was found have been isolated. However, many of these fungi have not been identified as to species because the sexual structures are necessary for the determination. Unfortunately, the isolates have not as yet been induced to produce them. However, the genus in all cases has been determined. Where species have not been established, the use of numbers

Figure 1

*Rhizidium* sp., a Unicellular Aquatic Phycomycete Growing on Nutrient Agar

A. Germinating zoospore showing formation of rhizoidal system

B–D. Further growth of rhizoidal system and enlargement of zoospore cyst to form sporangium

E. Cytoplasm of sporangium is cleaved out to form zoospores

F. Empty sporangia following zoospore discharge

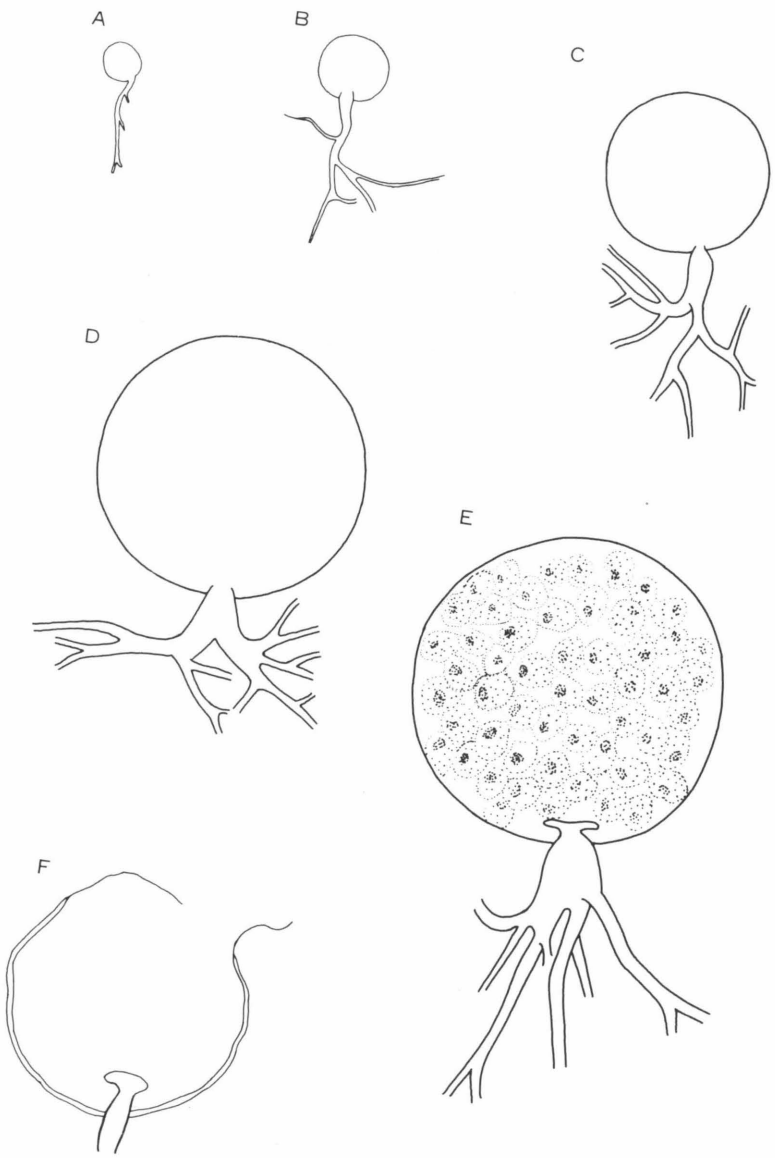

A
B
C
D
E
F

Figure 2

*Achlya caroliniana*, a Filmentous Aquatic Phycomycete

A. A hemp seed colonized by the fungus

B. A sporangium

C–F. Stages in development of the oogonium. C and D show the young oogonia, E an oogonium with oospheres, and F an oogonium with oospores

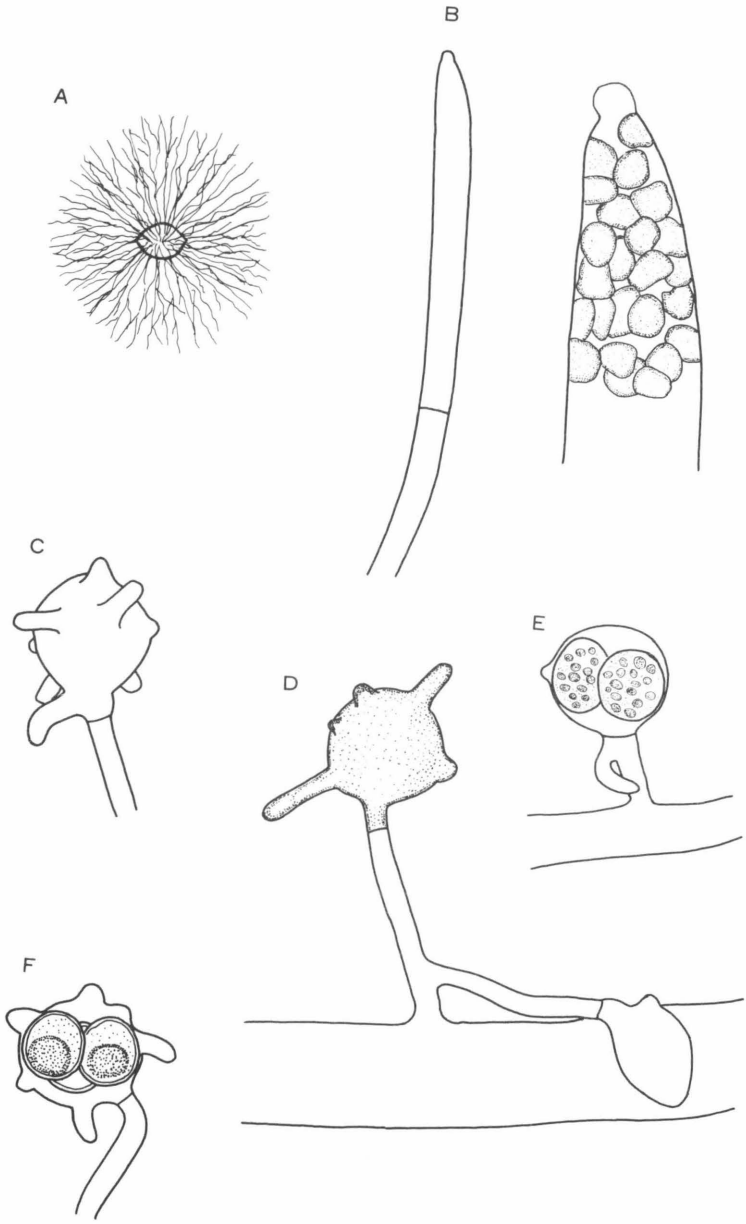

A
B
C
D
E
F

Table 3

Aquatic Phycomycetes Found in Little Stony Creek

| | Upland | Edge | Main Channel |
|---|---|---|---|
| *Achlya* sp. | – | + | – |
| *A. americana* | – | + | + |
| *A. conspicua* | – | – | + |
| *A. glomerata* | – | + | – |
| *A. klebsiana* | – | – | + |
| *Aphanomyces* sp. | – | + | + |
| *Catenaria* sp. | + | – | – |
| *Catenomyces persicinus* | – | – | + |
| *Chytriomyces appendiculatus* | – | + | + |
| *C. hyalinus* | – | + | + |
| *C. poculatus* | – | + | – |
| *Dictyuchus sp* | – | + | + |
| *Karlingiomyces sp* | + | – | – |
| *Leptomitus* sp. | – | + | + |
| *Nowakowskiella* sp. | – | + | + |
| *Phlyctochytrium* #1 | – | + | + |
| *Phlyctochytrium* #2 | – | + | + |
| *Phlyctochytrium* #3 | – | + | + |
| *Phlyctochytrium* #4 | + | – | – |
| *Phlyctorhiza variabilis* | – | + | + |
| *Pythium* #1 | – | + | + |
| *Pythium* #2 | + | + | + |
| *Rhizidium* sp. | – | + | + |
| *Rhizophlyctis rosea* | + | – | – |
| *Rhizophyctis* #4 | – | + | + |

Table 3
(Continued)

| | Upland | Edge | Main Channel |
|---|---|---|---|
| *Rhizophydium* #3 | – | + | + |
| *Rhizophydium* #4 | – | + | + |
| *Rhizophydium stipitatum* | + | – | – |
| *Saprolegnia* sp. | – | + | + |
| *Septosperma rhizophidii* | + | – | – |

after the genus will designate what is believed to be a distinct taxon. The taxonomic descriptions and discussions will be presented in a future paper.

Nine taxa in five genera of the Chytridiales, members of three genera of the Saprolegniales, and species of the genus Pythium were found in the New River cultures. Members of the chytrid genus *Rhizophlyctis* were found more commonly in the upland soils than in the river edge and bottom sediments. They occurred in 60% of the upland samples and in only 9% of the edge and bottom samples. In addition, four of the five samples from the river that contained species of *Rhizophlyctis* came from the edge rather than the bottom sediments. Species of *Rhizophlyctis* have been found by other workers to be ubiquitous soil organisms (Sparrow, 1960 and Willoughby, 1965).

Three other fungi were found more often in cultures from the upland soils than from those in the river edge and bottom. *Rhizophydium* #2 was found in 10% of the upland samples and 5% of the river samples. Two of the three occurrences in the river were from the edge. Two fungi were not found in the river—*Rhizophydium* was encountered only in the upland soils in 38% of the samples and *Catenaria* sp. was recorded from one soil sample from the upland area.

The chytrids *Phlyctorhiza variabilis* Karling and *Nowakowskiella hemisphaerospora* Shanor occurred more often in samples from the river than other species of the group.

*Phlyctorhiza variabilis* was found in 50% of the upland soil samples and 28% of the river samples. Within the river samples, half of the occurrences were in the main channel and half were at the edge. Only *Nowakowskiella hemisphaerospora* was found exclusively in the river. It occurred in 11% of the samples, all from the main channel.

In the New River area, filamentous fungi were found in all samples collected. *Aphanomyces* sp. was most common, being present in 42% of the upland and 70% of the river samples. Pythium #1 was present in 69% of the upland and 57% of the river samples. *Saprolegnia* sp. had values of 37% upland and 47% river, and *Achlya* sp. 38% and 35%, respectively. *Achlya caroliniana* Coker (3 collections), *A. flagellata* Coker (4 collections), and *Saprolegnia ferax* (1 collection) were the only water molds found in the river which could be identified to species.

In terms of number of taxa, the effect of the effluent was not conspicuous. Thirteen taxa were found at Station 1, ten taxa at Station 2, and twelve taxa

at Station 3. *Saprolegnia ferax* was recorded only at Station 2. This fungus has been previously reported from polluted situations.

Table 4

Number of Taxa per Collection from River

| Station | Edge | Main Channel | All Collections |
|---|---|---|---|
| 1 | 3.3 | 3.2 | 3.2 |
| 2 | 2.8 | 1.3 | 2.2 |
| 3 | 3.8 | 3.2 | 3.3 |

Table 4 indicates that the number of taxa per collection was reduced at Station 2 as compared to the other two stations. This reduction in diversity was especially evident within the main channel. Qualitative observations of the growth of the fungi in the culture dishes from Station 2 samples also indicated suboptimal growth conditions. Growth was slow and never as extensive as that in the dishes from the other two stations.

In the investigation of fungi in the Little Stony Creek area, 15 chytrids were found. With the exception of one species of *Phlyctochytrium* all chytridiaceous fungi were found in the creek edge and bottom sediments and were absent from samples collected at the upland sites. It is possible that the species of *Phlyctochytrium* from the upland area is the same as one collected from the creek soils. Further study is required in the determination of these forms.

There were 4.4 taxa of the Chytridiales found per collection in the main channel and 4.8 taxa per sample at the edge of the creek. The most commonly occurring fungi were *Phlyctorhiza variabilis* which occurred in 16% of the samples, *Chytriomyces hyalinus* Karling in 77%, *Phlyctochytrium* #2 in 50%, *Nowakowskiella* sp. in 44%, *Chytriomyces appendiculatus* Karling in 40%, and *Rhizophydium* #3 in 44% of the samples. There did not appear to be any distinction in terms of individual species or between the edge and main channel samples in the creek.

Filamentous fungi were commonly encountered. Eleven taxa were found in the creek with 2.2 taxa per collection (2.1 in the edge and 2.3 in the main channel).

In the genus *Achlya* four species were identified. *A. americana* was found in 6 of the 9 samples collected from Little Stony Creek on October 15, 1971.

**III. Summary and Conclusions**

With regard to the field study on the occurrence of aquatic Phycomycetes in the New River and Little Stony Creek areas the following general comments can be made:

1. Nine chytridiaceous fungi were found in the New River area. Only one of these, *Nowakowskiella hemisphaerospora*, occurred exclusively in the river sediments. The others were found more often in the upland soils and two occurred only in the latter location. *Phlyctorhiza variabilis* was consistently found in the river as well as from the upland soil. Its frequency in the river suggested that it may carry out its life history within the river. The other chytrids found in the river are most likely misplaced soil fungi. The situation was quite different in the Little Stony Creek area. Of the 15 chytrids found, all but one or possibly two fungi occurred exclusively in the creek sediments. There was a difference in the composition of species between the two habitats. Twelve chytrids found in Little Stony Creek were not observed in samples from the New River.

2. Filamentous fungi were commonly encountered in both habitats. Eleven taxa were found in Little Stony Creek with 2.2 taxa per collection. This is similar to the figure of 2.6 taxa per collection reported from the New River. *Pythium* #2 was the only filamentous form found in the upland samples. It also occurred in 22% of the stream samples, as compared to 57% in the New River. In the genus *Achlya* four species were identified, as compared with two species in the New River. The only species common to the two streams was *A. americana*. It was found in one sample from the New River as compared to its occurrence in 6 of the 9 samples collected from Little Stony Creek on October 15, 1971. The *Saprolegnia ferax* found in the New River was not present in the Little Stony Creek collections.

3. The largest number of taxa, both filamentous and chytridiaceous, from any one stream collection was 12 in Little Stony Creek and 6 in the New River.

4. In the New River, the effect of the effluent from the Radford Army Ammunition Plant on the numbers of taxa was not conspicuous. However, there was a reduction in the number of taxa per collection at the station near the effluent when compared with the stations above and below the outflow area.

5. It is concluded that there was a difference in the numbers of organisms found in the two habitats. The enriched New River contained fewer species than the mountain stream. The specific influence of the effluent from the Radford Army Ammunition Plant was not clearly shown. Further study should be done, to include more extensive sampling for a longer time.

# LABORATORY STUDIES

Laboratory studies were undertaken to determine what effects toxic chemicals might have on growth and reproduction of aquatic fungi. Much of the previous work on toxic substances has focused on higher organisms such as fish, but little attention has been devoted to the decomposers, particularly the fungi. The field studies indicated that these organisms occurred commonly in the New River and Little Stony Creek. Laboratory studies would provide information as to whether the pollutant directly kills the fungus or whether the effect is more subtle, e.g., changing the autotrophic community which brings about a change in the community structure of the decomposers. Also, toxicity studies would help to evaluate the role of the fungi in the purification of streams.

Specific effluents from the Radford Army Ammunition Plant were not used in the laboratory studies for two primary reasons. There was great variation in the effluent depending on the predominant manufacturing process occurring within the plant at any particular time. This variation would make replication difficult and the data could be misleading in terms of extrapolation to the field condition. Furthermore, pure culture studies would be unsatisfactory since autoclaving might change the effluent composition. In addition, the components of the media might cause precipitation of substances in the effluent. Thus a series of compounds was examined which were representative of the major types of pollutants that might be encountered. Zinc was used as an example of heavy metal poisoning, cyanide as a respiratory inhibitor, mannitol as osmotic stress, and linear do- and tridecylbenzenesulfonate as surfactants.

While this methodology has the disadvantage of not being directly applicable to the field situation, there are advantages to this approach. The substances used in this study are representative of the kinds of materials in the Plant effluent. Furthermore, repeatable observations can be made. Comparisons with organisms used in other studies, such as fish, invertebrates, and algae can be done. In addition, it can be argued that an initial study of a simple system is more reasonable than a complex one.

**I. Materials and Methods**

a. Growth Studies

*Achlya caroliniana* Coker was isolated from the New River collections where it was found growing on hemp seeds. Zoospore cysts were removed from the

tip of a discharged sporangium and placed on Bacto-Cornmeal Agar (Difco). Growth of the filaments from cysts was rapid enough to outgrow any bacterial colonies which developed. Hyphal tips free of bacteria were transferred to new agar dishes. From this pure culture a single zoospore cyst was removed and used to start a new culture. This culture, derived from a single spore, was used in all of the laboratory work on *Achlya.*

For the growth studies, a liquid synthetic nutrient medium was utilized. This medium was basically that used by Barksdale (1962, 1963) in her work on *Achlya* and contained the following ingredients:

| | gm/liter |
|---|---|
| monosodium glutamate | 0.5 |
| glucose | 3.0 |
| L-cystine | 0.02 |
| Tris (hydroxymethyl) aminomethane | 1.2 |

| | molar |
|---|---|
| $KH_2PO_4$ | 0.0015 |
| KCL | 0.002 |
| $MgSO_4 \cdot 7H_2O$ | 0.0005 |
| $CaCl_2$ | 0.0005 |

| | mg/100ml |
|---|---|
| EDTA — Disodium Ethylenediamine-tetraacetate | 2.0 |
| Fe as $Fe(NH_4)2(SO_4)_2 \cdot 6H_2O$ | 0.1 |
| Zn as $ZnSO_4 \cdot 7H_2O$ | 0.1 |
| Mn as $MnSO_4 \cdot H_2O$ | 0.05 |
| Cu as $CuSO_4 \cdot 5H_2O$ | 0.01 |
| Co as $CoSO_4 \cdot 7H_2O$ | 0.02 |
| B as $H_3BO_3$ | 0.05 |
| Mo as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ | 0.02 |

The pH was adjusted to 6.8, and 50-ml portions were placed in 250-ml Erlenmeyer flasks. The flasks with medium were sterilized by autoclaving at 15 pounds pressure for 15 minutes.

Growth was initiated by adding a zoospore suspension, obtained as follows. Petri dishes containing 30 ml of the synthetic growth medium plus 2% agar were inoculated with a mycelial plug 6 mm in diameter. These dishes were incubated at 25°C for 5 to 7 days. Two mycelial plugs were removed from

the margin of one of these dishes and each was placed in a 250-ml Erlenmeyer flask containing 50 ml of sterilized growth medium diluted 1:10. These flasks were incubated at 25°C for 24 hours. The plugs were then removed and placed in flasks containing 50 ml sterilized glass distilled water. After 10 hours, abundant zoospore release had occurred. One-ml aliquots containing approximately 10,000 zoospores were utilized as inoculum in the growth studies.

At 20°C incubation, maximum growth was obtained in 7 days, at which time each experiment was terminated. Growth was measured as mg of dry weight. Whatman No. 1 filter papers were dried at 60 to 70°C, brought to room temperature in a dessicator, and weighed. The cultures were filtered with the mycelium being collected on the filter paper. After being washed with distilled water, the paper was again dried and weighed. The difference in the two weights represented the dry weight of the mycelium.

All glassware was acid washed and rinsed in distilled-deionized water. Glass distilled water was utilized in all media. Three to five replicate flasks were utilized at each chemical concentration level.

Another organism used in the study was a *Rhizidium* sp., obtained from samples collected in Little Stony Creek. The fungus was originally found growing on snake skin bait. Sporangia were removed from the snake skin, washed in sterile water, and placed on nutrient agar medium containing streptomycin and penicillin. The medium was a modification of that originally proposed by Koch (1957) and contained the following ingredients:

$\frac{1}{2}K_1$ Medium

| | gm/liter |
|---|---|
| Difco Bacto-peptone | 0.33 |
| Difco Yeast Extract | 0.22 |
| Dextrose | 1.00 |
| Difco Bacto-agar | 15.0 |
| Penicillin | 0.10 |
| Streptomycin | 0.10 |

After the sporangia were placed on the agar plate, they were pushed through the agar to remove some of the adhering bacteria. A drop of sterile water was placed over the sporangium, which generally stimulated the discharge of the zoospores. The drop of water, now containing zoospores, was spread over the surface of the agar medium. After 24 to 48 hours, the zoospores had

developed into young thalli. Those thalli free from bacterial growth were transferred to new dishes of medium which did not contain the antibiotics. After several days of growth to be sure that no bacteria were present, zoospores were again spread over the surface of the dish. A single developing plant was removed and used to start the culture which was utilized in all of the growth studies.

Growth studies were conducted in a liquid medium of the following composition:

Modified $K_1$ Medium

| | gm/liter |
|---|---|
| Difco Bacto-peptone | 0.66 |
| Difco Yeast Extract | 0.04 |
| Glucose | 5.0 |

Erlenmeyer flasks of 250-ml capacity were filled with 50 ml medium, and inoculated with a zoospore suspension. The suspension was obtained by covering a Petri dish containing the $\frac{1}{2}K_1$ medium (minus antibiotics) with zoospores. After 5 to 7 days of growth at 20°C, the agar and associated fungi were transferred to sterile glass distilled water. Within 1 to 2 hours, abundant zoospore release had occurred. This suspension was adjusted to contain approximately 100,000 zoospores per ml.

In all tests, the flasks were incubated at 20°C for 9 days on a reciprocating shaker (100 excursions per minute). All other procedures were the same as those described in the studies on *Achyla*.

b. Sexual Reproduction Studies

*Achlya caroliniana* was utilized in the studies of the effect of toxic compounds on sexual reproduction. The procedure here was similar to that used for producing a zoospore suspension. Mycelial plugs were placed in 250-ml Erlenmeyer flasks containing 50 ml of a 1:10 dilution of the nutrient medium and incubated at 25°C for 24 hours. The plugs were then transferred to 250-ml Erlenmeyer flasks with 50 ml of a 0.01 molar solution of tris buffer and within 24 hours the development of the typical sexual structures was initiated. The pH of the buffer solution was adjusted to 6.8 prior to autoclaving. The various test chemicals were added to the distilled water prior to the addition of the mycelial plugs to determine their effect on the sexual process.

While this methodology allowed for abundant sexual reproduction, it had the disadvantage of not being easily quantified. Therefore, the results are presented on a relative basis with the control, non-stressed results being used as the standard.

c. Toxic Chemicals Utilized

1. Cyanide. The cyanide was added as NaCN. The cyanide solutions were filter sterilized and added aseptically to the growth medium after autoclaving.

2. Zinc. Zinc was added as $ZnSO_4 \cdot 7H_2O$. The zinc solutions were autoclaved separately from the growth medium and then added aseptically.

At concentrations above 6 to 7 ppm, precipitation of the zinc occurred in the synthetic medium used for the *Achyla* growth studies. This led to the following semi-quantitative procedure. It was found that up to 60 ppm of zinc could be added to a 2% water agar solution without any observable precipitation occurring. Petri dishes containing 2% water agar with the specified amount of zinc added were prepared and zoospore suspension of 0.3 ml was added to the surface. After 36 hours and 4 days, the amount of growth as measured in length of mycelium from each zoospore was observed.

Similar precipitation of zinc did not occur in the medium used for the growth studies of *Rhizidium*.

3. Mannitol. The mannitol was added to the medium before autoclaving.

4. Detergents. Surfactants of the alkylbenzenesulfonate type are the most widely used (Swisher, 1970). The two surfactants used in this study are of the linear, non-branched type. These are new formulations currently under study by Dr. John Cairns in the Biology Department of Virginia Polytechnic Institute and State University.

Linear tridecylbenzenesulfonate

| | |
|---|---|
| Solids | 49.1% |
| Free oil | 0.4% |
| Alcohol insol | 3.6% |
| Active ingredient | 45.3% |
| Chain length | $C_{14}$ |

Linear dodecylbenzenesulfonate

| | |
|---|---|
| Solids | 44.7% |
| Free oil | 0.8% |
| Alcohol insol | 2.2% |
| Active ingredient | 41.6% |
| Chain length | $C_{12}$ |

The surfactants were obtained in a liquid condition and the concentrations used were based on the relationship of dry weight to wet weight. Thus, 1 gm wet weight of linear dodecylbenzenesulfonate gave 0.45 gm of dry weight. The 0.45 figure was used in preparing the concentrations used in these studies. However, a more common method is based on the active ingredients. One gram wet weight of linear dodeclybenzenesulfonate has 0.41 gm of active ingredients and this figure can be used in calculating the concentrations. The set of numbers on each surfactant graph (Figure 5) shows the concentration used based on active ingredients.

**II. Results and Discussion**

The effects of the toxic chemicals on the vegetative growth of *Achlya caroliniana* are shown in Figures 3 through 5 and Table 5.

Figure 3 shows the influence of cyanide on the vegetative growth of *Achlya*. Concentrations up to 15 ppm were used and even at the higher concentrations, growth was never completely inhibited, although growth was not great enough to provide any dry weight values.

The osmotic stress (Figure 4) introduced by solutions of up to 5% mannitol had only a slightly depressing effect on the growth of *Achlya*.

The longer the carbon chain surfactant was found to be the most toxic of the two surfactants (Figure 5).

Table 5 presents the results of the zinc experiment. As a check on this experimental technique, a similar procedure with cyanide was utilized, and this gave a good correlation with the results of the growth in liquid cultures to which cyanide had been added.

When the experiment with zinc was set up, it was anticipated that each successively higher toxicant concentration would be lethal to a large number of zoospores and that this higher toxicity would be manifested through a

Figure 3

Growth of *Achlya caroliniana* on Defined Medium After 7 Days with Varying Concentrations of Cyanide

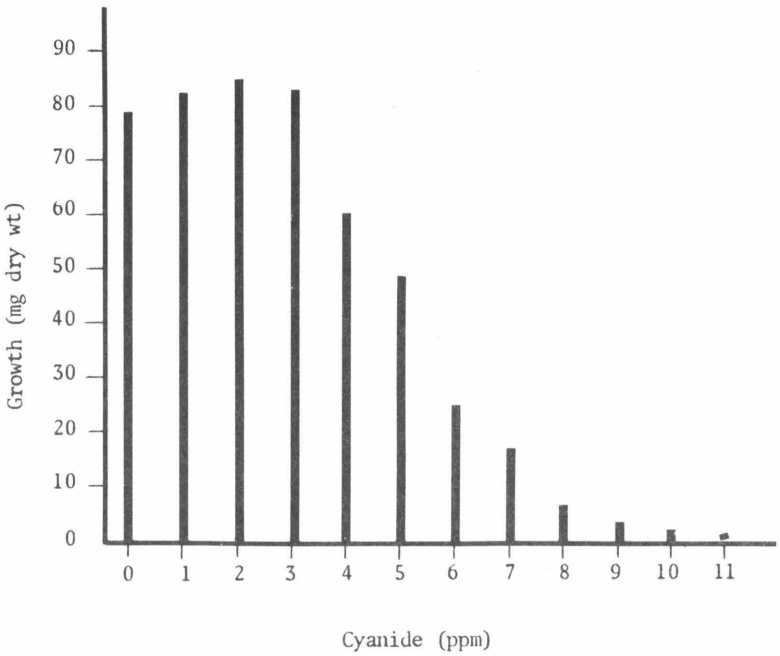


Figure 4

Growth of *Achlya caroliniana* on Defined Medium After 7 Days with Varying Concentrations of Mannitol

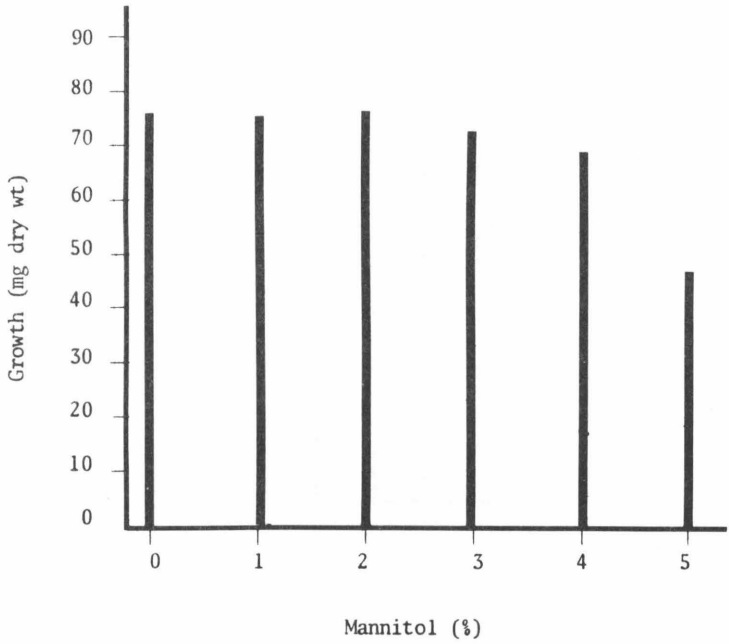
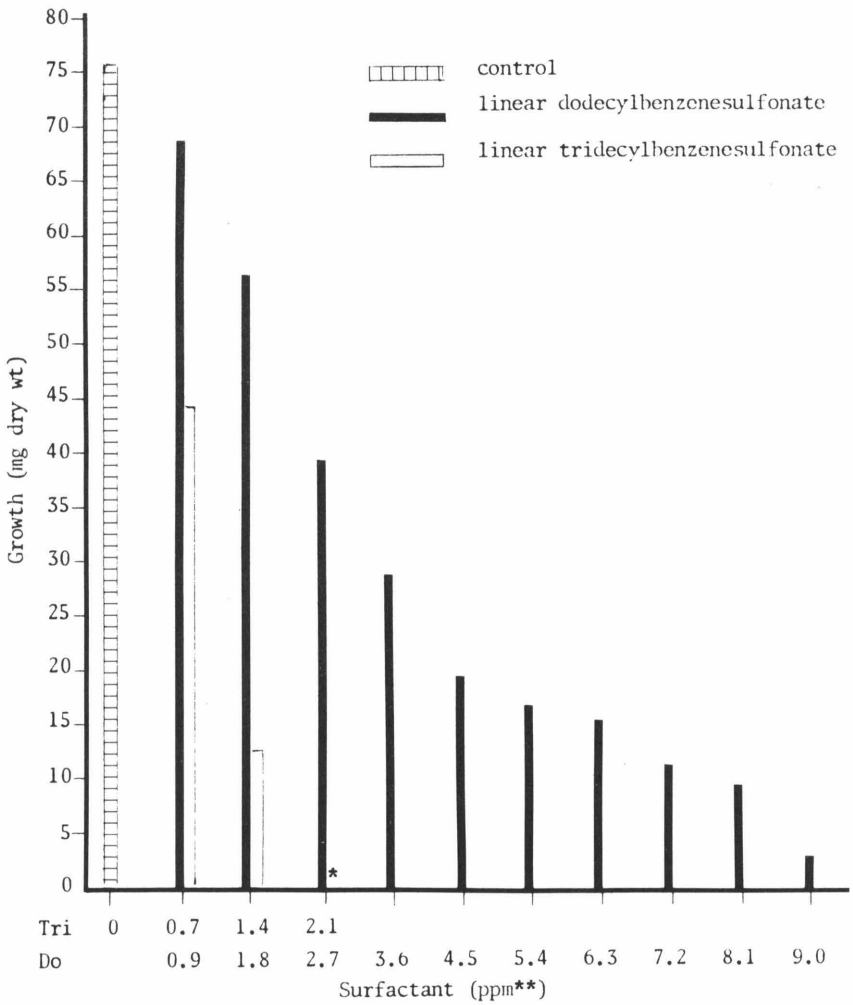


Figure 5

Growth of *Achlya caroliniana* on Defined Medium After 7 Days with Varying Concentrations of Surfactants




*Limited growth. Insufficient material to calculate weight.
**See text for interpretation.

Table 5

*Achlya caroliniana*
Mycelial Length from Zoospore Germination on 2% Water Agar with Varying Concentrations of Zinc and Cyanide

| | Zinc (growth, μ) | | Cyanide (growth, μ) | | Cyanide plus 5ppm Zinc (growth, μ) | |
|---|---|---|---|---|---|---|
| ppm | 36 hours | 4 days | 36 hours | 4 days | 36 hours | 4 days |
| 0 | 358 | exten. | 331 | exten. | 368 | exten. |
| 2 | X | X | 120 | 266 | 147 | 248 |
| 4 | X | X | 82 | 128 | 37 | 37 |
| 5 | 110 | exten. | X | X | X | X |
| 6 | X | X | 37 | 37 | – | – |
| 8 | X | X | – | 37 | – | – |
| 10 | 101 | 386 | – | – | – | – |
| 12 | X | X | – | – | – | – |
| 14 | X | X | – | – | X | X |
| 15 | 92 | 202 | X | X | X | X |
| 20 | 55 | 120 | X | X | X | X |
| 40 | 27 | 46 | X | X | X | X |
| 60 | – | 18 | X | X | X | X |

– No observable growth
X No test run

difference in the number of zoospores germinating at each concentration. This was not the case. Equal numbers of zoospores germinated at each concentration until at the higher concentrations no germination occurred. This was the situation with both the cyanide and the zinc. The zoospore cysts could be observed on the surface of the agar and still appeared to be viable although no germination had occurred. Fourteen zoospore cysts which had been placed on a dish containing 14 ppm of cyanide 7 days earlier were thus removed and placed on freshwater agar. In all cases, germination occurred within a few hours and vigorous growth ensued. This indicates that concentrations which prevent germination and growth are not lethal to the zoospore. The zoospore would thus seem to be capable of withstanding at least short term exposure to toxicants without losing its viability.

Figures 6 through 20 present the experiments performed on the chytrid *Rhizidium* sp. Preliminary studies were carried out at different zinc concentrations with another chytrid, *Chytriomyces hyalinus*. In this study, the fungus was grown on pieces of purified chitin in a soil extract solution which contained various concentrations of zinc. This made it possible to observe directly the effects of the zinc through a life cycle. The reduced growth at concentrations above 10 ppm resulted from an interruption of zoospore discharge. Sporangial growth appeared normal but after zoospores were formed inside the sporangium, they were either not released, or if released, they immediately encysted at the orifice of the sporangium and eventually died without germinating.

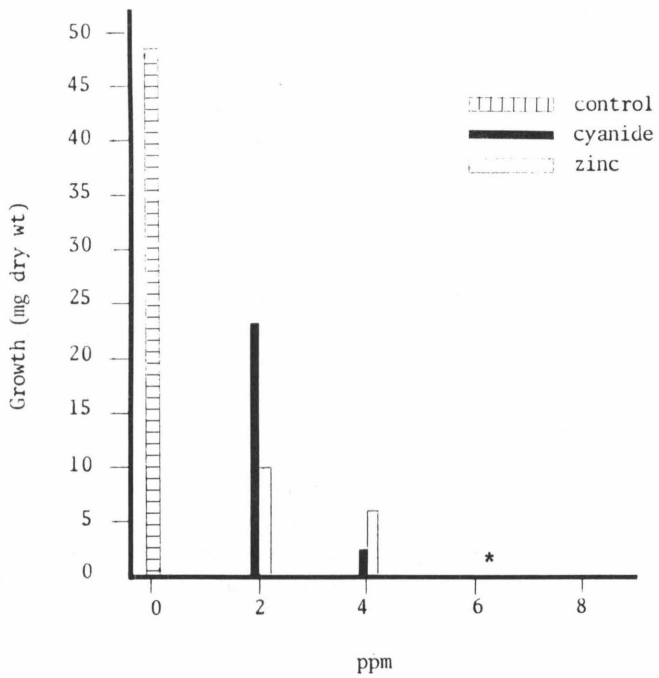
Figure 10 presents a comparison between the *Achlya* and the *Rhizidium* sp. in the growth responses to the four test chemicals. The *Achlya* showed a greater level of tolerance to the zinc, mannitol, and cyanide, while the *Rhizidium* sp. had a greater tolerance to the surfactant.

Figure 11 summarizes the effects of the test chemicals on sexual reproduction in *Achlya*. Oospore production is disrupted at much lower concentrations as compared to their effect on vegetative growth. At levels of toxins which still allow significant vegetative growth, sexual reproduction is completely repressed. The production of oospores is important because it permits genetic recombination and also provides the organism with a spore capable of withstanding harsh environmental conditions. Rather low levels of toxicants thus interfere with this important process.

Studies are being conducted on the effects of the test chemicals on zoospore mortality in both the *Achlya* and the chytrid, *Chytriomyces hyalinus*. The zoospores may be the stage most sensitive to stress conditions since (1) they are surrounded by only a membrane as compared to the cell wall surrounding

Figure 6

Growth of *Rhizidium* sp. on Modified $K_1$ Medium After 9 Days with Varying Concentrations of Cyanide and Zinc




*Limited growth. Insufficient material to calculate weight.

Figure 7

Growth of *Rhizidium* sp. on Modified $K_1$ Medium After 9 Days with Varying Concentrations of Mannitol

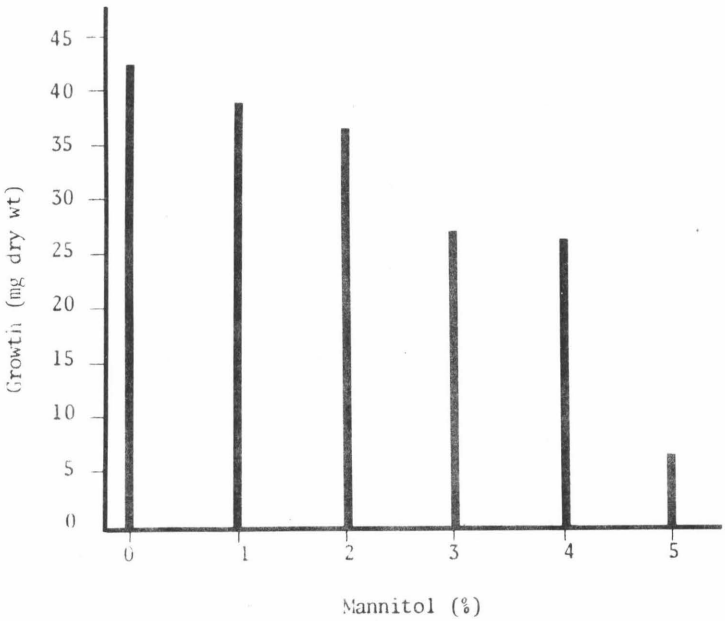
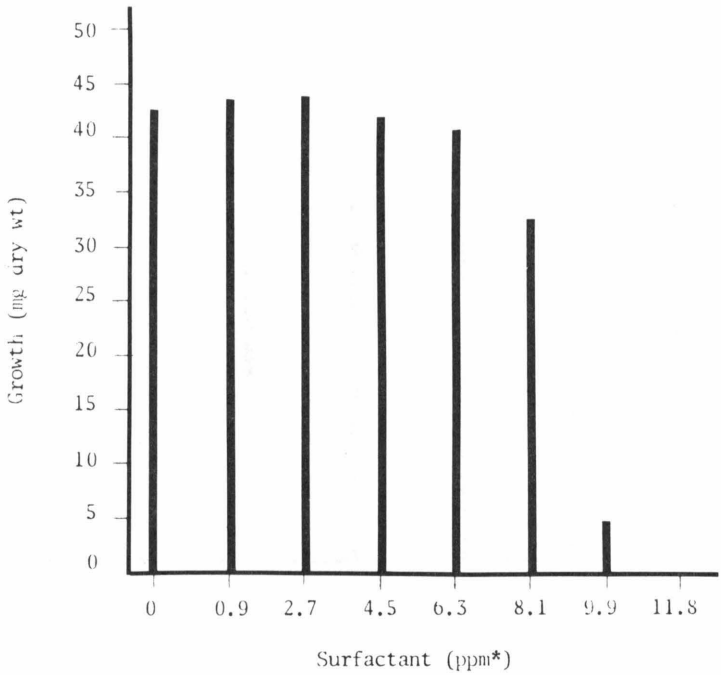


Figure 8

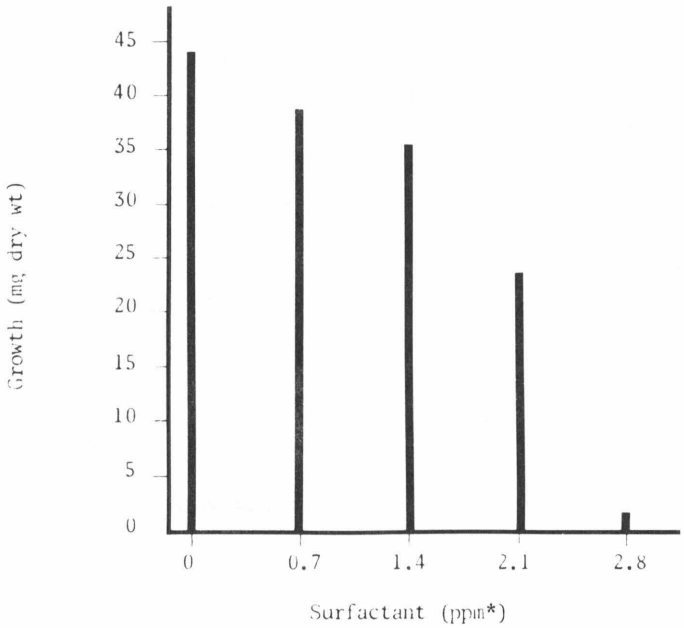
Growth of *Rhizidium* sp. on Modified $K_1$ Medium After 9 Days with Varying Concentations of the Surfactant, Linear Dodecylbenzenesulfonate




*See text for interpretation.

Figure 9

Growth of *Rhizidium* sp. on Modified $K_1$ Medium After 9 Days with Varying Concentrations of the Surfactant, Linear Tridecylbenzenesulfonate




*See text for interpretation.

Figure 10

A Comparison of the Effect of Various Compounds on the Growth of *Achlya* and *Rhizidium*

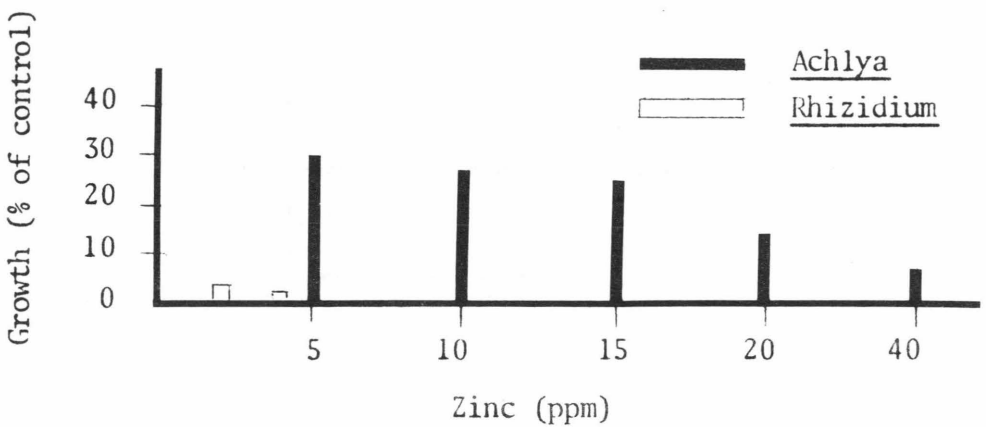


Figure 10
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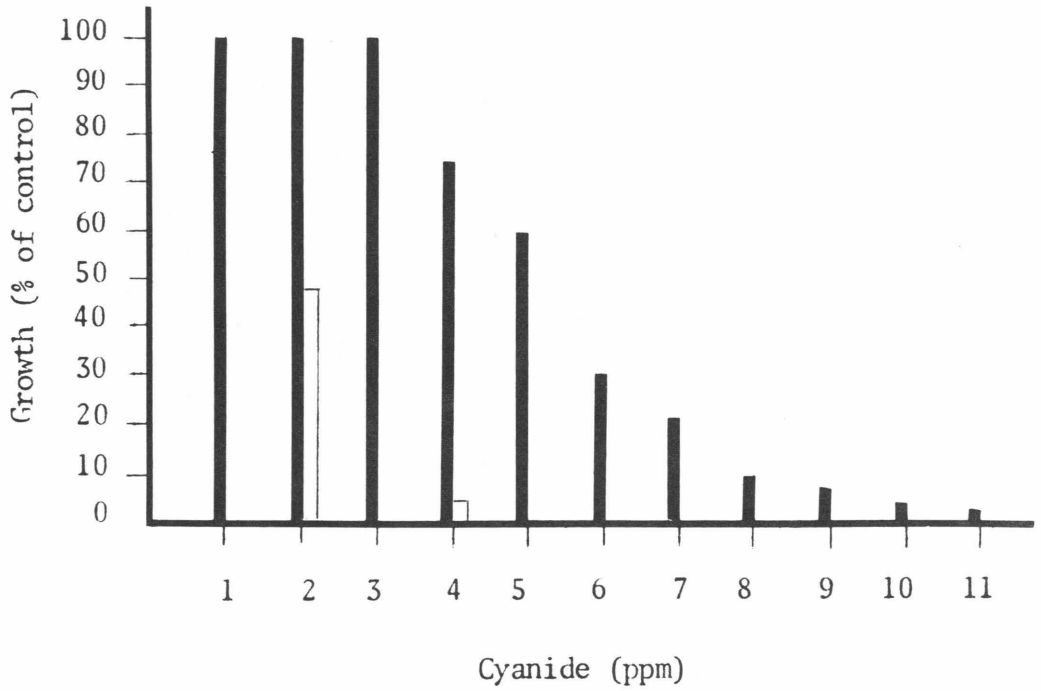

Figure 10
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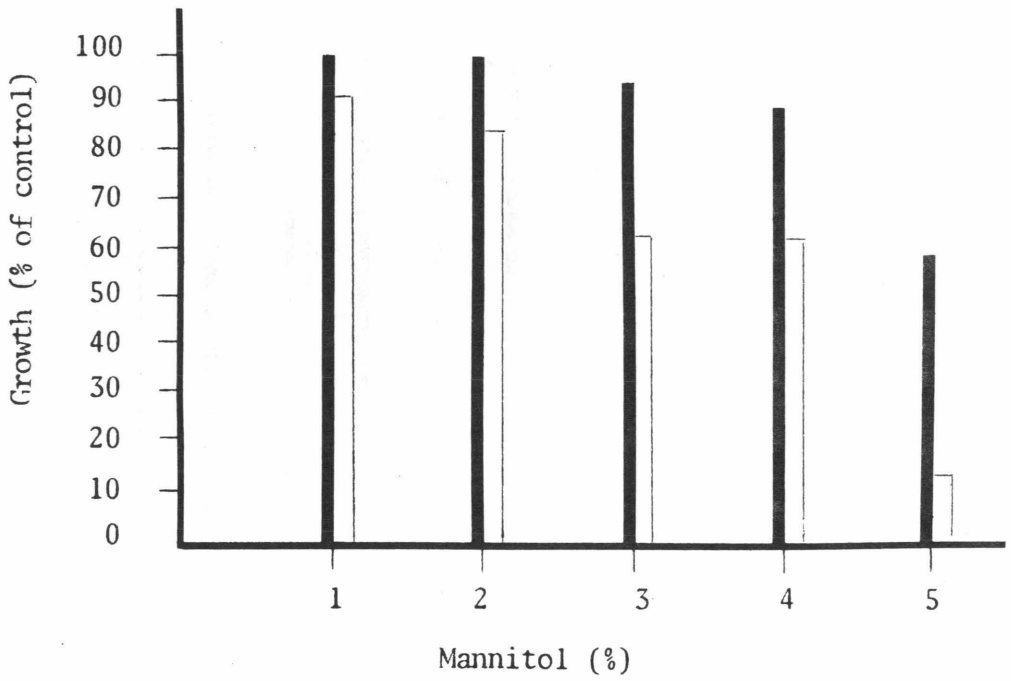

Figure 10
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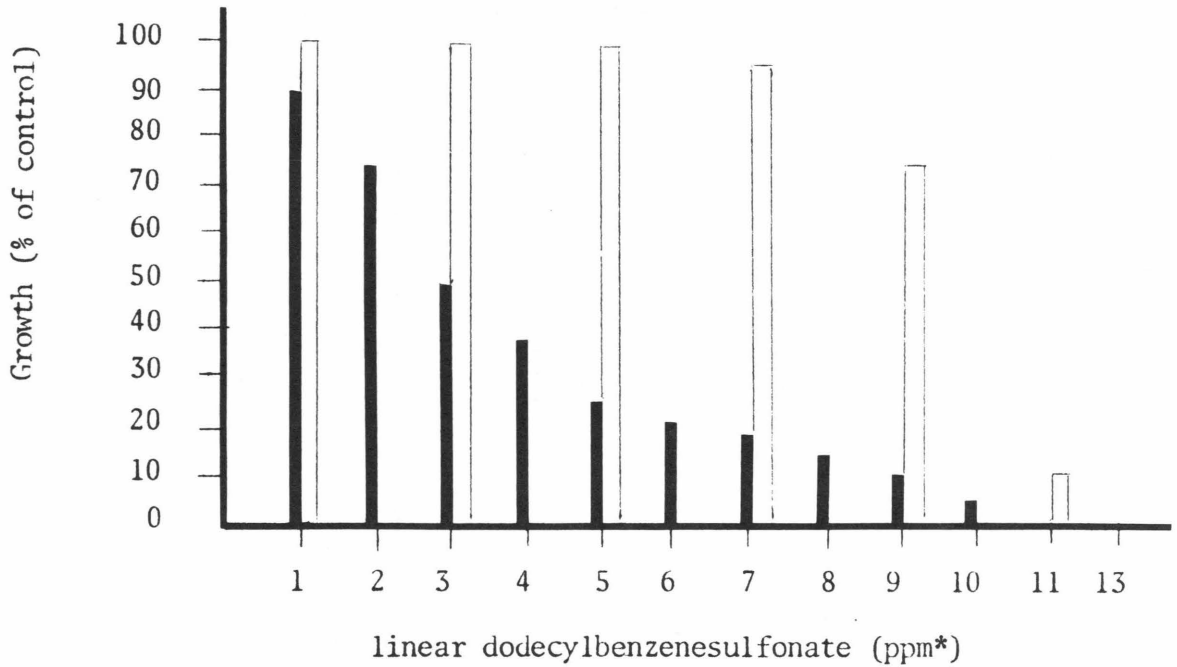


*See text for interpretation.

Figure 10
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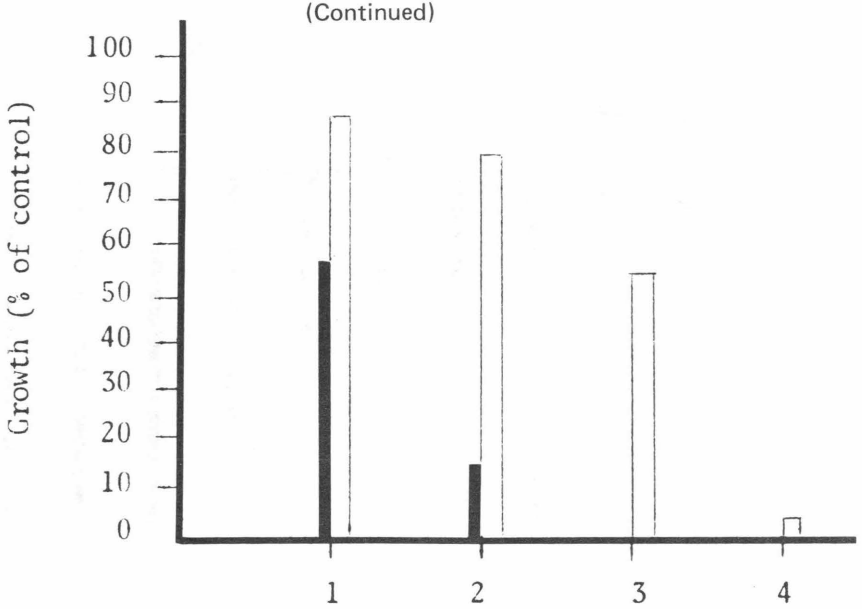


*See text for intrepretation.

Figure 11

The Effect of Various Compounds on the Formation of Oospores in *Achlya caroliniana*

| pH | | Zinc | |
|---|---|---|---|
| 4 | -- | 1 ppm | ++ |
| 5 | +++ | 2 ppm | -- |
| 6 | +++ | 3 ppm | -- |
| 7 | +++ | 4 ppm | -- |
| 8 | +++ | 5 ppm | -- |
| 9 | +++ | | |
| 10 | +++ | | |
| 11 | -- | | |

| Cyanide | | Mannitol | |
|---|---|---|---|
| 1 ppm | + | 1% | ++ |
| 2 ppm | + | 2% | +++ |
| 3 ppm | + | 3% | +++ |
| 4 ppm | + | 4% | + |
| 5 ppm | -- | 5% | -- |

| Linear docecyl-benezene sulfonate | | Linear tridecyl-benzenesulfonate | |
|---|---|---|---|
| 1 ppm | ++++ | 1 ppm | ++++ |
| 2 ppm | ++++ | 2 ppm | +++ |
| 3 ppm | ++++ | 3 ppm | ++ |
| 4 ppm | +++ | 4 ppm | + |
| 5 ppm | +++ | 5 ppm | -- |
| 6 ppm | ++ | | |
| 7 ppm | + | | |
| 8 ppm | + | | |
| 9 ppm | -- | | |
| 10 ppm | -- | | |

the sporangial stage and (2) they have only a limited food supply which must suffice until a new source of food is reached.

These experiments are still in progress, but some general trends are evident. As in the growth studies, the zoospores of *Achlya* sp. are more resistant to the test chemicals than those of the *Chytriomyces hyalinus*. For example, at 5 ppm of zinc, after an exposure of 6 hours, 25% of the original zoospore suspension of *Achlya* is viable, whereas in the *Chytriomyces* only 2 to 3% of the zoospores remain viable.

Comparing the growth responses reported here with those of other organisms is difficult for two reasons.

1. Toxicity studies of this type have not been conducted on any other aquatic fungi.

2. The experimental conditions under which toxicity tests are conducted in other groups of organisms are quite different from those employed in this study.

Most toxicity studies have utilized fish (Kemp et al., 1971). Generally the responses reported here are similar to those reported for fish. Exceptions to this would be the responses of the *Achlya* sp. to zinc and cyanide. In many fish, less than 1 ppm of cyanide is sufficient to cause death of 50% of the population within a few hours. Likewise, zinc at concentrations of approximately 10 ppm will be lethal to 50% of the population in four days. The few reports for organisms other than fish generally indicate greater tolerances to toxic chemicals (Kemp et al., 1971). Thus, 3.4 ppm of cyanide or 48 ppm of zinc were necessary to cause immobility in *Daphnia magna.* To reduce the BOD of "sewage organisms" values by 50% required 920 ppm of zinc. Williams and Mount (1965) also report tolerances of three fungi, *Leptomitus lacteus* and two Deuteromycetes, to levels of zinc which reduced the number of dominant species and species diversity in the autotrophic periphyton community. Responses to zinc and cyanide similar to those of *Achlya caroliniana* are also found in various terrestrial fungi (Allen and Strobel, 1966; Throneberry, 1973).

It is also important to remember that the experimental conditions under which the *Achlya* sp. and *Rhizidium* sp. were tested represent an optimal growth situation for the fungus. The fungus has an abundant and balanced nutrient supply, is provided with a stable optimal temperature, and is not competing with other organisms.

## III. Conclusions

1. In zinc, cyanide, and mannitol, *Achlya caroliniana* is tolerant of higher concentrations than the *Rhizidium* sp.

2. In the surfactants, the *Rhizidium* sp. is more tolerant than the *Achlya caroliniana*.

3. In *Achlya caroliniana* the sexual reproductive process was interrupted at much lower concentrations of toxic chemicals as compared with vegetative growth.

4. Although comparisons with other organisms are difficult, it appears that the responses of the two fungi studied are not radically different from insects and other microorganisms. Fish are more sensitive, especially in zinc and cyanide, as compared with the *Achlya caroliniana*.

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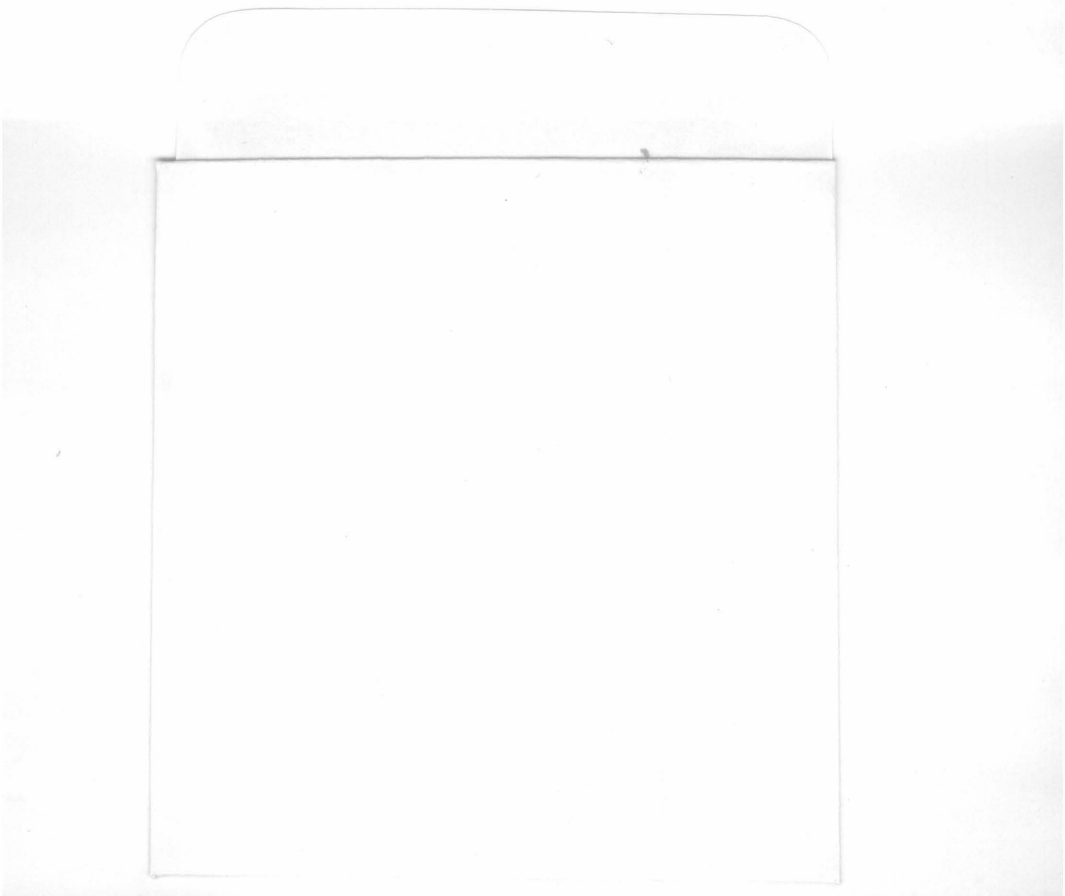

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