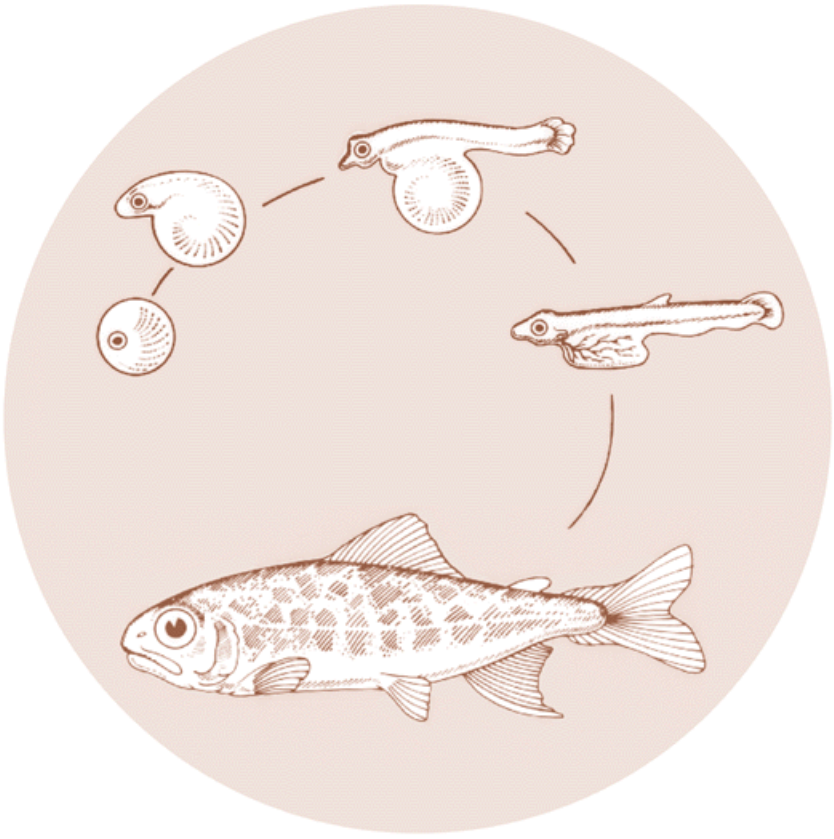
April 1994

SALMON SAPROLEGNIASIS



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SALMON SAPROLEGNIASIS

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Salmon Saprolegniasis

Proceedings of the symposium ***Saprolegnia*** in Salmon held at the 1992 annual meeting of the Mycological Society of America on August 8, 1992, in the Portland Convention Center, Portland, Oregon.

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PREFACE

The chapters in this compilation were presented orally at the symposium ***Saprolegnia*** in Salmon, on August 8, 1992, at the annual meeting of the Mycological Society of America. The symposium was organized by Howard C. Whisler and funded by the Bonneville Power Administration. This is the first international conference on salmon saprolegniasis in this century. The symposium provided a unique opportunity for an international group of scientists to share their ideas directly with others across discipline boundries. The symposium resulted in a state of the art picture of this disease of salmonid fishes.

The symposium was composed of four parts. The first characterized the problem of saprolegniasis from historical, taxonomic, and salmonid physiology perspectives. Pathogenicity and control measures were the subject of the second and third parts respectively. While all authors presented new findings or ideas pertinent to their geographical region, the fourth part of the conference emphasized current regional research. This symposium volume is organized in the order the talks were presented, but, with an additional part. The informal gatherings of the symposium participants included discussions of what research was needed in the field. It seems appropriate that this list of needed research be included in the volume. The usefulness of a comprehensive bibliography was also recognized. Since Gil Hughes had the bibliography in preparation it was decided that each of the authors would submit citations to him and the finished product included in the volume. One of the symposium participants chose not to submit a manuscript, however, the abstract of the talk has been included.

The manuscripts have been included in the volume with only minimal editing. The skill and style of each author's presentation have been retained in the hopes of giving the reader a slight sense of the spirit of the symposium.

I would like to thank Ms. Mary Piper, Ms. Rene Fester and Ms. Dan-Lu Zhang for their help in ensuring that the conference proceeded smoothly. Ms. M. Kay Suiter deserves special thanks for her efforts in bringing the manuscripts together into a single format suitable for submission as a single volume.

George J. Mueller, Editor

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SECTION I

BACKGROUND

chapter 1

SAPROLEGNIASIS, THEN AND NOW: A RETROSPECTIVE'

Gilbert C. Hughes2

> **"What is the Salmon Disease?** However absurd it may seem to start with a question to which an answer might readily be given, on the faith of a lamentable experience, by a host of individuals, yet it is always advisable to proceed in matters of this kind from some common ground as a starting point."
>
> *Mordecai Cubitt Cooke, 1880*

'This paper is dedicated to the memory of Prof. A.W. Ziegler, who introduced me to the Saprolegniaceae and to the idea that studying aquatic fungi could be fun as well as scientifically rewarding.

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ABSTRACT

Over 240 years ago Arderon (1748) first figured what was undoubtedly a saprolegniasis of Roach and more than 100 years ago the notorious "Salmon disease" of English and Scottish rivers (1880-82) attracted much attention from biologists, sportsmen, and fishermen alike. Investigations of "Salmon disease" left no doubt that species of Saprolegniaceae are commonly associated with salmonid fish in nature and might well be important pathogens of these commercially important animals. Despite the years involved and numerous studies of water molds by mycologists, it remains paradoxical that we still know so little of the true nature of fungal-salmonid associations. Even today, we still can't identify most of these "fish fungi" with real confidence! Why do we know so little after so long? This paper examines the past assumptions, presumptions, and ideas that have so hampered acquisition of a better understanding of salmonid-fungal associations over the past century. New ways of studying saprolegniasis which hold promise for real understanding of fungal-salmonid symbioses and ultimately for control of the pathogenic water molds will also be considered.

Key words: *Saprolegnia parasitica,* Salmon parasites, Oomycetes, Saprolegniaceae, saprolegniasis, taxonomy, malachite green.

INTRODUCTION

Symbiotic associations between fishes and saprolegnian fungi have been known for many years. I would suspect that saprolegnian fungi have been associated with living fishes as parasites or perthotrophs[3] so long as fishes and fungi have existed. In 1748 William Arderon reported what was clearly saprolegniasis[4] of roach, *Rutilus rutilus* L. in England (see Fig. 1). Ainsworth (1976) considered Arderon's letter the first record of a mycotic disease of any vertebrate animal. From 1748 to the present time, reports of saprolegnian fungi in or on various fishes have been frequent in both the mycological and fisheries literature. Historically the fungi involved in these associations were not necessarily considered either as primary parasites or as pathogens of their fish hosts. The nutritional opportunism of the Saprolegniaceae suggests that saprobes, perthotrophs, and parasites might all be found on various fish species. The particular fungus or fungi one might actually find on a particular fish would depend largely on environmental conditions and the general health of the fish at the time of infection or infestation by fungi.

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[3] Hawksworth et *al.* (1983) define a *perthotroph as* a saprobe using as its food the dead tissues of living hosts.

[4] Throughout this paper the term saprolegniasis refers to any disease of fishes or fish eggs caused by any fungus species in the family Saprolegniaceae (Oomycotina), a practical usage of the term suggested by Nolard-Tintiger (1974).

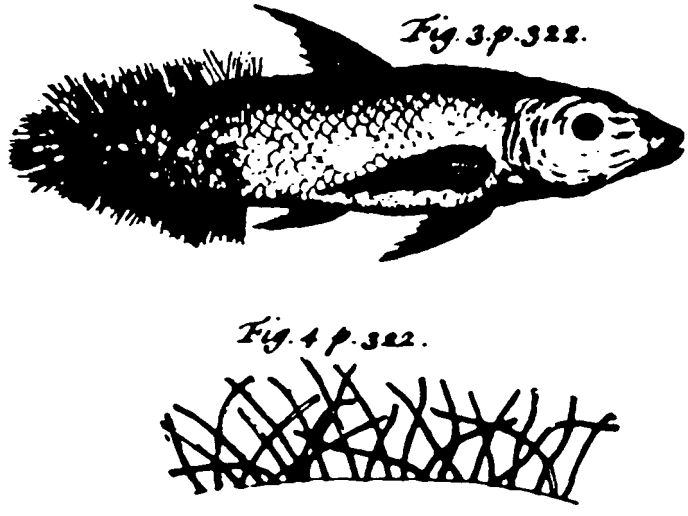


FIGURE 1. Saprolegniasis of a roach, *Rutilus rutilus* L. (W. Arderon, 1748).

Saprolegnian fungi have been known particularly as symbionts of cultured salmonids and salmonid game fishes in fresh waters for many years. It may seem that anadromous salmonids (see Table 1) are especially susceptible to saprolegniasis, but, so far as I am aware, this aspect of the associations has never been studied. It is paradoxical, however, that despite the considerable interest in saprolegniasis over many years, we still know so little about the biological aspects of these symbioses or the fungi involved in them. For example, how many species of the Saprolegniaceae are capable of growth on or in living fishes? Which of them are parasites, even pathogens producing characteristic disease symptoms? Which of the fungi are more likely to be nutritional opportunists with perthotrophic life styles? Why is it that despite decades of study it is still virtually impossible to identify confidently the species of the Saprolegniaceae that live on or in fishes? What events or attitudes or findings of mycologists and fisheries biologists in the past have been primary contributors to the general confusions that still prevail after nearly **250 years?**

In 1880, Cooke (see above) suggested that a better understanding of the problem of saprolegniasis of fishes is dependent on finding some common ground as a starting point for investigating it. Much more recently Peters (1991) suggested that "if we want to get someplace, then we must know where we are and where we want to go." Peters continued, "For similar reasons, science progresses more easily when present limits and future goals are known." I would submit that students of associations between fishes and fungi have, through the years, paid too little attention to the "present limits" in considering these symbioses and have failed to plan their "future goals" in any systematic way. Too often the mycologists have had little background in basic fisheries biology and

TABLE 1. A general classification of salmonid fishes based on Sanford, 1990. Individual species listed are those most frequently reported as hosts for members of the Saprolegniaceae.

SALMONID FISHES

Suborder Salmonoidei sensu Sanford, 1990

Family **Salmonidae (10** genera, 65-70 species)

Subfamily Salmoninae (6 genera)

Onchorhynchus - Pacific Salmon, Trout (1 1 - 12 species)
- *0. gorbuscha* (Walbaurn) - Pink Salmon
- 0. *keta* (Walbaum) - Chum Salmon
- *0. kisutch* (Walbaurn) - Coho Salmon
- *0. masou* (Brevoort) - Masou Salmon
- 0. nerka (Walbaum) - Sockeye & Kokanee Salmon
- *0. rhodurus* Jordan et McGregor - Amago Salmon
- *0. rshawyrscha* (Walbaum) - Chinook Salmon
- *0. clarki* (Richardson) - Cutthroat Trout
- *0. mykiss* Walbaum - Rainbow Trout, Steelhead Trout, Kamchatka Trout

Salmo - Atlantic Salmon, Trout (5-6 species)
- *S. salar* Linnaeus - Atlantic Salmon
- S. *rrutta* Linnaeus - Brown Trout

Salvelinus - Char, Trout (8-9 species)
- *S. alpinus* (Linnaeus) - Arctic Char
- *S. pluvius* (Hilgendorf) - Japanese Char
- *S. fonrinalis* (Mitchill) - Brook Trout
- *S. malma* (Walbaum) - Dolly Varden
- *S. namaycush* (Walbaum) - Lake Trout

Subfamily Thymallinae (1 genus, 4 species)

Thymallus - Graylings

Family **Coregonidae** - Ciscoes, Kiyis, Whitefish

(3 genera, 32 species)

approach these fish fungi primarily as mycological problems. Just as often, fisheries biologists are interested primarily in solving immediate practical problems. For example, in cases where high fish mortalities are attributable to a particular fungus, the main emphasis is on quickly eliminating the fungus causing the problem. There is often much less interest in understanding its more fundamental relationships to the fishes. In this paper I shall attempt to point out why I think the "present limits" of our knowledge place us in such an embarrassingly poor position to explain the biological relationships between fishes and saprolegnian fungi. I would also like to propose some courses of action that might be useful in finding the kind of "common ground" that Cooke (1880) had in mind. This could eventually lead to better definitions of our future goals and objectives. Ultimately it should all lead to a better understanding of the organisms involved in these symbioses and of the fish diseases that can result from them. **This is important; for, as Yogi Berra might say, if we *don't know where we are going, there's a very good chance that we'll end up somewhere else!***

PRESENT LIMITS

How does one explain the present limitations to our understanding of the *Saprolegnial* Salmonid pathosystem? What are the primary events over the past two and a half centuries that have led to the state of confusion that we find regarding this subject today? I would submit that there are three, perhaps four, major topics that warrant our attention in this regard:

1. First, the Report of the Royal Commission on the Salmon Disease in England and Scotland in the late 19th century (1877-l 879) did more than any other single publication over the past hundred years or so to determine attitudes and approaches to saprolegniasis and its treatment. The published report of this commission (Buckland, Walpole & Young, 1880) represents the first exhaustive study of saprolegniasis of salmonids anywhere in the world. Despite its shortcomings, it must be considered pivotal in our present attempts to understand or solve the problems these fungi create for fishes or biologists under natural and fish culture conditions.

2. I think the major part of the overall saprolegniasis problem, particularly as it applies to salmonid fishes, is a long-standing taxonomic problem related to the family Saprolegniaceae, a problem that rests almost certainly on both taxonomic practice and practitioners alike. These taxonomic problems have invariably led to confusions in the interpretations of the biological information we have about saprolegniasis and its control. In biology generally, an organism's name is the "hook" on which we tend to "hang" all that is known about the organism. A name can also be thought of as the "door" through which one gains access to all information available about the organism. If the name is not known or the organism is given the wrong name, then all the accumulated knowledge, all that we know about the organism, is denied to the worker. Even worse, the information

obtained can relate to a completely different organism than the one in hand. The ability to apply names correctly to both fishes and the fungi associated with them is crucial to assessing what is known of them as individual species or as partners in associations of hosts and perthotrophs, parasites, or pathogens. This is especially true in our attempts to understand that highly questionable species, *Saprolegnia parasitica* Coker and its role or roles in saprolegniasis.

3. Finally, I would submit that a lot of our difftculty at the present time can be placed squarely on that chemical compound known to every fisheries biologist as malachite green oxalate. There are, of course, other culprits associated with our present problems in dealing with saprolegniasis.

I shall explain below why I think these three deserve a special place in our considerations of the current problems and their solution.

Salmon Disease Commission, U.K. 1880

In 1879, the British Government of the day appointed a Royal Commission to make inquiries ". ..into the causes of the disease which has recently appeared among the salmon of the Tweed, Eden, and other rivers of England and Scotland, and into the steps which it may be expedient to take to prevent its propagation in the rivers in which it has already appeared, and its extension to other rivers" (Buckland, et *al.*, 1880). Appointed to the Commission were Messrs. Frank Buckland, Spencer Walpole, and Archibald Young. At the time, Buckland and Walpole were Inspectors of Fisheries for England and Wales, and Young was Commissioner of Scotch Salmon Fisheries. Over a one year period the Commission conducted public hearings in about a dozen localities where the disease had been prevalent from 1877 to 1879. They also solicited additional opinions, comments, and recommendations from various experts. This group included M.C. Cooke, C.L. Jackson, Henry Lee, Prof. George Rolleston, David Milne Home, and Erasmus Wilson. Cooke was the only professional mycologist in the group. Jackson and Lee were both aquarium naturalists; Rolleston was Anatomy Professor at Oxford; Milne Home was Chairman of the River Tweed Commissioners; and Dr. Wilson is not further identified in the Report (BickIand, et *al.* 1880). Reports or letters from all of these experts plus a paper on salmon disease by A.B. Stirling (Anatomy Department, University of Edinburgh) read before the Royal Society of Endinburgh in 1879 (see also, Part 1 Stirling, 1880a) are included as Appendices to the final Commission Report. The Commission received reports and depositions on the disease from as far field as Siberia (Stirling, 1880b), New Brunswick in eastern Canada (Milne Home, 1880), British Columbia in western Canada, and the San Francisco area of California (Bickland, et *al.*, 1880).

This large amount of information from such diverse sources comprises the most thorough treatment of saprolegniasis that we have seen. Unfortunately its very

thoroughness was also the report's greatest weakness. It is difficult in reading the Report to get a really clear idea of the disease or the role of the fungi in it. Virtually every possible explanation or solution is proposed at one point or another in this Report. I am reminded of one of John Ramsbottom's observations. Ramsbottom (1941) made the statement in quite a different context, but, with only a slight addition, it is nonetheless most appropriate here: "It is difficult to rid oneself of the impression that although many of the foremost scientists of the period interested themselves in *[salmon disease]* fungi, most of them merely added to the confusion, made worse by the fact that no matter how unlikely their suggestions they were readily taken up in the attempt to understand this anomalous group of organisms" (Ramsbottom, 1941, p. 33 1, my addition in [ ] and italics).

So far as descriptions of the fishes at various stages of infection are concerned the Commission Report is a valuable contribution. Rarely will an interested reader find more detailed discussion of all aspects of the behavior of the infected host organism. Likewise, details concerning the macroscopic features of the fungi involved are extensive. There is less useful information about the extent to which the fungi might penetrate into host tissues or damage internal organs. The Report itself warned that, "The symptoms affecting live fish do not seem to have been observed so carefully as the appearances on the dead fish..." The Commissioners, largely on A.B. Stirling's (1878, 1880a) advice, accepted that this "salmon disease" fungus *was Saprolegnia ferax. Saprolegnia ferax* (Gruithuisen) Thuret, 1850, is one of the most common and well-known species in the genus *Saprolegnia.* It can be isolated virtually anywhere one might want to look for water molds and almost certainly would have been present in the rivers where the salmon disease was found. There is also no doubt that it can and probably does live as a perthotroph on fishes, frogs, or other aquatic animals. That S. *ferax* could be the causative agent of virulent saprolegniasis or salmon disease is more questionable. Nonetheless this species was cast in this role by the Salmon Commission in 1880. The Report even included a fairly accurate illustration of the species that is reproduced here as Figure 2. This fungus is so common and so distinctive that I suspect most students of these fungi would be able to identify it with little more information than is given in the illustration, despite its inaccuracies. Identification of the various structural components of the fungus shown in Figure 3 will be assisted by reference to Figure 4, and the legend accompanying it. There is little chance that any knowledgeable mycologist would *confuse Saprolegnia ferax* with Coker's *Saprolegnia parasitica.*

With regard to prevention or control of saprolegniasis, the Commission Report had less to offer. They concluded that the disease cannot be eradicated since the fungus is so ubiquitous and spreads so rapidly. Furthermore, the commissioners expressed the view that salmon disease could only be controlled in a limited sense by removing dead fish from the rivers or by improving the riverine environments. The means suggested for accomplishing these improvements included reducing obstructions to fish passage,

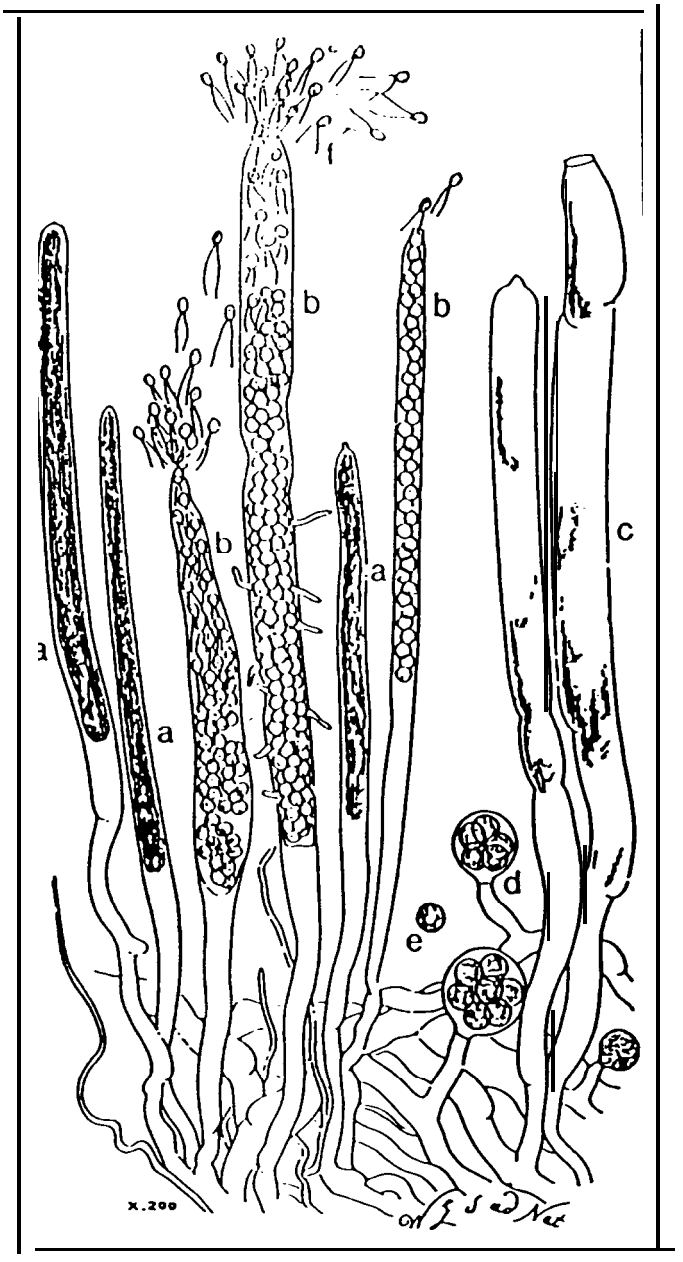


FIGURE *2. Saprolegnia* ferax (Gruity.) Thuret, 1850, as illustrated in *the Report on the Disease Which Has Recently Prevailed Among the Salmon of the Tweed, Eden, and Other Rivers in England and Scotland* (Buckland, *et al.*, 1880). [This figure originally appeared in *the Gardeners' Chronicle.]* a) young zoosporangia; b) mature zoosporangia, releasing zoospores; c) empty zoosporangia; d) oogonium, with oospores (note absence of antheridia); e) mature oospore; f) biflagellate primary zoospores.

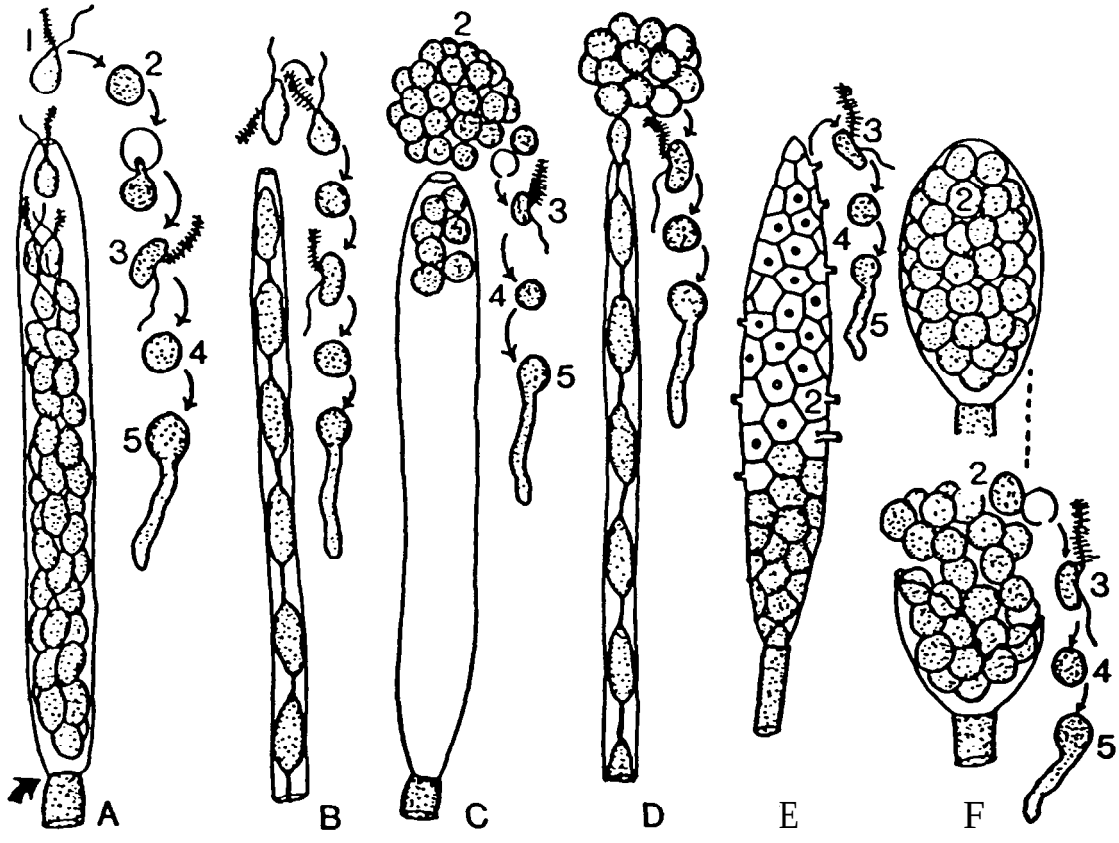
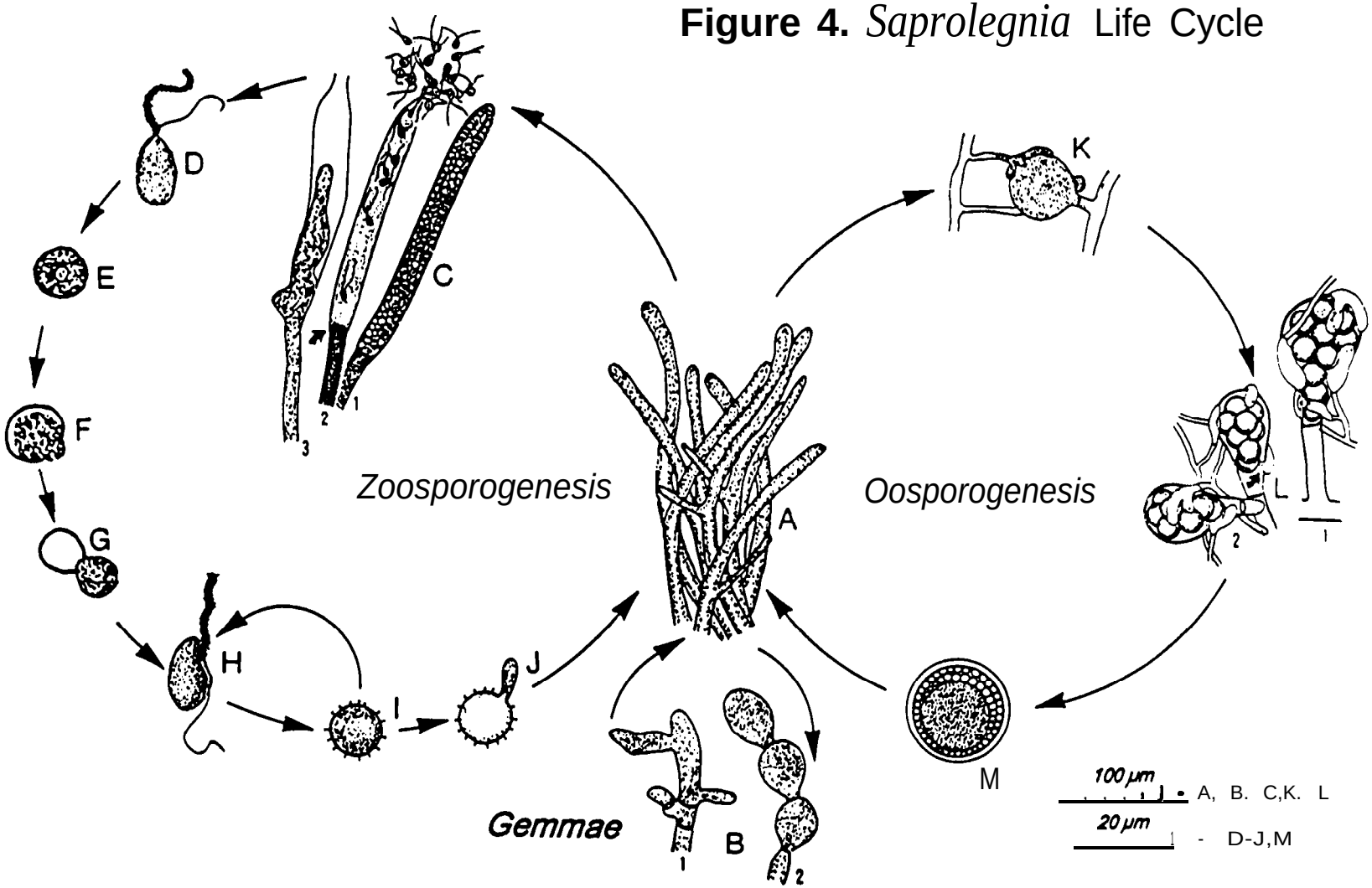


FIGURE 3. Sporangial types and zoospore escape patterns in six genera of the Saprolegniaceae. A) *Saprolegnia,* characterized by clavate zoosporangia and diplanetic zoospores; primary zoospores (Al) swim away from the sporangium on release, B) ***Leptolegnia*** has narrow, filamentous zoosporangia but a *Saprolegnia-like* diplanetism, *C) Achlya,* zoosporangial shape resembles *Saprolegnia* but primary zoospores encyst (C2) immediately on exiting the sporangial tip, D) *Aphanomyces* has narrow filamentous sporangia *(as* in *Leptolegnia)* but a zoospore escape pattern like *Achilya,* E) ***Dictyuchus*** possesses clavate sporangia, *as* in *Saprolegnia,* but primary zoospores encyst within the zoosporangium and do not escape, instead forming a net of cells within the sporangium and germinating to release secondary zoospores (E3) singly through papillae in *the* sporangial walls, *F) Thraustotheca,* zoosporangia disintegrate to release encysted primary zoospores (F2) which then germinate to release typical reniform secondary zoospores. Al) primary zoospore, A2) primary zoospore cyst, A3) secondary zoospore, A4) secondary zoospore cyst, A5) germinating secondary cyst. A, C, D, & E, are the genera most commonly implicated in saprolegniasis of fishes (after Wolf & Wolf, 1947).

Figure 4. *Saprolegnia* Life Cycle



eliminating pollution, or taking steps to ensure that the rivers are not overstocked with fishes. A number of people who made presentation to the Commission felt that overstocking and overcrowding of fishes was the primary "cause" of the disease.

The Salmon Commission Report concluded that "every **cause without exception, which has been assigned as the true origin of the salmon disease in infected rivers, is to be found in rivers where no disease exists, or has ever been known to exist."** This statement reveals not only the level of frustration that characterized the general conclusions of the Salmon Disease Commission but also the degree of frustration that was to characterize many of the studies that followed over the next century.

The lasting ideas to come from the work of the Salmon Commission were not so much from the Commission Report itself, as from the notions of the various experts who advised the Commission and other scientists who contributed views to the discussion, stimulated by the work of the Commission, Many of these individuals not only submitted reports to the Commission but also published papers on salmon disease in the popular scientific press of the day (e.g., The *Gardeners' Chronicle and Gardening Illustrated) as* well as in various traditional scientific journals.

The most notable of the ideas concerning saprolegniasis which appear to have resulted from the 1877- 1882 epizootic in Great Britain are the following:

1. The fungus is a secondary parasite, only able to survive where a primary bacterial infection has conditioned the fish tissues for its growth. This view was espoused by Rutherford in 188 1, during the time of Huxley's work on the disease. In this scenario, bacteria kill fish tissues on which the fungi establish themselves and then

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FIGURE 4. Typical *Saprolegnia* Life Cycle. A) Vegetative mycelium (2n), **B)** Gemrnae; **B-l,** irregularly shaped gemmae, B-2, catenulate chain of gemmae; C) Zoosporangia. **C-l,** nearly mature, zoospores visable internally; C-2, Mature zoosporangium, releasing primary zoospores; C-3, Empty zoosporangium with new zoosporangium forming by internal proliferation; **D)** Primary, pyriform zoospore; E) Encysted primary zoospore; **F,G)** Germinating primary cyst; H) Secondary, reniform zoospore, note insertion of flagella; I) encysted secondary zoospore, note hooked projections on surface of cyst; J) Germinating secondary cyst. Note: Secondary zoospores may go through several cycles of encystment to produce the hyphal element seen here; **K)** Immature oogonium with immature diclinous antheridia; **L),** Mature oogonia, with mature oospores (zygotes). Note fertilization tube at arrow, L-2; M) Mature, subcentric oospore.

begin to grow on epidermal tissues (i.e., the fungi are perthotrophs). Rutherford's (1881) views were supported by the work of Hume Patterson (1903) whose studies appeared to demonstrate conclusively that the salmon disease was caused by a motile, gram-negative bacillus, which Hume Patterson named *Bacillus Salmonis Pestis.* For most of the early years of the twentieth century it was generally accepted by many workers that the salmon disease was caused by this bacterium and that the fungi were at worst opportunistic, secondary parasites.

2. The disease is caused by a single species of *Saprolegnia,* generally thought to *be Saprolegnia ferax* (Gruith.) Thuret. This conclusion is largely attributable to the papers of Sterling (1878, 1880a,b, 1881) in Scotland. Huxley (1882a) was prepared to recognize the causative fungus as one of the "S. *ferux* group" of Pringsheim and De Bary but did not further identify it. Subsequently *Saprolegnia parasitica* Coker fell heir to this rather unfortunate, and poorly documented generalization.

3. Saprolegnia species are unable to act as primary pathogens of fishes. This view was challenged by both Stirling (1880a) and Huxley (1882a,b). They were both convinced that the fungus could and did act as a primary pathogen.

Stirling (1880a) concluded his paper by noting that, "I feel convinced that the so-called salmon disease is the *fungus* itself, and that no structural disturbance in the fish is necessary to cause fungus attack... The origin and cure or prevention of the plague must be sought for in the life history of the plant." T.H. Huxley, the eminent zoologist and evolutionist, had succeeded Frank Buckland as Inspector of Fisheries for England and Wales in 188 1 following Buckland's untimely death. Huxley devoted considerable time and effort to studying saprolegniasis personally and in 1882 published two significant papers on the subject (Huxley, 1882ab). From his various observations and experiments with cultures Huxley (1882a) concluded that ". ..*the Saprolegnia* attacks the healthy living salmon exactly in the same way as it attacks the dead insect, and that it is the sole cause of the disease, whatever circumstances may, in a secondary manner, assist its operations." We don't know just how studies of saprolegniasis might have developed if the views of Huxley and Stirling had been accepted in the 1880's. As it turned out they were both expressing views that most others, then and later, seemed determined to disregard.

All three of the ideas mentioned above became well-entrenched in the "dogma" of saprolegniasis since the 1877-l 880 epizootic in the U.K. although for many years now we have had good reason to question each of them.

Taxonomy-Saprolegniaceae

Taxonomy, in the sense that the term refers to the principles and philosophic basis where by our classifications are constructed, has long been a problem in the Saprolegniaceae.

This family, prominent in the Oomycotina and consisting of some 12-l 5 genera and 125-150 species (Table 2), was first studied seriously in the 19th century. Mycologists have continued to show interest in these fungi ever since. These days the Oomycotina and thus the Saprolegniaceae, are considered better placed in either the Kingdom Protoctista or the Kingdom Chromista and, regardless of which, are not considered very closely related to the real fungi. Today they are perhaps more correctly treated *as pseudofungi.* There was once a time, in the nineteenth century and early years of the twentieth century, when it seems that almost every mycologist worthy of the name devoted some time to studying the Saprolegniaceae. For example, Brefeld, Comu, de Bary, Fischer, Hartog, Hildebrand, Humphrey, Leitgeb, Maurizio, Pieters, Pringsheim, Schroeter, von Minden, and Zopf all made significant contributions to our knowledge of the family. This degree of interest is certainly not the case as we move into the final years of the twentieth century.

The Saprolegniaceae (water molds) are a family of filamentous, coenocytic organisms (Figs. 2, 3) living in fresh water habitats or in wet soils. Nutritionally they live as saprobes, perthotrophs, or parasites or (in some cases) as all three, depending on circumstances. A number of species that one might nominally consider as saprobes can live as perthotrophs given suitable dead cells or tissues on the bodies of living, prospective hosts. There is little question that under the right conditions some of these perthotrophs invade the living tissues of the host and thus become true parasites. The jump from parasite to pathogen is a likely next step since few of them would qualify as very good biotrophic parasites. At the risk of being a bit technical, the taxonomically important features of the developmental cycle are described below and variously illustrated in Figures 2, 3, and 4. An understanding of this much of the morphology and life cycle of the Saprolegniaceae is essential to understanding just how the taxonomic system for the family can be considered part of our present problems.

Asexual reproduction is accomplished by means of unicellular, biflagellate zoospores (planots) (Fig. 3A-1, 4D, 4H) produced in (usually) terminal sporangia separated from the hyphal filaments by basal septa (arrow, Fig. 3A, Fig. 4C-2). Zoospores (Fig. 3A) are usually both diplanetic and dimorphic, i.e., there are two different swimming stages involving two different kinds of zoospores: pyriform primary zoospores (Fig. 3A-1, Fig. 4D) and reniform secondary zoospores (Fig. 3A-3, Fig. 4H). Each type of zoospore not only has a different shape but also a different point of flagellar insertion (cfr. Fig. 3A-1, Fig. 3A-3, Fig. 4D, Fig. 4H). Furthermore, either or both of the two swimming stages may be suppressed to varying degrees in some genera in the family (Figs. 3C, 3E, 3F). Both zoospore types encyst, in some cases immediately, in others after swimming for varying periods of time. Primary zoospore cysts (Fig. 3A-2, Fig. 4E) germinate (Fig. 4F, G) to release secondary zoospores (Fig. 3A-3, Fig. 4H). Secondary zoospore cysts may bear hooked hairs on their surfaces. Pickering, Willoughby and McGrory (1979) found that parasitic *Saprolegnia* spp. from fishes have secondary cysts with groups or bundles of long, hooked hairs, whereas saprobic *Saprolegnia* isolates produced secondary cysts with short, single, hooked hairs on their surfaces. Few species

TABLE 2. A general classification of the Saprolegniaceae *sensu* Dick, 1973. Individual species listed are those most frequently implicated as causing saprolegniasis of salmonid fishes.

SAPROLEGNIAN FUNGI

Order Saprolegniales sensu Dick, 1973

Family **Saprolegniaceae - (14** genera, 126-146 species)

- **Saprolegnia* - (20-22 species)
  - *S. ferax* (Gruith.) Thuret 1850
  - *S. monoica* Pringsheim 1858
  - *S. torulosa* de Bary 188 1
  - *S. diclina* Humphrey 1893
  - *S. parasitica* Coker 1923
  - *S. delica* Coker 1923
  - *S. australis* Elliott 1968
  - *S. shikotsuensis* Hatai, Egusa & Awakura 1977
- **Aphanomyces* - (22-25 species)
  - *A. laevis* de Bary 1860
- **Achlya* - (46-56 species)
  - *A. prolifera* Nees von Esenbeck 1823
  - *A. polyandra* Hildebrand 1867
  - *A. racemosa* Hildebrand 1867
  - *A. flagellata* Coker 1923
- *Brevilegnia* - (12-13 species)
- * *Dictyuchus* - (5-6 species)
  - *D. monosporus* Leitgeb 1860
- **Leptolegnia* - (5-6 species)
- *Scolicolegnia* - (4 species)
- *Geo legnia* - (2-3 species)
- *Aplanopsis* - (2 species)
- *Calyptralegnia* - (2 species)
- *Plectospira* - (2 species)
- *Phythiopsis* - (l-2 species)
- * *Thraustotheca* - (l-2 species)
- *Sommerstorffia* - (1 species)

*See Figure 3 for zoosporangial features.

in the family have been examined by transmission electron microscopy to ascertain the nature of the surface of their secondary zoospore cysts. Secondary zoospores may go through several cycles of encystment-release-encystment before they finally germinate to produce a hyphal element (Fig. 3A-5, Fig. 4J) terminated by a zoosporangium.

Gemmae or chlamydospores (asexual aplanospores) formed through modification of hyphal segments (Fig. 4B) may be produced abundantly by saprolegnian fungi. They are especially common in isolates obtained from fishes, although most members of the family produce them when growth conditions begin to stale and oxygen availability is reduced. Gemmae may be irregular in shape (Fig. 4B-1) or often occur in catenulate chains (Fig. 4B-2). They germinate to produce hyphae, or a hypha bearing a terminal zoosporangium or even may become transformed into zoosporangia or oogonia in some parthenogenetic species.

Sexual reproduction is by means of gametangial contact (Fig. 4K) which leads to fusion of haploid oospheres (eggs) produced in lateral (Fig. 4K, 4L-1) or terminal (Fig. 4L-2) female gametangia (oogonia) with sperm nuclei carried to the oogonia by antheridial branches (Fig. 4K, 4L) and to the oospheres by fertilization tubes (arrow, Fig. 4L-2). Both gametangial types (oogonia and antheridia) are separated from hyphal filaments by septa (Fig. 4K). The zygotes (oospores, Fig. 4M) may undergo a resting period but in time they usually germinate to produce hyphal filaments that soon form terminal zoosporangia and zoospores. A generalized life cycle (Fig. *4)* for *Saprolegnia* shows the sequential relationships of these structures in the developmental cycle of the family.

Taxonomically, genera in the Saprolegniaceae are delimited by the morphological and developmental features of the zoosporangia and the manner of zoospore formation and escape (or failure to escape) from the zoosporangia. This persists as a primary character for separating genera even though Salvin (1941, 1942) demonstrated over fifty years ago that environmental conditions, particularly temperature, can affect the morphology and development of zoosporangia and the pattern of zoospore release and activity. In one experiment, he showed that cultures of a single *Brevilegnia* isolate exhibited **the asexual** and **sexual** characteristics of five different species of *Brevilegnia* when the growth conditions were varied. He *also* showed that a number of *Brevilegnia* isolates essentially "*became Thrausrorheca* isolates" when grown at different temperatures. Salvin's findings, needless to say, cast considerable doubt on a taxonomy that makes such variable and changeable characters so important in delimiting genera. It will be especially suspect if other genera in the family, *e.g. Suprolegniu, Achlya,* or *Aphanomyces,* are shown to have similar morphological responses to temperature variations or other environmental factors as those Salvin found for *Brevilegnia.* In the most recent monograph of *Suprolegnia,* Seymour (1970) based his taxonomic revisions primarily on cultures of species grown on a standard medium under standard conditions.

His use of 18°C as the standard growth temperature for all cultures of all isolates pretty well excluded any findings for *Saprolegnia* of the sort Salvin noted for *Brevilegnia.*

It doesn't get much better when one examines the criteria for delimiting species. These are based largely on the morphology and development of the oogonia and their contents. Such features as oogonial size, presence and types of ornamentations on oogonial walls, presence of pits (thin spots) in oogonial walls, number and size of oospores in oogonia, or visual differences in stored lipid-like globules are all important in this regard. The origins of antheridia are also of some importance here; whether they arise from the same hypha as the oogonia (androgynous, Fig. 4L-2) or from distant hyphae (diclinous, Fig. 4K) is an important character. Various of these taxonomically important features and some of their relationships to one another are illustrated in Figures 2, 3, and 4. Figure 3 shows the general zoosporangial morphology and stages in zoospore release for six characteristic genera in the Saprolegniaceae. The six genera shown in Fig. 3 also serve to illustrate the complete range of zoospore release patterns found in the family (see legend, Fig. 3). Included in the group are the four genera most commonly found on fishes: *Saprolegnia, Achlya, Aphanomyces,* and *Dictyuchus* (Cfr. Table 2). Figure 4 presents a typical *Saprolegnia* life cycle. Scale bars in Figure 4 indicate the relative sizes of the various structures shown. Scale bars are not included in Figure 3 nor are the various zoosporangia shown there drawn to the same scale.

Taxonomy—*Saprolegnia parasitica* Coker

Unquestionably the greatest single taxonomic confusion in studying, explaining, or understanding saprolegniasis is what I'll call "the *Saprolegnia parasitica* problem." With it not only comes the difficulty in recognizing this particular species but also the problems it exemplifies in attempting to identity any species of the Saprolegniaceae on fishes.

Saprolegnia parasitica was one of four new species of *Saprolegnia* described by W. C. Coker in his 1923 monograph of the Saprolegniaceae (p. 57-59), a volume providing the first major taxonomic treatment of the family in North America since Humphrey's assessment in 1893. The fungal isolates were received by Coker from the fish hatchery at Wytheville, Virginia and from the aquarium of the U.S. Fish Commission in Washington, D. C. Coker noted that "in each case the fungus was growing as a parasite on fish." The full description of the species given by Coker (p. 57-58) is as follows:

> **"Growth rather delicate on insects and other usual media, moderately dense, not long, rarely reaching 1 cm on a mushroom**

> grub[5]. **Gemmae abundant, size and shape very variable; often in chains, mostly terminating hyphae, but sometimes intercalary. Sporangia variable but usually bent and irregular, at times up to 0.7 mm long, very often proliferating from the side below as in Achlya; when growing through others sometimes discharging spores through the side wall of the old sporangium; spores 9-11.5μ thick, in our form swimming in two stages as usual in the genus (Not swimming in Huxley's form). Sexual reproduction not observed so far in our form and very rarely observed by others (Huxley)." (Coker, 1923, p. 57-58)**

Several points from the original description should be emphasized:

(1) The fungus was originally found on fishes but the host organisms were not identified further. Not even common names were given.

(2) Zoosporangial renewal was often more typical for the genus *Achlya* than for *Suprolegnia,* and zoospore release was occasionally like that seen in *Dictyuchus* (Fig. 2E).

(3) Sexual reproduction was not observed. No oogonia or associated structures were seen (emphasis mine).

On the basis of these three points, one might wonder why Coker put this obviously variable fungus in *Suprolegnia* at all. The observation of two actively swimming zoospore stages in his cultures probably accounts for this, despite the fact that diplanetism had not been observed by earlier workers studying very similar isolates in England, cfr. Huxley (1882a,b). However, it is even more curious that Coker chose to describe his fish fungus as a **new** species. He clearly had not seen any of the sexual structures on which species delimitations were (and are) traditionally based. Furthermore, to describe a new species of any organism primarily on the basis of structures or features *if does not have* is quite simply bad taxonomy. It was not acceptable taxonomic practice then nor is it acceptable practice now. However, Coker did make the absence of sexual reproductive structures one of the primary distinguishing characteristics of his new species. He was, himself, therefore. the principal source of the notion that any *Saprolegnia* growing on a living fish. and not producing sexual reproductive structures, is, by defmition, *Saprolegnia parasirica.* This idea is still with us today (see Hatai & Willoughby, 1988) and is a prominent cause of much of the disarray in assessing the identities of all Saprolegniaceae that may be associated with fishes as perthotrophs, parasites or pathogens. This is particularly true in light of the fact that a number of the saprolegnian species that grow on fishes as parasites or perthotrophs do not normally produce sexual structures in abundance on the hosts.

[5]Coker often grew his cultures on houseflies, other insects, and various larvae, or grubs as well as a variety of seeds.

The next important development in the S. *parasitica* story came in 1932 when Bessie B. Kanouse published her studies of S. *parasitica* isolates from various fishes and fish eggs [especially whitefish *(Coregonus)* and rainbow trout *(Oncorhynchus)]* from localities in Michigan. She was able, through manipulations of various culture conditions, to obtain in her cultures oogonia, antheridia, and all the other sexual cycle structures so important to species delimitations in the Saprolegniaceae. She emended the description of *S. parasitica* Coker incorporating these results. so that features of the sexual cycle could be added to the formal description of the species:

> "Sexual reproduction by means of oogonia and antheridia. **Oogonia terminal or intercalary; pyriform,** clavate, subspherical to **spherical in shape: 65- 135 x 60-75 μ or 65-95 μ in diameter. Walls very thin. colorless, smooth, unpitted. Oospores variable in number. 3-25(35) usually filling the oogonium; subcentric[6]; coloured rich golden-brown... Antheridial branches diclinous or androgynous in origin, the latter long, slender. winding filaments. Antheridia small clavate to subcylndric; l to 5 attached to each oogonium." (Kanouse. 1932. p. 447)**

Unfortunately. we must view Kanouse's work with a degree of caution. Unlike Coker (1923), she did identify the host fishes that were the source of her *Saprolegnia* isolates and her taxonomy carefully followed standard taxonomic practice. However, we have no real way of knowing that the fungus Kanouse studied in Michigan was the same fungus species that Coker described in North Carolina from a Virginia fish. The problem is not made easier by the fact that Coker left no type material for S. *parasitica,* nor did he leave any reference slides of the fungus. Coker did deposit a type culture in the Centraalbureau voor Schimmelcultures, Baam, but this culture has long since been lost. I have found no mention of it in the literature aside from Tiffney's (1939a,b) references to it in his papers. This general lack of type or reference material is a continuing problem that frustrates all workers who are interested in the taxonomy of the Saprolegniaceae, no matter what genus or species they may be studying (Seymour, 1970).

Kanouse (1932) considered her S. *parasitica* identical with the species described by Coker (1923) and that would normally be sufficient. Unfortunately, Coker's S. *parasitica* is based about as much on features it doesn't have as on those it does have. The issue is complicated further in this case by the fact that in Coker's last fling at the taxonomy of the Saprolegniaceae (Coker & Matthews, 1937) there was no nomenclatural recognition of Kanouse's 1932 emendation of *S. parasitica* nor was there any mention of Kanouse's

[6] A characteristic of oospores in which the lipid-like storage globules in the cytoplasm consists of one layer of globules on one side and two layers on the other side. (See fig. 4M.)

paper. Coker & Matthews still cited the species as "*Saprolegnia parasitica* Coker, Saproleg. 57. 1923." They did. however. include a description of the oogonia, antheridia, and related structures that very closely follows Kanouse's description (see above) but there is no direct indication that her work was its source. Coker & Matthews' only mention of Kanouse's paper is found under "Illustrationsl" where reference is made to "Mycologia 24: pl. 12,13." Examination of these plates in Volume 24 of Mycologia reveals that they illustrate Kanouse's 1932 study of *S. parasitica.*

Kanouse's paper not only presented the first description of the sexual structures of a fungus she considered conspecific with *S. parasitica* Coker. She also noted (1932, p. 43 1): 1) "The species of water mold that is found commonly on fish and fish eggs in fish hatcheries and in the fresh water lakes and streams belongs *to* the genus *Suprolegnia"; 2)* "The fungus is widespread not only in America but also in Europe"; 3) "Coker proposed the name *Saprolegnia parasitica* Coker for the fungus associated with fish and fish eggs." These comments leave little doubt that Kanouse considered *S. parasitica* the only water mold living on fishes and fishes eggs.

Soon after, Tiffney (1939a), in an often cited paper, reported on his studies of Coker's *Saprolegniaparasitica* and its ability to parasitize a variety of different fishes and amphibians from the New England region of the United States. Tiffney's study is especially interesting since he used a sub-culture of Coker's *type* of *S. parusitica* in his infection experiments. Five of the 16 fish species included in the tests, three of them salmonids *(Oncorhynchus mykiss Salmo trutta,* and *Salmo salar),* proved to have virtually no resistance to the fungus. All individuals in this group, uninjured and injured fishes alike, were 100% susceptible to infection and all of them died as a result. Ten of the remaining fish species were less susceptible to the fungus although there was a high mortality (>75%) in fishes that were injured before exposure to the fungus. Only the eel. *Anguilla rostruta* (Lesueur). was immune to the fungus throughout the infection experiments.

Three points made by Tiffney seem especially pertinent to this discussion. First, he did not consider that *S. parasitica* was the only species of the Saprolegniaceae parasitizing fishes and other aquatic animals; second, he found that injury of the hosts greatly lowered their resistance to the fungus; and. third, he found the symptoms of the disease identical in all fish species he studied. Tiffney's discussion of the behavior of the infected fishes is also important. He noted that soon after infection the individual fish, "evidently irritated. rubbed the infected region by vigorously swimming against some object". a behavior noted by a number of respondents to the Salmon Commission in Great Britain (Buckland, *et al.,* 1880). Tiffhey (1939a) found that, "During the second to fourth day after inoculation . . . the trunk muscles seemed to become more and more paralyzed until the fish was incapable of moving its body and finally of moving even its fins, and in this state floated to the surface of the water or lay on the bottom of the tank." This is in sharp contrast to the kind of behavior seen in infected fishes in Great Britain, where most

reports considered the fungus infection a superficial one, with little or no damage to fish tissues below the epidermis and no mention of any involvement of the musculature or motor activities. It is also interesting that Tiffney, on autopsy of floating fishes, found their internal organs essentially normal and their hearts beating. This is not the typical etiology of the "salmon disease" seen in England and Scotland (Buckland, *et al.* 1880; Stirling 1878, 1880a,b; Huxley. 1882a.b).

In addition to his work with *Saprolegnia parasitica* Tiffney (1939b) investigated the identities of other fungal species living as parasites of fishes. He recorded 128 isolates of "... pathogenic members of the Saprolegniaceae . . . from diseased aquatic animals in southern New England." His isolates were all in the genera *Saprolegnia, Achlya*, and *Dictyuchus. Suprolegnia* was the most important genus of the trio. *S. parasitica sensu* Coker was the most common parasitic species in his collections, comprising 122 of 128 total fungi. Tiffney was well aware of the problems posed by his inability to identify 95% of his isolates precisely. on the basis of oogonial characters. He concluded his discussion of them by suggesting that "... at present it seems best to consider *Saprolegnia parasitica* a species of convenience. and it should be treated as such until further studies have demonstrated its exact nature."

The modem concept of *Saprolegnia parasitica* dates from the 1930's (Neish & Hughes, 1980). after which time the name should never have been applied in Coker's sense again. Unfortunately. it didn't work out that way and over the years numerous authors have continued to apply the name *S. parasitica* in the original sense. This practice did not go completely unnoticed. In 1960, O'Bier wrote, "The use of asexual characters and the parasitic habit as the diagnostic features for this species have made the taxon a catch-all for all non-fruiting isolates of *Saprolegnia* even though reproductive structures have been described for the species." Two years later, Scott & O'Bier (1962) reiterated this and added. "It is difficult to understand the persistence of such an error in the literature and the perpetuation of it by well-informed workers." Scott & O'Bier recommended a more conservative approach to the interpretation of the species limits of *S. parasitica*, commenting that, ". . .Coker's (1923) original description of *Suprolegnia parasitica* is erroneous and untenable..." O'Bier (1960) and Scott & O'Bier (1962) both proposed that a desirable temporary solution would be to place in *S. parasitica* only those isolates that exhibited sexuality *and* possessed the diagnostic features of the taxon.

The practice of automatically identifying sterile Saprolegnia isolates from fishes as *S. parasitica* continued through the next few years. This ultimately led Neish (1976) to propose rejection of *Saprolegnia parasitica* Coker *as* a *nomen ambiguum* in accordance with Article 69 of the International Code of Botanical Nomenclature. This proposal was accompanied by the observation that all fruiting isolates of *S. parasitica* could be correctly assigned *to Saprolegnia diclina* Humphrey without any change in Humphrey's (1893) original concept of that species. Thus *was* born the *Saprolegnia diclina-Saprolegnia parasitica* complex. This species complex (including *S. kauffmaniana, S.*

shikotsuensis, and *S. australis.* in addition to *S. diclina* and S. *parasitica)* was almost immediately adopted by Willoughby (1978) in his work on saprolegniasis in England. He considered the taxonomic implications of the complex in some detail and accepted Neish's (1976) view that *S. parasitica* Coker is a synonym of *Saprolegnia diclina* Humphrey. Most notably. Willoughby (1978) divided *S. diclina* into three subspecific groups on the basis of the length/breadth ratios of the oogonia: *S diclina Type I.* occurring as a parasite of salmonid fishes; *S. diclina* Type 2. occurring as a parasite of perch, and *S. diclina* Type 3, a saprobic form that does not occur as a parasite. In their papers up to 1990 the British researchers of saprolegniasis adopted this scheme fairly consistently (cfr. Pickering. Willoughby & McGrory, 1979; Beakes, 1983; Willoughby, Pickering & Johnson. 1984; Wood & Willoughby, 1986; Wood. Willoughby & Beakes. 1988: Cross & Willoughby, 1989). The first indication of any change came in 1988, when Hatai & Willoughby referred the fungus back to *Suprolegnia parasitica* Coker. They justified this disposition as follows: "Isolates were made from six separate lesions and in sub-culture the fungi were all identified as *Suprolegnia,* but no oogonia were obtained. The isolates were confirmed as being pathogenic to fish and *were therefore regarded as S. parusiticu* Coker (1923)." (italics mine). With this one short statement the whole taxonomic question was put on its way back to square one, or perhaps I should say back to 1923. From about 1990 Willoughby and his co-workers in the U.K. began to abandon the idea of the *Suprolgenia diclina-Saprolegnia parasitica* complex in their taxonomic treatments and move back to Coker's views of *Saprolegnia* species parasitic on salmonids. For example. Hatai. Willoughby & Beakes (1990) observed that "In the U.K.. *S. parasitica* has usually been the sole representative of the genus isolated as a pathogen from salmonid fish..." There was no mention that *S. diclina* in any guise had ever been found on a fish in the U.K. Old ideas not only die hard, they seem to "change hard" as well. By 1992. \Villoughby & Roberts could assert that, "When the salmonid fish Atlantic salmon.. *Salmo salar* L.. brown trout. *Salmo trutta* L.. and char. *Salvelinus alpinus* L., are attacked by aseptate fungi of the family Saprolegniaceae, the infection *is almost invariably caused* by a single taxon. *Suprolegnia parasitica* Coker." (italics mine). Following this astounding statement are some fifteen lines of descriptive text referring only to asexual characteristics of the taxon. Nothing at all is said of the sexual cycle. which suggests that the authors have really gone back to Coker (1923). They have not considered either *S. parusiticu* as emended by Kanouse (1932) or as described by Seymour (1970) as possible alternatives. It should be remembered, however, that Coker's original description of the taxon is just as "erroneous and untenable" (to echo Scott & O'Bier. 1962) in 1993 as it was seventy years ago. The addition of more detailed descriptions of the development. morphology, or behavior of the zoospores and the zoospore cysts does not alter this fact. I submit that. despite the extremely interesting and unique features of the *Suprolegnia* that occurs "almost invariably" on salmonids in the U.K., there is no way that anyone. anywhere, can know for certain that it is conspecific with the taxon that Coker (1923) called *Suprolegnia parasitica.* Considering S. *parasitica* as a component of an *S. diclinu-S. parasitica* complex may not have been the ideal solution to the vexing taxonomic problems of the taxon but it was a progressive move.

Going back to Coker's (1923) concepts can only be considered as retrogressive in the extreme!

T.W. Johnson and R.L. Seymour are presently completing a new monograph of the Saprolegniaceae which will be published in the near future (T.W. Johnson. pers. comm.). Johnson has kindly provided me with advance copy of the textual portions of this new monograph dealing with *S. purasiticu* for mention in this discussion. In the new monograph one will find all of the fish parasites in *Suprolegniu* reduced to synonymy with *Saprolegnia diclina* Humphrey. This includes not only *Saprolegniu purusitica* Coker but also *Isoachlya purusitica* (Coker) Nagai. *Saprolegnia parasitica* Coker *emend* Kanouse. and *Saprolegnia shikotwensis* Hatai, Egusa et Awakura. A new circumscription of *S. diclina.* based on Seymour's (1970) monograph of *Suprolegnia.* plus additional data from the recent literature and new study- of specimens. has convinced Johnson & Seymour that *S. diclina* Humphrey. 1893. and *S. parasitica sensu* Kanouse, 1932. can no longer be retained as separate species. As regards *S. purasitica* Coker. Johnson & Seymour conclude their discussion by noting that. "In the final analysis. it appears to us best to identify any non-oogonial isolates of *Saprolegniu* from fish simply as *Suprolegnia* sp. and *all accounts* ***of*** *nonsexual S. parasitica* referred to in the literature subsequent to Kanouse's redefinition of the taxon should be so treated." (italics mine).

The various contradictor? and conflicting interpretations of species concepts and the taxonomy of saprolegnian fungi found on fishes as perthotrophs. parasites. or pathogens. pose major obstacles to a complete understanding of saprolegniasis and its biological implications for fisheries biology. As is painfully obvious from the discussion above, taxonomic issues have been at the heart of the confusions afflicting studies of saprolegniasis over many years and they remain major contributors to the confusions that exist today.

Malachite Green

Malachite green is probably the most widely applied chemical control compound ever used to reduce the incidence of saprolegniasis in fish hatcheries and fish farms around the world. It and its present status are thoroughly discussed elsewhere in this volume (Alderman, Chap. 6. this volume). I shall only mention malachite green here in terms of its contribution to the present problems we face with a burgeoning incidence of saprolegniasis in fish culture and enhancement facilities. How can I take the position that malachite green is part of the problem? Almost everyone knows that malachite green was the solution to saprolegniasis in fish culture for years. Yet. from the mycologist's point of view, it should be considered as contributing to the current problems. Why? It's simple. Malachite green worked so well. with such efftciency, and did such an effective job of controlling serious incidence of saprolegniasis in hatcheries, spawning facilities, holding ponds. etc.. that all of us who should have been working away trying to find a

biologically based solution to the problems of saprolegniasis were lulled into thinking that the problem was solved. Only a small group of dedicated scientists continued to work at and study the mycological and fisheries problems associated with saprolegniasis. In the days of malachite green. most fisheries biologists thought of saprolegniasis only rarely, if they thought of it at all. Too many years passed. years when we should have been studying these fungi more rather than studying them less. Mycologists even neglected saprolegniasis in natural populations of fishes of all kinds and so missed the great opportunities they had to study these unique symbioses in nature and without the pressures associated with epizootics in hatcheries and fish farms. Unfortunately, for too long mycologists and fisheries biologists alike tended to consider malachite green as the final solution to saprolegniasis rather than a temporary solution that was actually giving us the time to really understand the disease. So we were all caught flat-footed when use of malachite green was prohibited in North America. It is not prohibited everywhere yet but I suspect that day will come eventually. And in the meantime our problems with saprolegniasis become more and more widespread and complicated.

FUTURE GOALS

The foregoing discussion of saprolegniasis is rather patchy. and is not in any sense comprehensive. but it does indicate major aspects of the inconsistencies that still impede the study of fungi and their interactions with fishes. A number of generalizations that might serve to guide future studies of these associations would be appropriate, even though details of procedures can not be included here.

First. there is every reason to suspect that more species of the Saprolegniaceae than *Suprolegnia parasiticu* [whatever it is!] can live and probably do live as parasites and pathogens of fishes. It also seems significant that students of saprolegniasis through the years since Munch (1929) coined the term *perthophyte (= perthotroph,* in modern usage) have failed to consider in any detail a perthotrophic nutrition for fungi associated with fishes. A look at the Table 1. (p. 13- 16) in Neish & Hughes (1980) suggests that of the forty-two species in the Saprolegniaceae reported to 1980 as parasites of fishes, many of them could have been living on the various fishes as perthotrophs rather than as parasites or pathogens. Furthermore. I suspect most water molds are potentially good perthotrophs, a fact that would greatly complicate the recognition of true parasites or pathogens. If several perthotrophs were sharing a host fish with a pathogen it could be difficult to determine just which fungus was the pathogen and which were the perthotrophs. I suspect it was the number of perthotrophs on fishes that led so many early workers to accept so readily the idea that saprolegniasis was a secondary disease and that fungi were not likely to be found on fishes as primary parasites or pathogens.

Second. I think the old notions concerning the roles of injury or bacterial preconditioning of fishes in fungal infection, and other such involvement of “third party”

organisms or events in saprolegniasis should be extensively reexamined. The evidence at hand suggests that perhaps too much as been made of such explanations. tending to diminish the involvement of fungi in fish health. This would seem especially true in fish hatcheries and fish culture environments.

Third. we have to get serious about resolving existing taxonomic problems in the Saprolegniaceae. particularly in the group of species most likely involved in saprolegniasis. A new taxonomy for the entire family, would be mycologically desirable and a good move. but a new taxonomy for the fish parasites and pathogens is essential **if** solutions to the problems created by saprolegniasis will ever be properly understood or solved. As I have said above. if we can't put correct names on the fungi in question, it will be impossible to access the considerable information that has accumulated regarding these fungi. It will also be impossible to interpret correctly any new findings relative to their activities as pathogens

From a broad perspective. a new look at the taxonomic problems in the Saprolegniaceae using both new approaches and new attitudes. is of necessity, a first priority job. Without a workable and consistent means of identifying and naming the fungi we are never going to understand them or what we think we already know of their biology and lifestyle in relation to fishes. This is especially true of fungi associated **with** salmonid fishes.

Already work is under way along some of the most promising avenues. In the 1993 meetings of the Mycological Society of America, Blackuell & Powell (1993) reported on their studies of phenetic taxonomic relationships within the Saprolegniaceae as determined by applying various methods of multivariate analysis. Multivariate approaches to taxonomic problems in fungi generally are long overdue. They have much to offer, however and in this case promise a much better understanding of generic relationships within the Saprolegniaceae.

We also badly need to convince the researchers one might call the "biotechno-mycologists" that their methodologies of DNA or RNA sequencing; DNA or RNA replications. amplifications. and hybridizations; riboprinting; RFLP; etc.. provide a valuable array of techniques that can **be** focused profitably on taxonomic muddles such as we find with these fish parasites. The morphological and cytological features and the culture characteristics of the Saprolegniaceae uould seem to make them especially amenable to applications of many of these molecular techniques. It often seems to me that these workers are reluctant to target groups of fungi whose alpha taxonomy is poorly known or contentious. for their biochemical or molecular taxonomic studies. However. it is in just such groups as the Saprolegniaceae that they will find not only the challenges but also the promise of exciting and useful results at the end of the road. It is even possible that these new methodologies applied in combination with older taxonomic methodologies (including cytochemistry and electron microscopy) will provide us with

the best possible change to sort out the taxonomic disarray that is so typical of some of these water mold genera.

As a starting step in a search for new taxonomies, I would propose that we concentrate on two avenues of study. First, detailed investigations, using all available methodologies. should be directed to the fungi in the world's culture collections that were isolated from fishes, most especially those isolates identified as *"Suprolegnia parasitica"*. Since international culture collections are a primary source for cultures studied in experimental laboratories around the world, it would be very useful and enlightening to find out just how much taxonomic variability exists in this array of *Saprolegnia* isolates. How many different species of Saprolegniaceae are hiding in the world's culture collections under the name *Saprolegnia parasitica* or *Saprolegnia diclina* Type I? How many of these "impostors" are being used in studies of saprolegniasis and providing false information to further obscure the problems at hand? There may well be no misidentifications in this group and each of these cultures may be the organism its label says it is. Second. fungi isolated directly from infected salmonids should be subjected to the same sorts of critical scrutiny, using the same methodologies. In time this two-pronged attack will give us a much better idea of just what we are dealing with in terms of the diversity of fungal species living as perthotrophs and parasites on fishes. This approach will also make it possible to distinguish the organisms that are really involved in the fish disease syndrome from the organisms that have been mistakenly implicated. It should also, eventually, make possible development of a simple, easy to apply method for identifying the fungi as they are found on the host fishes, a method that works without an elaborate research laboratory and several months time between the fish and a name for the fungus. I must agree with Stirling's (1880a) proposal that the "salmon disease is the fungus itself' and that the origin and cure or prevention of saprolegniasis must be sought for in studies of the fungus or fungi causing the disease.

There is. however, no question but that attempts to solve the present taxonomic problems in the Saprolegniaceae will offer little in terms of immediate, suitable or effective control of the infections that afflict fish hatcheries and fish culture facilities around the world to an increasingly disturbing extent. I realize that one might just as easily advocate a search for some environmentally safe, super-chemical that will provide control (i.e.. eradication) of the fungi appearing routinely on fishes in hatchery ponds, or on eggs in the hatcheries. If it would eliminate the problems the fungi cause for the fish and fish culturists alike. then it probably shouldn't matter too much whether or not the relationships between the fungi and the fishes are completely understood. In this scenario, it is the results that would count. i.e.. the fungus infections would be eliminated or significantly reduced. Studies of the biological interactions between the fishes and the fungi would again be relegated to the inquisitiveness of a few dedicated biologists. This is certainly an optional strategy for dealing with saprolegniasis but as a biologist and a mycologist I can not accept it as a desirable one. Real control of saprolegniasis will come from carefully conducted studies of the fishes and the fungi to better understand their

interactions with one another. It will not come from a “quick-fix” solution like a broad spectrum anti-fungal chemical compound that deals with the problem without the necessity to understand it. After all. we had something a lot like that with malachite green and look where we are left now that it can no longer be used.

It has been 113 years. but I suspect Mordecai Cubitt Cooke would still be urging us today to make sure of the common ground as the starting point for our continuing studies of saprolegniasis.

ACKNOWLEDGMENTS

I should like to express my appreciation to the Bonneville Power Authority for their generous support of the symposium and for making it possible for this group of mycologists and fisheries biologists to gather in Portland to consider the present status of saprolegniasis around the world. I would especially like to thank Howard Whisler for his kind invitation to come to Portland and share my ideas about fish fungi with the members of the symposium. Howard, George Mueller. and the graduate students from Seattle deserve our thanks for their hospitality and for providing such a fine venue for our discussions. My thanks also go to Terry Johnson for so kindly providing me with unpublished material from his forthcoming monography of the Saprolegniaceae. His taxonomic views and conclusions are important in the context of this paper. I reserve my final appreciation for George Mueller, as editor of this volume. George, with the patience of some latter-day Job, dealt with all of the delays in the completion of this manuscript with a quiet calm that I must confess usually left me somewhere between “disconcerted” and “inspired”. Without his kindness and encouragement this paper never would have seen the light of day.

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chapter 2

FEATURES WHICH CHARACTERIZE *SAPROLEGNIA* ISOLATES FROM SALMONID FISH LESIONS - A REVIEW

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Sarah E. Wood
Andrew W. Burr[2]

ABSTRACT

The features which characterize *Suprolegnia* isolates associated with fungal infections of live salmonid fish are reviewed. Specific identification of many fish-lesion isolates using traditional morphological criteria is difficult and has resulted in considerable confusion as to the true identity of fish pathogenic species of *Suprolegnia*. However. fish-lesion isolates from wide geographical origins are all characterized by their distinctive cyst coat morphology. germtube growth. and esterase isoqme patterns. A CLUSTAN analysis based on over 130 characters was used to group 90 isolates of *Suprolegnia*. approximately a third of which were of fish origin. The salmonid fish-lesion isolates all grouped together into a single major cluster, suggesting that they form a coherent separate taxon (Saprolegnia parasitica).

The status of the fish pathogen in lakes and rivers is discussed and the annual occurrence of Saprolegniaceae in an important salmon river. the Tyne and its tributaries. is described. Although *S. parasitica* is relatively scarce in the river. it was always isolated from lesions taken from live fish.

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Comparative investigations into the behaviour. surface structure and chemistry of zoospores and cysts of *S. parasitica* and *S. diclina* have been undertaken to try and elucidate the basis of fish pathogenecity. Although zoospores and cysts of both species react similarly to the lectin Con A, significant differences were observed in the binding patterns of the lectin WGA, which also failed to induce encystment of *S. parasitica* zoospores. A series of monoclonal antibodies (MAb's) were raised to a fish lesion isolate, vvhich all reacted with the immunodominant surface matrix material. Spores of both S. *parasitica* and *S. diclina* reacted strongly with these MAbs. but those of S. *ferax less so.*

The significance of all these observations and the implications for future research are discussed.

Key words: *Saprolegnia parasitica.* Oomycetes. fish, salmon. trout, parasites, systematics. pathogenicity. lectins. monoclonal antibodies.

INTRODUCTION

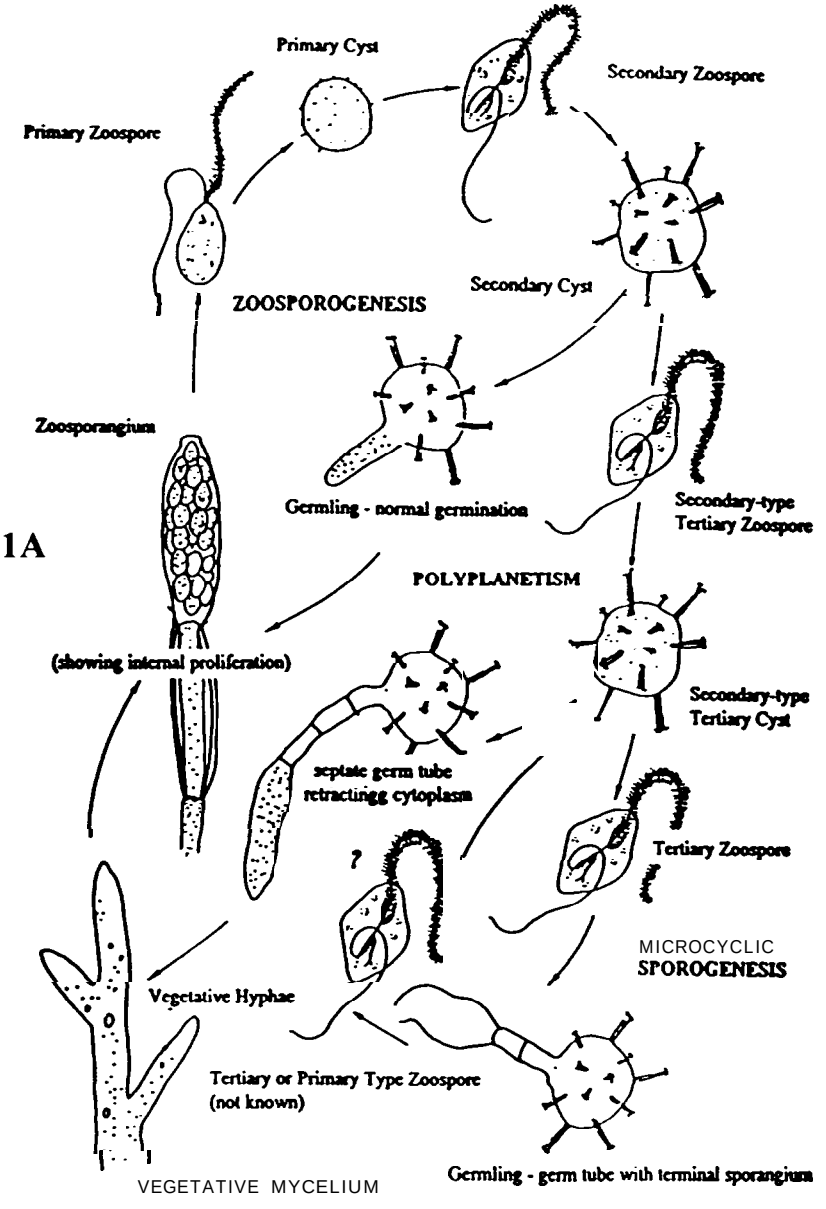
It was the association of saprolegnian water moulds with the so-called "salmon disease" which devastated salmon stocks in British rivers in the late 1870's which first focussed attention on these organisms as major pathogens of wild salmonids. However, the precise aetiology of the salmon disease. more recently known as Ulcerative Dermal Necrosis (UDN) has, and continues to be. the subject of much speculation (see Neish and Hughes, 1980). Whilst not the causal agents of the initial UDN skin lesions, it is the subsequent fungal infection of the skin ulcers which leads to the ultimate death of the fish. *Saprolegnia* species and their close water mould relatives invade epidermal tissues of a wide range of freshwater fish and readily infest moribund eggs (for reviews see Wilson, 1976; Neish and Hughes, 1980; Pickering and Willoughby, 1982). In salmonids the physiological state of the fish is always crucial in determining whether successful fungal infection will occur and it is always sexually mature, stressed or damaged fish which are most susceptible to *Saprolegnia* (Richards and Pickering, 1978; Pickering and Christie, 1980; Cross and Willoughby, 1989). Such infections are usually categorized under the heading of "saprolegniasis" and it is generally considered that these moulds behave as "opportunistic" necrotrophs. In addition, mycotic infections arising from *Saprolegnia are* reasonably easily controlled by the application of the chemotherapeutant malachite green either on its own or in combination with other biocides (Olah and Farkas, 1977; Alderman, 1982). As a result, the importance of saprolegniasis as a major fish disease has tended to be minimalized (Neish and Hughes, 1980). Unfortunately, recent concerns about the safety of malachite has meant that it is no longer available for use as a fisheries chemotherapeutant in many parts of the world. Without a cheap and environmentally acceptable chemical control, problems in hatcheries arising from infections with *Saprolegnia* are likely to increase in the future.

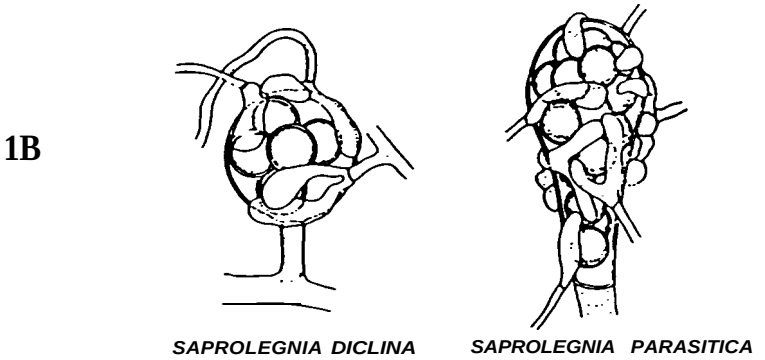
Saprolegnia belongs to the oomycetous fungi. which have their phylogenetic roots with the chromophyte algae (which includes the diatoms. chrysophytes and brown seaweeds) rather than with the main evolutionary line of chitin containing fungi (Beakes. 1989). This should be born in mind when suitable fungicides against these organisms are being sought. since many fungicides which are effective against higher fungi are ineffective against oomyeetes and *vice versa* The vegetative mycelium of the fungus is usually made up of a series of thick (< 30 μm diameter). branched. coenocytic filaments which form a whitish cotton wool or felt-like mat on the surface of the fish. However *Saprolegnia* has quite a complex life cycle (a summary of the asexual stages is given in Fig. IA). The pyriform primary zoospores which are released from the zoosporangia are weak swimmers and function simply to disperse the spores from the immediate vicinity of the sporangium and parent colony. These primary zoospores settle and round up to produce *ca.* 10 μm diameter. thin-walled. cysts (syn. "encysted zoospores" or "cystospores"). This primary cyst acts as a miniature sporangium and releases a reniform secondary zoospore (Fig. IA). which is the main motile stage. The secondary zoospore can maintain motility for many hours. even days. until it also encysts to produce a seconday cyst (Fig. 1 A). Under ordinary light microscope optics these secondary cysts are indistinguishable from their primary counterparts. although electron-microscop> reveals subtle differences (Fig. 2) in cyst coat architecture (Beakes. 1983). Although the main infective spore is not known it has often been assumed to be the secondary zoospore. If enecysted secondary zoospores fail to locate a suitable host/substrate they can release another secondary-type zoospore which in turn settles to form another "secondary type" of cyst (Fig. IA). These spores should be more correctly referred to as "tertiary" types and this phenomenon of repeated cycles of encystment and emergence is known as polyplanetism This behaviour is clearly an important adaptive feature enabling *Saprolegnia* propagules to have several attempts at locating and attaching to suitable food substrates. Therefore. in nature. fish are likely to be challenged by a variety of secondary and tertiary zoospore and cyst types including germlings. Throughout the rest of this account when zoospores and cysts are referred to they will always be the morphologically identical secondary/tertiary types.

Over the past decade we have tried to address several major questions related to these fish parasites. Do the fungal isolates taken from lesions on live fish represent a distinct "pathogenic" tason? What is the normal status of potential fish pathogenic water moulds in a typical salmonid-rich river system? If only a single species/isolate is generally able to infect live (stressed) fish. what is the basis of its pathogenicity? Each of these questions will now be addressed in turn.

DO FISH LESION ISOLATES REPRESENT A DISTINCT TAXON?

Studies on the pathogenicity to fish of saprolegnian fungi has undoubtedly been complicated by considerable confusion surrounding the systematics of these organisms. It is





1B

SAPROLEGNIA DICLINA *SAPROLEGNIA PARASITICA*

often unclear from the literature precisely which species of water mould is involved in the mycotic infections described. Unfortunately. many *Saprolegnia* isolates taken from fish are asexual and cannot be identified to the species level since specific identification in *Saprolegnia* has been largely based upon the morphology of the sexual organs, i.e. the oogonia, antheridia and oospores (Seymour. 1970). In his major taxonomic treatment of the genus. Coker (1923) erected the species *Saprolegnia parasitica* Coker to describe asexual isolates taken either from fish or moribund fish eggs. The oogonial characteristics of this species were however later described by Kanouse (1932) but not based on the same material as that described by Coker. The diagnostic features of these isolates were their thin-walled. unpitted. clavate oogonia (16-28 urn), abundant diclinous antheridia (i.e. arising from separate hyphae). and small (18-22 μm) oospores (Fig. 1 B). However in more recent taxonomic re-examinations of this group. it was suggested. on the basis of their oogonial characteristics, that this taxon was probably synonymous with the common saprophytic species *S. diclina* Humphrey (Neish. 1976; Willoughby 1978). Even so isolates from salmonid fish fell into their own distinct subgroup which Willoughby (1978) defined as “Type 1 ". A further sub-group “Type 2” encompassed isolates from coarse fish, whilst the saprophytic isolates, clearly belonging to *S. diclina* (sensu *stricta).* were placed in the third “Type 3” group.

Inspite of the obvious confusion arising from the application of traditional taxonomic characters to define *Saprolegnia* species the secondary cysts of salmonid lesion isolates (Type I of Willoughby, 1978) when examined as whole mount preparations in the transmission electron microscope were found to be ornamented by bundles (consisting of 4-16 spines) of elongate (2.5 to ca. 14 μm in length) bifurcately hooked hairs or spines (Fig. 2E, F, H; Meier and Webster, 1957; Pickering et *al. 1979;* Hallett and Dick. 1986; Beakes. 1983; Beakes and Ford, 1983; Willoughby *et al.. 1984;* Wood. 1987; Puckridge et *al..* 1989; Hatai et *al.,* 1990). These spines have been compared to and indeed are often referred to as “boathooks” (Meir and Webster. 1957). Comparable cysts of saprophytic species such as *S. ferax* and *S. diclina sense stricta* (Fig. 2D) possess similar spines but they are only OS-l.0 μm in length and normally occur singly or very occasionally in groups of no more than 3 spines. In *S. hypogyna* (Pringsheim) de Bary the secondary cysts are decorated by single, thicker, spines <3.0 μm in length, whereas all *Achlya* species have smooth undecorated cyst walls (Fig. 2C.G; Pickering et *al.,* 1979; Willoughby et *al.* 1984; Hallett and Dick, 1986; Hatai *et al.. 1990).*

Figure 1. A. Schematic asexual life cycle of *Saprolegnia parasitica* showing varied patterns of cyst germination. B. Drawings of typical and representative sexual organs (oogonia, antheridia and oospores) of *S. diclina* and *S. parasitica.* Adapted from Seymour 1970. Nova Hedwigia 19: l-l 24.

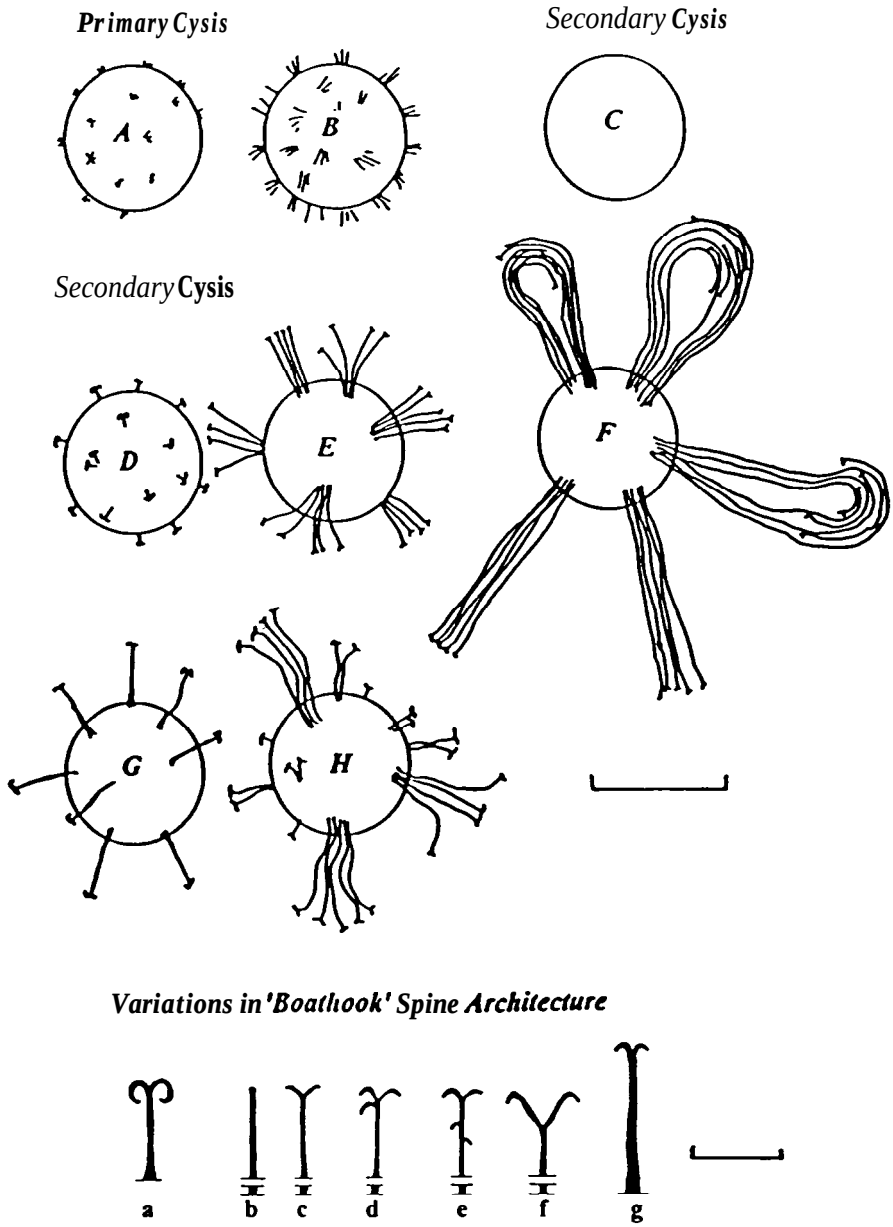


Figure 2. Schematic diagrams summarizing cyst coat ornamentation in *Saprolegnia* spp. and other oomycete genera. Primary cyst ornamentation of S. ***diclina/ferax*** (A) and S. *parasifica* (B). Secondary cyst coat ornamentions: Smooth walled genera such as *Achlya, Aphanomyces* etc. (C); short spined-species such as S. *ferax, S. diclina* Type 3 (D); extremes of cyst ornamentation in S. *parasitica* Type I (E e.g. isolate TP41; F e.g. Australian isolates, H e.g. Japanese isolate H2); *S. hypogyna (G).* Scale Bar = 10 μm.

Although in fish-lesion isolates these spines can often be >lO μm in length, the bundles of spines are often interdigitated so as to form characteristic hooped appendages (see Fig. 2F) which are more substantial structures than the individual spines. Nevertheless, there is considerable variation both in the number and length of the spines in these bundles (Figs. 2E, F, H; 3) and even in the individual spine micro-archictecture (Fig. 2 a-g). Rather curiously it has been consistently found that there is a linear relationship between the number of spines in the bundle and absolute length of spines (Fig. 3; Pickering *et al.,* 1979; Puckridge *et al.,* 1989; Hatai *et al.,* 1990). Whilst the majority of isolates have secondary cysts with a reasonably constant spine number and length (Figs. 2E, F; 3), a few isolates have cysts with very varied ornamentation (Fig. 2H) which nevertheless shows a similar regression of cyst spine/length to that observed between individual isolates. A good example of such an isolate was H2, isolated in 1986 from silver salmon from Miyagi prefecture, Japan (see Fig. 13 in Hatai *et al.,* 1990). It also appears that size of these spines is not necessarily an indicator of pathogenicity, since some of the most aggressive parasitic isolates (Hatai, 1980) produce cysts with spines that are at the lower end of bundle size-length distribution (Fig. 3; again exemplified by isolate H2 from Japan, whose mean spine length is only around ca. 2.5 μm Hatai *et al.,* 1990). Inspite of their size these bundles of spines are remarkably difficult to see in the light microscope using normal brightfield optics, but can be detected quite readily under negative (anopteral) phase contrast. It should be pointed out that similar optical systems from different microscope manufacturers are not equally good at differentiating these structures and positive phase contrast and differential interference contrast (Nomarski) optics also reveal these spines, but often less clearly than with anopteral optics. This is certainly the simplest, quickest and, providing a suitable microscope available, cheapest way of ascertaining if an unknown asexual *Saprolegnia* isolate belongs to the likely fish pathogenic group (Willoughby, 1985). Care has to be taken in cultures heavily infested with bacteria not to confuse attached bacteria with spine bundles (Wood, 1988). It is interesting that the observation of this distinctive cyst ornamentation with isolates taken from live fish is remarkably cosmopolitan and has been observed in collections of isolates from many parts of the world (Fig. 4).

Fig. 2. cont.

Lower panel illustrates variation in spine tip microarchitecture (scale bar = 0.5 μm). Typical short highly reflexed spines of saprophytic isolates (a). Variations observed in the tip architecture amongst *S. parasitica* isolates, from spines with no hooks (b), shallow bifurcations (c), multiple hooks (d,e) to deep "gull winged" bifurcations (f). Thick rigid spine typical of S. *hypogyna (g).*

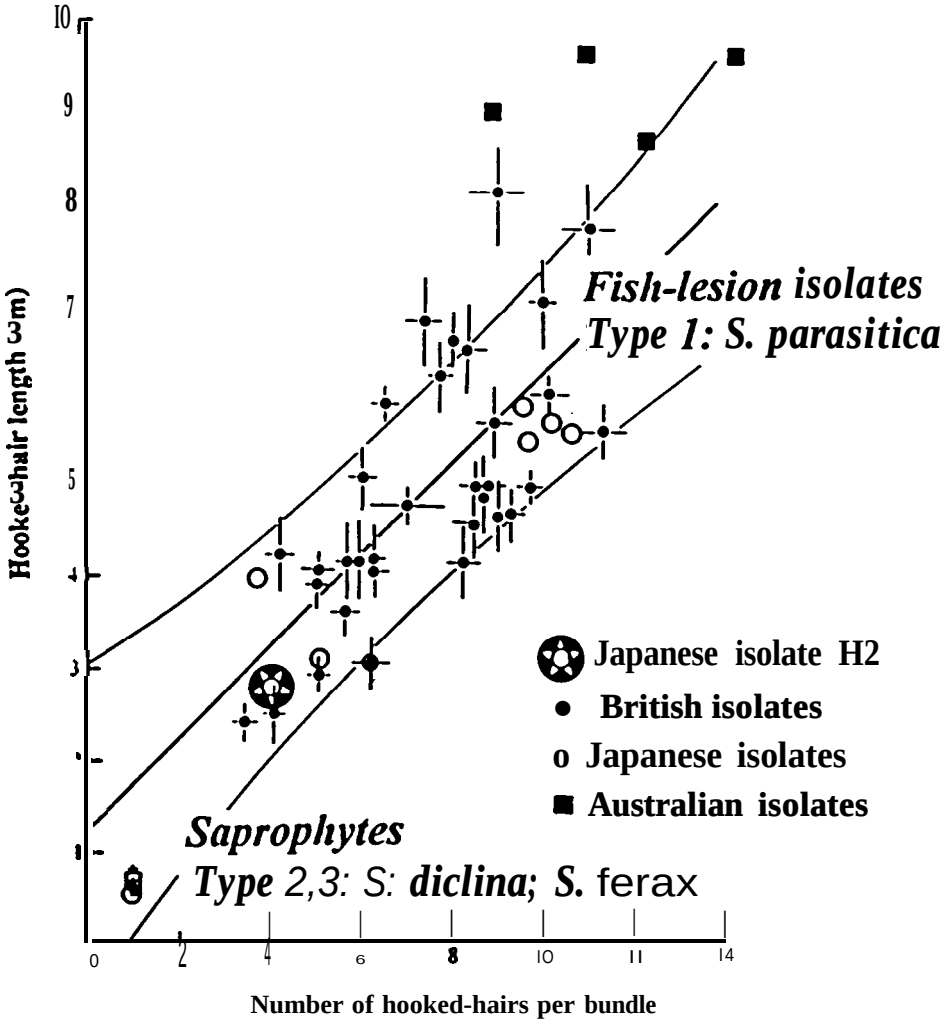


Figure 3. Plot showing regression of mean hooked-hair length against number of hairs per bundle. Modified from Pickering et al. (1979). Trans. Br. mycol. Soc. 72: 427-436. Each point is the mean of approximately 50 measurements.

It has also been noted that the cysts of the British salmonid-lesion species **of** *Saprolegnia* often germinate in a distinctive way (Willoughby, 1977; Willoughby et al., 1983; Willoughby, 1987; Willoughby and Roberts, 1992). When cysts were suspended in autoclaved lakewater, or even fish mucus, a significant proportion, germinate by producing rather narrow uniform germtubes, which became regularly septate so maintaining the cytoplasm within the apical growing compartment (Fig. 5). Many of these germtubes after 20-500 pm of this narrow septate growth resume the more typical

Figure 4. Map of the world showing the geographical origins of fish lesion-isolates examined in the EM and showing characteristic cyst-coat ornamentation of *S. parasitica.*

wider coenocytic germtube growth, sometimes branching in a bipolar fashion to form distinctive T-shaped germlings (Fig. 5, Willoughby, 1977; Willoughby et al., 1983). This distinctive growth pattern enables these cysts to achieve extremely fast extension rates (estimated at around 15-20 μm h-1 in different natural waters and up to ca. 40 μm h-1 in trout mucus) under relatively low nutrient conditions, such as provided by hatchery fish pond water, sterilized lake water and fish mucus itself (Willoughby et *al., 1983;* Willoughby, 1987; Wood et al., 1987). In natural waters, some of these cysts eventually form small apical zoosporangia which release one or two zoospores (Fig. 5). This "microcyclic" behaviour was not induced in germlings growing in mucus (Willoughby et *al.*. 1983).

The distinctive septate pattern of germination appears to be regulated by the nutrient status of the growth medium with normal germ tube development occurring both at the extremely low and higher nutrient levels (Willoughby et al., 1983). In contrast the spores of saprophytic species (S. *diclina* and S. ferax [Gruith.] Thuret) did not usually germinate at all in sterile lake water (Willoughby et al., 1983). Such spores have, however, been induced to germinate in a septate fashion by critical manipulation of nutrient levels (Wood, pers. comm.), which indicates that this is not a morphogenetic pathway unique to fish parasites.

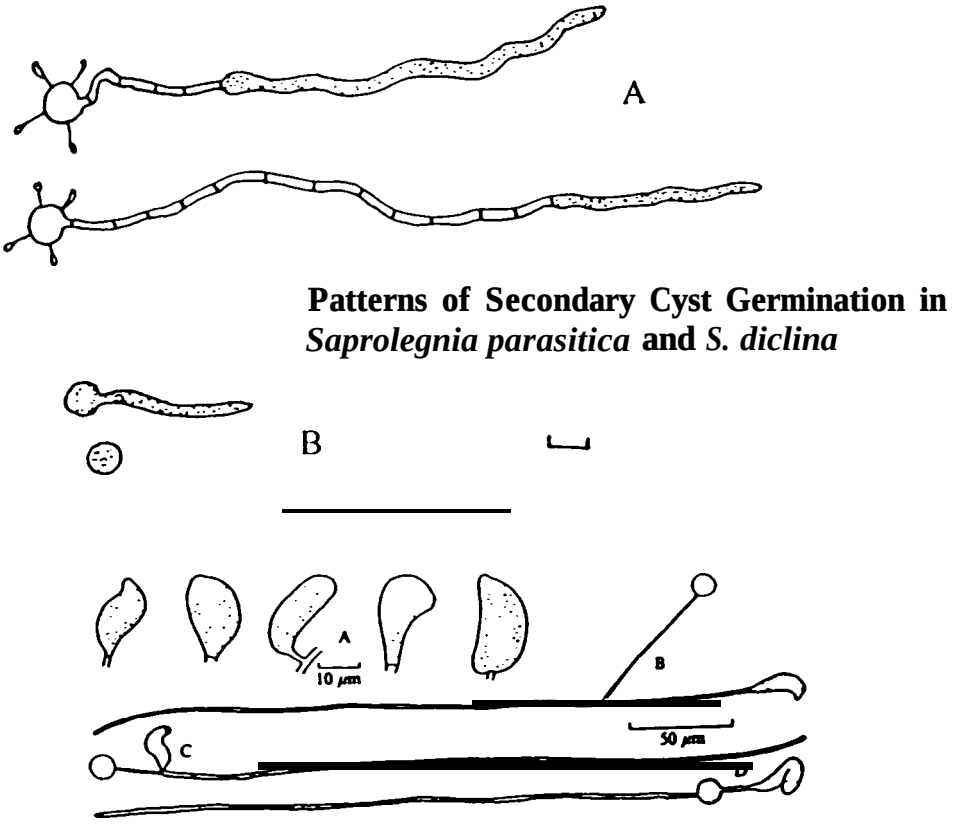


Figure 5. Upper section: schematic diagrams of germimating cysts of *S. parasitica* (A) and *S. diclina* (B) showing differences in germination pattern in sterilized lake water.

Lower section: Drawings of starved germlings of S. *parasitica* LIClO, showing typical sporangia which form as part of the microcyclic sporogenesis cycle (A) and general morphology of these sporulating germlings (B,C,D). From Willoughby (1977). Trans. Br. mycol. Soc. 69:133-166.

The fish-lesion isolates also had distinctive esterase isozyme profiles (Beakes and Ford, 1983; Wood, 1988). The majority of isolates taken from live salmonid fish can be characterized by having a number of fast-running major esterase isozyme bands compared with *S. diclina* and other asexual species (Fig. 6). Whilst the esterase isozyme pattern obtained from isolates taken from salmonid fish was extremely uniform, *Saprolegnia* isolates from coarse fish had much more variable esterase patterns (Fig. 6).

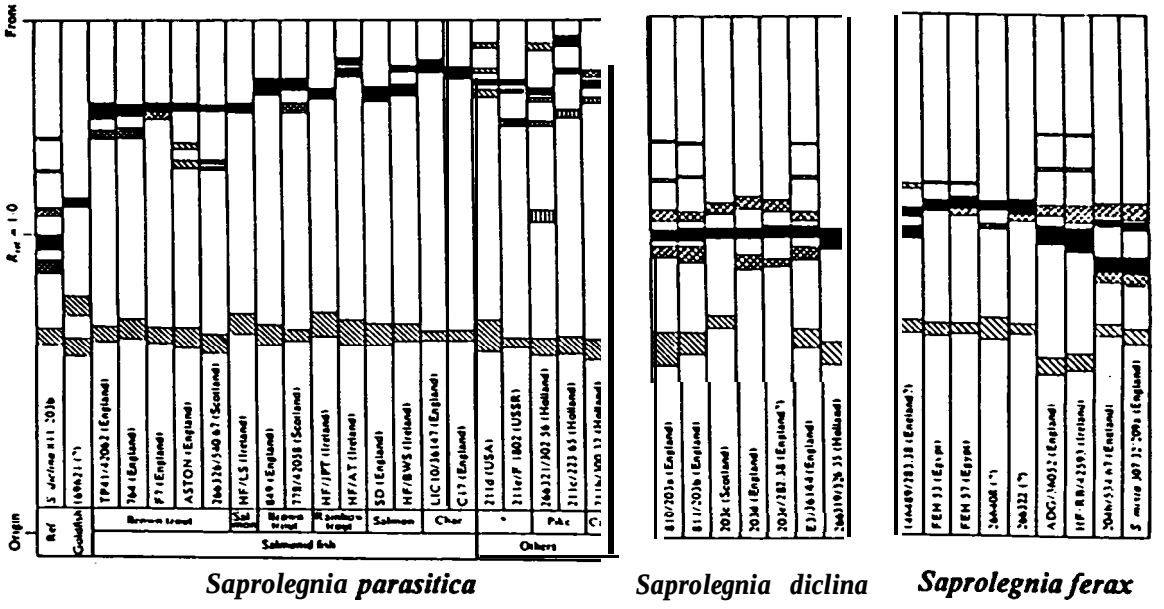
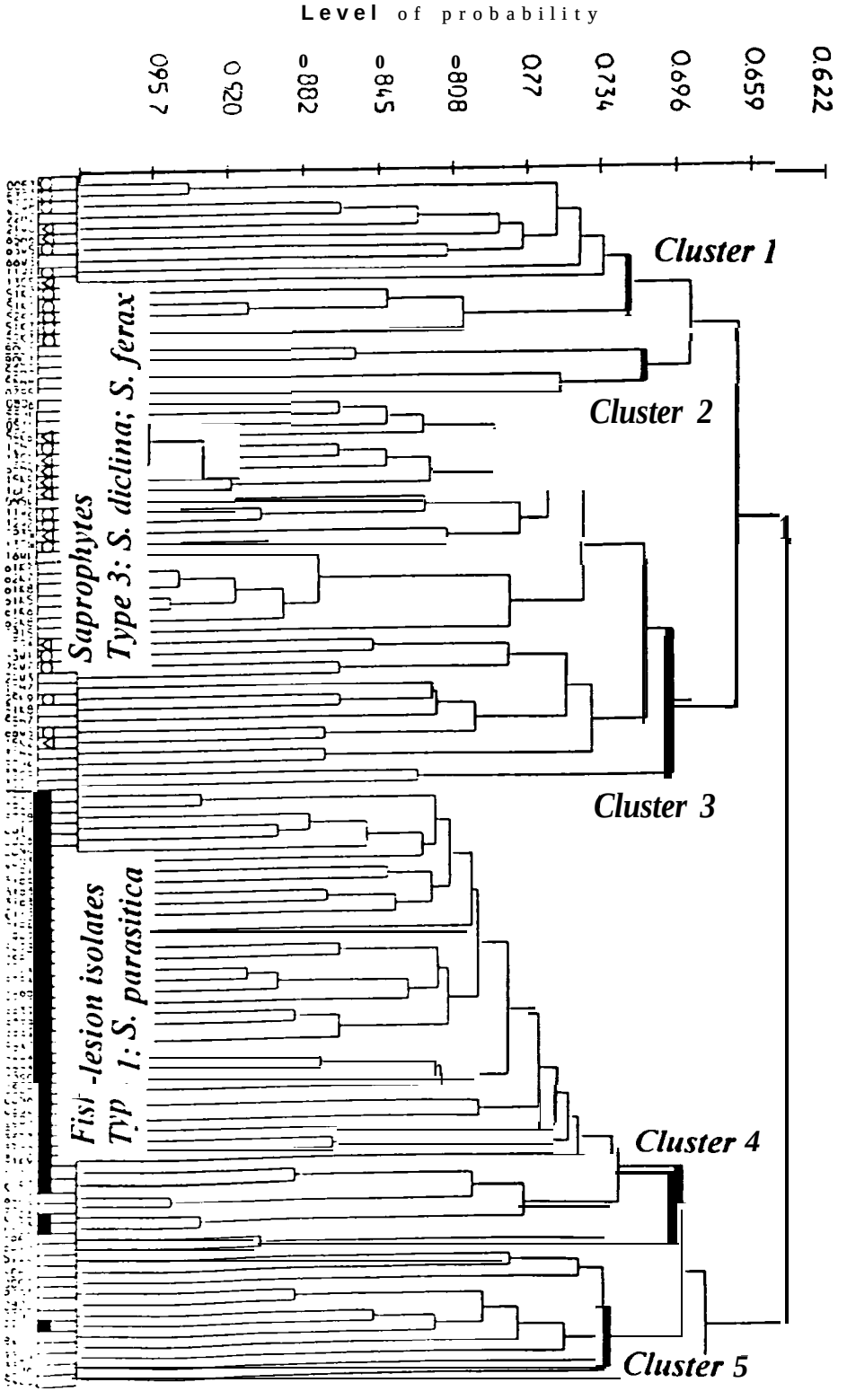


Figure 6. Diagrams of major esterase isozyme bands obtained from vegetative **mycelia** of long-spined ***Saprolegnia*** *parasitica* isolates (left-hand panel) compared with esterase bands from S. *diclina* (middle panel) and S. *ferax* (right-hand panel). Adapted from Beakes and Ford (1983). J. Gen. Microbial. 139:2605-2629.

We decided to see if *Saprolegniu* isolates (93 in total belonging to 12 recognizable taxa) could be separated using an objective numerical taxonomy approach based on coefficients of similarity. We also included a number of mycelial and physiological (growth rate/temperature optima) characters in this analysis, making a total of 132 in all. The data was analysed using a CLUSTAN analysis of similarity and dendrograms based both on a "Simple Matching Coefficient" (SM), in which both positive and negative matches were taken into account and the "Jaccard Coeffkient" (Jd) when only positive matches are considered (see Wood, 1988). In both analyses the typical long-spined fish lesion isolated *(S. parasitica)* clustered closely together and were clearly grouped on a different branch from the saprophytic species such *as S. ferax* and *S. diclina* (Fig. 7). This separation was most clearly shown when the Jaccard coefficient of similarity was used on all the characters measured (Fig. 7). All of the Type I (Willoughby, 1978) fish-lesion isolates were separated by first division of the tree and all except one fell within a cluster (Cluster 4) separated at a level probability of 0.696. The second cluster of this branch of the tree (Cluster 5), contained one "Type 1" salmonid isolate, a number of uncharacterized isolates, some coarse fish isolates and one of the so-called "filter paper" isolates (Beakes and Ford, 1983; Willoughby et *al.*, 1984). Within the second major branch of dendrogram there were three major clusters, separated at the 0.696 probability


Level of probability
0.957
0.920
0.882
0.845
0.808
0.77
0.734
0.696
0.659
0.622
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Saprophytes
Type 3: S. diclina; S. ferax
Fist -lesion isolates
Typ 1: S. parasitica

level (Clusters 1,2 and 3). A majority (1 O/l 8) of the saprophytic isolates belonging to S. *diclina* fell within the branch defining Clusters 1 and 2, together with 3/9 of S. *ferax. The* remainder of the isolates of these species were contained within Cluster 3 (Fig. 7). From this analysis the validity of considering the fish lesion isolates as a sufficiently distinct to form a taxon in their own right would seem to be confirmed. It is proposed that species grouped within Cluster 4 (Fig. 7) represent a natural group of isolates with the potential for infection of live fish (Types 1 and 2 of Willoughby, 1978) and defines the taxon referred to *as Saprolegnia parasitica* in this account.

WHAT IS THE STATUS OF *SAPROLEGNIA PARASITICA* IN THE NATURAL ENVIRONMENT?

Clearly it would be of considerable help in developing control strategies, particularly related to the efficacious application of potential fungicides, to know if there are any seasonal variations in the occurrence and abundance of potential pathogenic *Saprolegnia,* particularly in the vicinity of hatcheries. It might also be argued that the prevalent isolation of *S. parasitica* from fish lesions may simply be the result of this species being the most abundant in the water column, rather than it showing any specific pathogenicity towards susceptible fish. Whilst there have been many ecological surveys of water moulds in natural water systems (amongst some of the more recent or geographically pertinent studies are those of Willoughby, 1962; Willoughby and Collins, 1966; Hunter, 1975; Hallett and Dick, 1981; Klich & Tiffany, 1985). However, the determination of the status of potential fish pathogenic species has only rarely been addressed (Willoughby *et al.*. 1984). In most of these studies boiled seeds are used as baits to isolate the water moulds from the collected water samples. In some studies, direct dilution plating of the water samples has been carried out (Willoughby, 1962) or spores have been concentrated by centrifugation and directly counted (Hallett and Dick, 1982). The precise status of individual species is often difficult to establish because of the significant variations in water mould populations which can occur both over short (e.g. diurnal) and longer (e.g. seasonal) time scales (Hallett & Dick, 1981). Recently a considerable amount of effort has been devoted into trying to develop a selective assay for the fish pathogen, although with only limited success (Willoughby *et* al., 1984; Willoughby, 1985). Some of the most informative quantitative data has been obtained using dilution assays of water samples. The water sample is added to a dilute glucose-peptone medium, containing streptomycin

Figure 7. Dendrogram illustrating possible relationships between *Saprolegnia* isolates based upon the Jaccard Similarity coefficient using all classes of characters (morphological, biochemical and physiological) measured. The position of isolates classified as S. *ferax* (open triangles), S. *diclina/rype* 3 (open circles) and S. *parasitica*/Type 1 (solid squares) are indicated.

and penicillin to control bacterial growth, and gelled into a semi-solid medium using a carboxy-methyl cellulose based, fungicide free, Polycell-gel wallpaper paste (Willoughby *et* al.. 1984; Willoughby, 1986; Wood and Willoughby, 1986). Recently it has been suggested that recoveries of water moulds could be improved if hydroxyethyl cellulose (Natrosol 250) is used in place of the Polycell (Celio and Padgett. 1989). However, Willoughby (pers. comm.) reports that the use of semi-solid media tends to increase problems with bacterial contaminants and now often carries out his assays using just the dilute nutrient and antibiotic components.

Although it is probably unwise to draw too many sweeping generalizations from these ecological studies, in nearly all the surveys published to date saprophytic species such as S. *ferax* and *S. diclina* are reported to predominate amongst the identified *Suprolegnia* isolates (Hunter, 1975; Klich and Tiffany, 1985). However, because many fish-lesion isolates fail to produce oogonia they simply become grouped with other "unidentified" *Saprolegnia* spp. and their true status cannot be ascertained. Most studies also seem to be agreement that in lakes, water mould populations are higher nearer the margins and in closed environments such as hatchery pools (Willoughby, 1962, 1986; Willoughby and Collins, 1966; Wood and Willoughby. 1986). In quantitative studies water mould propagule numbers in most natural water bodies typically seem to range from 10^1 to 10^3 spores l^{-1}. However, in hatchery pools their levels could be dramatically increased by the presence of infected fish, with the number of propagules per litre increasing from 200 spores l^{-1} to over 20,000 spores l^{-1} (Willoughby and Pickering, 1977). In the spring and summer of 1982 (see Willoughby, 1986), the number of assayed *Suprolegnia* spores from the water around the margins of Lake Windermere ranged from 6-73 l^{-1}, with the fish pathogen apparently contributing a significant proportion (2-28 l^{-1}). In a later Windermere survey carried out in the autumn of 1984 (Wood and Willoughby, 1986). the total numbers of Saprolegniaceae propagules estimated were between 173 and 363 l^{-1}, with the fish pathogen contributing a much lower proportion, ca. 11 spores l^{-1} (Table I). **As** Willoughby (1986) points out, considering the enormous volume of a lake such as Windermere and the relative sparsity of infected fish, these estimated numbers of *S. parasitica* propagules still seem remarkably high. He suggested that this might be accounted for by the ability of this fungus to make significant growth in dilute medium such as lake water and its ability to maintain zoospore numbers by means of microcyclic zoosporogenesis (Figs. 1 A, 5). Interestingly, Willoughby (1986) discounted the widely held belief that *S. parasitica* may be able to sustain itself by growing as a necrotroph on the bodies of dead invertebrates, on the basis of the rarity of such infested animals amongst invertebrate collections made from Windermere.

Wood and Willoughby (1988) also followed the colonization of three uninfected sexually mature dead American brook trout *(Salvelinus alpinus* L.) at one station (Lake margin) and three similarly killed char at a second (Mitchell Wyke). Isolations of fungi from superficial and dermal fungal lesions were made after 6-9 days. The fungi that were isolated from the first site are summarized in Table 1, and clearly show that many of the

Saprolegnia species present in the lake will colonize newly killed fish. The second batch of char in Mitchell Wyke all became colonized by a single *asexual Achlya* isolate. S. *parasitica* was not recovered from any of the fish exposed in this way. This further illustrates that the fish *per se are* suitable growth substrates for many water moulds and a simple nutritional explanation (i.e. only parasites have the enzymes which enable them to effectively utilize fish tissues) cannot be invoked to explain specificity.

TABLE 1. Polycell-gel water assays for Saprolegniaceae spores in Windermere. Combined results from an experiment in which three dead American brook trout were exposed in Windermere. Mats of mycelium were used to establish isolates in every case.

			Constituent categories / Recoveries													
			Total Saprolegniaceae/	*Achlya* (asexual)	*A. radio sdlaurizio*	*Aphanomyces*	*Dictyuchus*	*Leptolegnia*	*S. diclina* Type I	*S. ferax*	*S. furcata*	*S. hypogyna*	*Saprolegnia* 'filter paper'	*Saprolegnia* pathogen	*Saprolegnia* sp.	Saprolegniacae
Propagules l-1	L akeargin	Means	363	79	8	–	17	54	–	13		46	40	–	90	17
	Mitchell Wyke Bay	Means	173	91	–	11	–	38	13	–		–	–	11	–	9

Days exposure	Colonization description	Numbers of isolates made								
6	Superficial	11	0			I	1	4	4			I
8	Superficial	16	1			3	2	6	2			2
8	Dermal	6	0			1	**0**	**0**	**2**			**3**
9	**Dermal**	3	0			0	1	0	1			1
Totals		36	1			5	4	10	9			7

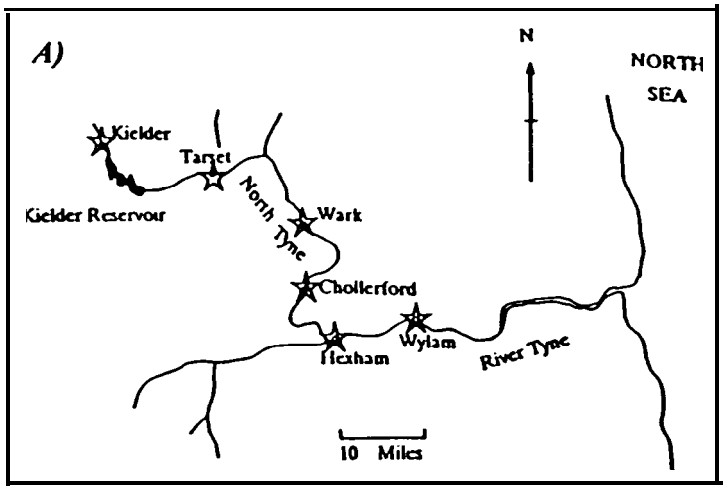
Adapted from Wood and Willoughby (1986). J. Appl. **Ecol**. 23:737-749.

In contrast to studies on water moulds in lentic (lake) systems there have been far fewer ecological studies on fast flowing (lotic) river systems. A bimonthly survey of water moulds in a productive salmon/trout river (the Tyne/North Tyne system) was carried out during 1985 (Wood and Beakes. 1993). Water samples from 6 stations along the River Tyne, from W!ylam in the east to (just above the point where the river becomes tidal) to just below the fish hatchery on the Kielder Bum, about 50 miles to the north-west (Fig. 8A) were assayed using the Polycell gel assay method. The seasonal pattern and distribution in water mould populations that were found is summarized in Fig. 8B. At all six sites there was a significant summer maxima in estimated water mould propagules, with *Suprolegniu* (Fig. 8B. dashed curves) being the dominant genus for most of the time. Again of all the water mould isolates identified, *S. ferax* and *S. diclina* were the most frequently encountered, although as often happens in such surveys, there were a significant number of *Saprolegnias* isolated which could not be identified to the species level. In spite of isolating into axenic culture many many hundreds of *Suprolegniu* isolates, only five with the characteristics of *S. parasitica* were positively identified. They were collected at Hexham in June. Tarset in August and at Chollerford and Hexham (2 isolates) in October. Therefore. inspite of a large influx of salmonids into the river from August onwards this did not dramatically increase the incidence of the pathogen recovered from the water samples. Therefore, in contrast to the Windetmere studies, S. *parasitica* does not appear to form a significant component of the water home water mould flora. However. as part of the annual electro-fishing survey of salmonids in The Tyne (and the removal of sexually mature fish to the Kielder hatchery), a number of isolations were also made in November 1985 from live Atlantic salmon and sea trout (mostly from small fin and tail lesions) and all turned out to *be* of *S. parasitica* (Wood, 1988). Clearly live fish represent a highly selective bait for the fish pathogen and routine surveys of river water samples for it may not. therefore, be particularly informative (Wood and Beakes, 1993).

WHAT IS THE BASIS OF THE PATHOGENICITY OF *SAPROLEGNIA PARASITICA* TO LIVE SALMONIDS?

Saprolegnia parasitica must possess some structural, physiological or biochemical features which governs its ability to colonise live fish so efficiently. In spite of attempts to try and characterize features of the fish lesion isolates which might confer pathogenicity very little unequivocal data has been obtained (Neish and Hughes, 1980). Several necrotrophic fish lesion isolates (falling into several taxa) have been shown to possess chemotrypsin-like proteolytic enzymes that their saprophytic counterparts did not (Pedusa and Bizzozero. 1977).

The proposition that the characteristic cyst coat of *S. parasitica* confers a selective advantage to these spores, enabling them to attach to and remain localized on the fish epidermis is an obvious one (Pickering and Willoughby, 1982). Inspite of the



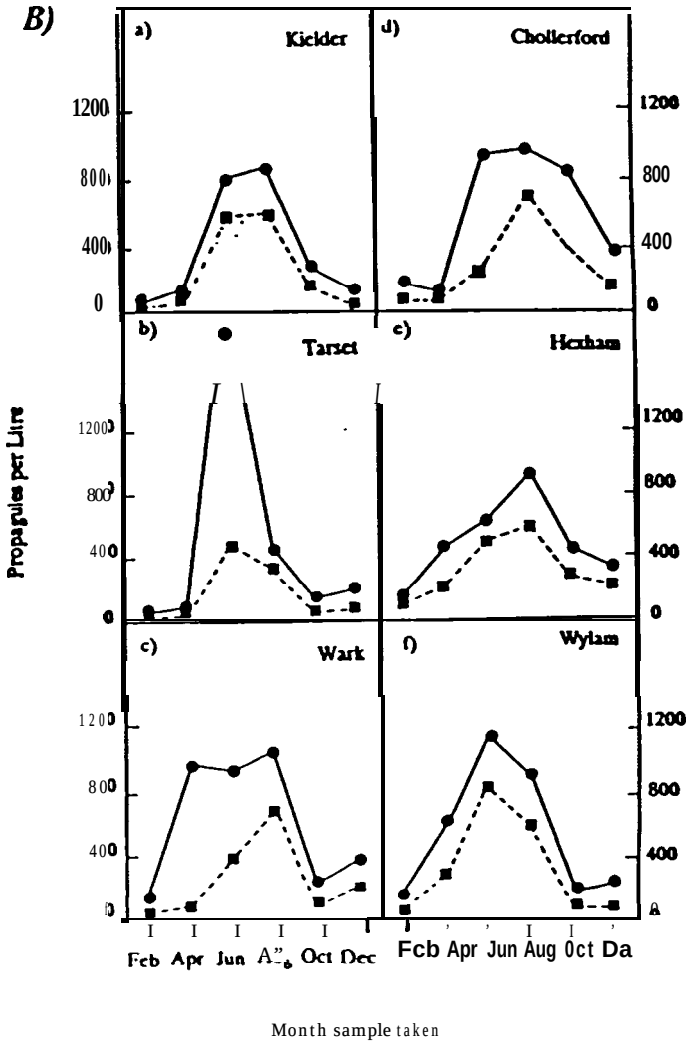


Figure 8. **A.** Sketchmap of the River Tyne and its tributaries showing the six sampling stations (starred) used to survey water moulds throughout 1985.

B. Graphical summaries of the water mould assays using Polycell gel method from the River Tyne during 1985. The upper (unbroken) curves are for total oomycete propagules and the lower (dashed) curves indicating the proportion of isolates belonging to the genus *Saprolegnia.*

From Wood & Beakes (1993), Nova Hedwigia, submitted for publication.

attractiveness of this suggestion it has, in practice proved a remarkably difficult hypothesis to test and prove. The attachment of pathogens to their hosts and successful host penetration are complex and subtle processes, particularly in zoosporic fungi. In plant pathogens it can involve stimuli involved in zoospore attraction (chemotaxis), entrapment (localization in vicinity of host), encystment triggers (often involving cross binding of specific ligands and receptors on the pathogen and host surfaces), adhesive production and attachment, suitable germination triggers and the production of the necessary enzymes or structures to facilitate successful1 penetration of the host tissues. A detailed comparison of zoospore and cyst structure and behaviour in a representative S. *parasitica* (Type 1 - TP41) and *S. diclina* (Type 3 - E3) isolates from Willoughby's 1978 study was undertaken to see if any differences could be found which might account for the pathogenicity of S. *parasitica* (Burr. 1992). To this end the following aspects were examined: a) encystment triggers: b) spore surface chemistry as defined by lectin and monoclonal antibody binding patterns; c) attachment of spores to artificial test substrates; d) accumulation of spores on fish surface.

As part of this study a panel of five monoclonal antibodies (MAbs. see Fig. 9d) were raised to a mixed zoospore and cyst suspension of the S. *parasitica* TP4 1, following the protocols which had been successfully established in peronosporalean oomycetes (see review by Hardham 1989). It was hoped that the use of MAb technology might result in obtaining a species specific antibody for the fish pathogen but in the event all the antibodies selected (5 in all from two separate fusions) were probably to the same immunodominant surface matrix component and were reasonably non-specific to a range of *Saprolegniu* species (Table 2). These antibodies also showed a little cross reaction with the saprolegniacean genus *Aphanomyces* but none with the peronosporalean genus *Pyhirtm.* These antibodies most strongly cross reacted with all isolates of the saprophyte *S. diclina* (Type 3) tested. but less intensely with species such as S. *ferax* (Table 2) This perhaps confirms that a close evolutionary relationship does exist between *S. parasitica* and *S. diclinu.*

Encystment triggers

It was found that physical agitation (vortexing) tended to have a more immediate effect upon the zoospores of the fish pathogen than the saprophyte, although after 10 minutes agitation all zoospores of both species were induced to encyst (Fig. 10 a,b). It therefore seems likely that in a normal fast flowing turbulent river system all the main infective propagules which the fish would be likely to encounter would be secondary/tertiary cysts or even germlings. Recently Willoughby and Richards (1992) have shown both the cysts and germlings of *S. parasitica* are very buoyant and sediment quite slowly in a still water column (perhaps the spines are important in increasing the spores drag co-efficient and reducing sedimentation see Fig. 11. or as with appendages in many planktonic algae

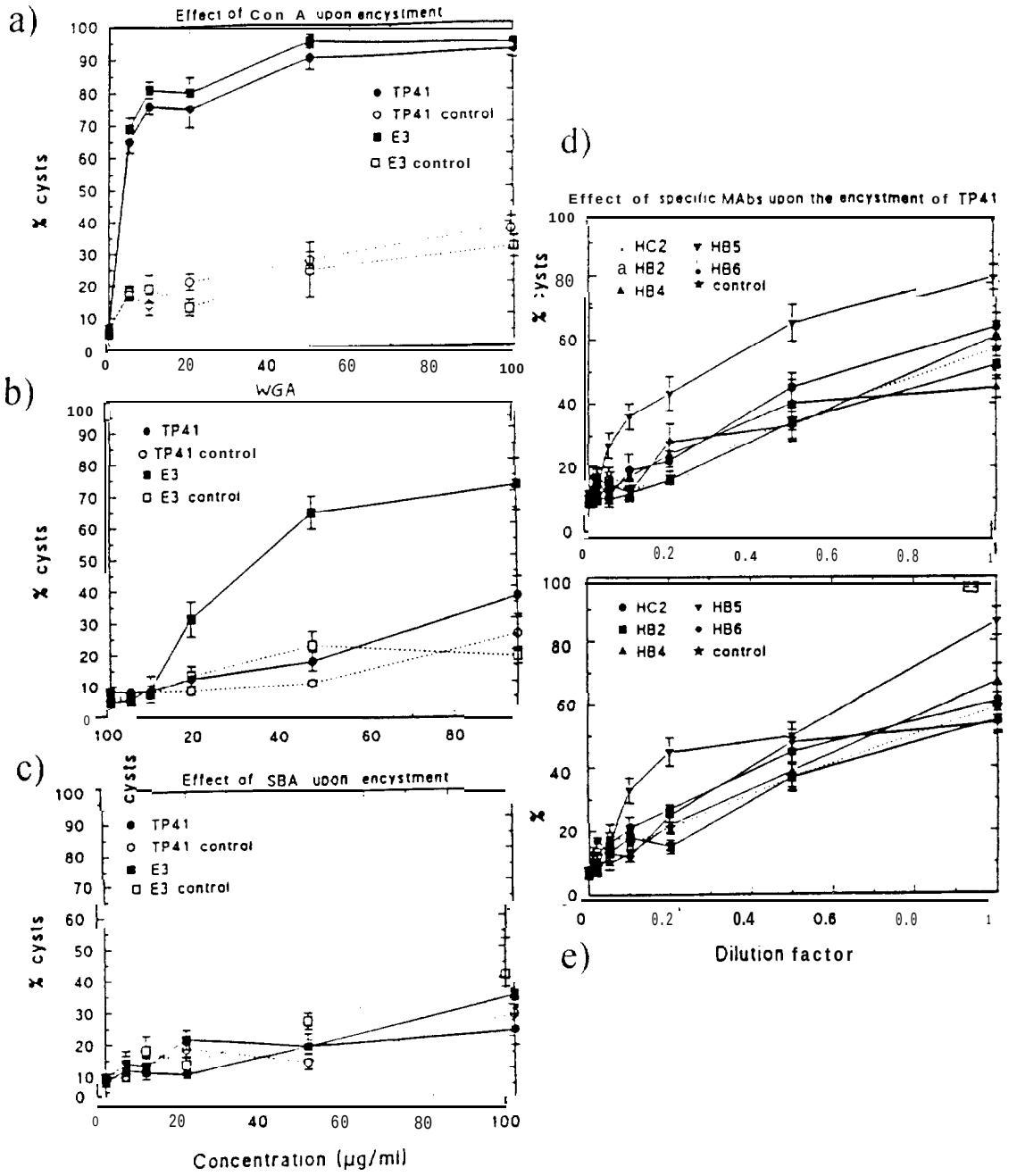


Figure 9. Plots summarizing the effects of lectins concanavalin A (a), wheat germ agglutinin (b), Soy bean agglutinin (c) and monoclonal antibodies (d, e), HC2, HB2, HB4, HB5 and HB6, (raised against zoospores and cysts of TP41) upon zoospore encystment of *S. parasitica* TP41 and *S. diclina* E3. All points are based upon the average of three independent replicate experiments. From Burr (1992), Ph.D. Thesis Newcastle University.

keeping the spores in circulation in the water currents). The sedimentation of spores of a saprophytic isolate was not made for comparison.

The effects of a dilution series of four natural organic materials/extracts (fish mucus. Fig. lOc, hemp seed extract Fig. 10d. bovine serum albumin. Fig. 10e and haemoglobin Fig. 1Of) upon zoospore encystment uere also determined. Neither fish mucus or haemoglobin differentially effected the spores of two isolates. and both induced rapid and fairly high levels of encystment (Fig. 10 c. f). The saprophyte E3 did however encyst more readily in the presence of hemp seed extract (Fig. 10d) and the parasite TP41 responded slightly more to the animal protein BSA (Fig. 10e). although this protein did not induce such high levels of encystment as the other extracts tested.

TABLE 2. Details of the isolates tested for their reactivity with the monoclonal antibodies HB5 and HB6. Amount of antibody binding is estimated from poor (+) to intense (++++). Abbreviations used: Freswater Biological Association, Ambleside (FBA): Lake Coniston (LC); Lake Windermere (LW); River Murray, Australia (RM); River Tyne (RT); South America (S.Am).

Isolate	Species	Origin	*Spine length (μm)	No. of spines per bundle	Fish Pathogen	Relative amount of MAb binding
TP4a	*S. purasitica*	*S. trutta* (FBA)	5.4	*8.5*	Yes	++++
H14	*S. purasitica*	Fish? (Japan)	*10.4*	*5.5*	Yes	++++
47A	*S. parasitica*	*S. trutta* (Spain)	*5.7*	*8.5*	Yes	++
co4	*S. parasitica*	*S. alpinus* (LC)	*5.4*	*20.5*	Yes	+++
E3	*S. diclina*	Water assay (LW)	<1.0	1.0	No	++++
66W5	*S. diclina*	Water assay (RT)	?	?	No	++++
1494	*S. ferax*	*N. erebi* (RM)	0.4	1.0	?	+
OGA2	*S. ferax*	Water assay (FBA)	*0.9*	1.0	No	+
Bol I	*S. auslralis*	*S. trutta (S.* Am)	*0.2*	I .o	Yes	None
55H7	*Saprolegnia ?*	Water assay (RT)	*?*	?	?	++++
3IHI4	*Saprolegnia ?*	Water assay (RT)	?	?	?No	+++
1504	*Saprolegnia* sp.	*N. erebi* (RM)	*13.3*	*12*	Yes	++

*See Fig. 2 secondary zoospore cyst ornamentation.
From Burr (1992). Ph.D. Thesis, Newcastle University.

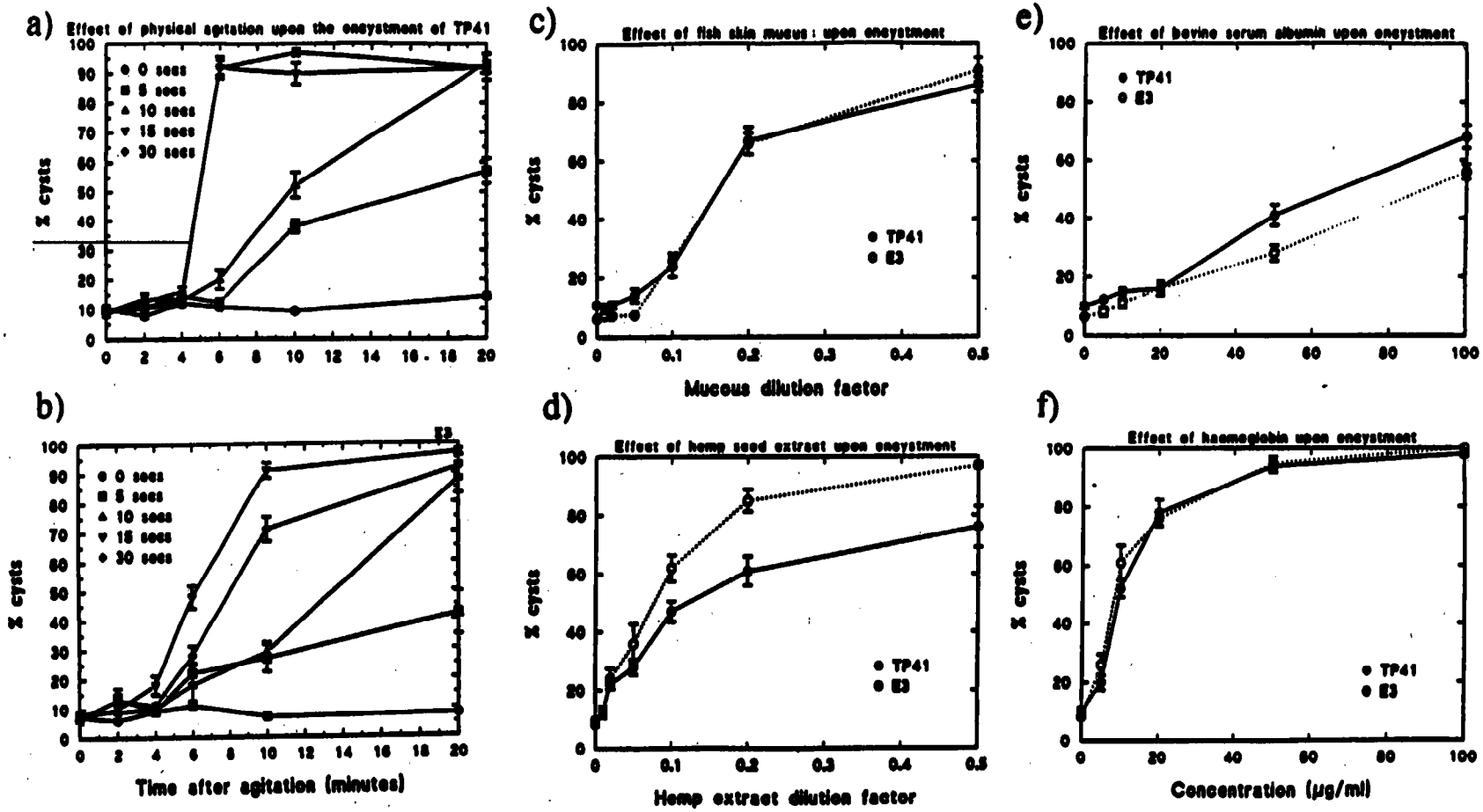


Figure 10. Plots summarizing experiments to examine the effects of physical agitation (vortexing a,b) and the addition of various natural animal and plant extracts (e-f) upon the encystment of zoospores of *S. diclina* (E3)[broken curves, c-f) and *S. parasitica* TP4 (solid curves, c-f). All data points are the average of three independent replicate experiments. From Burr (1992), Ph.D. Thesis Newcastle University.

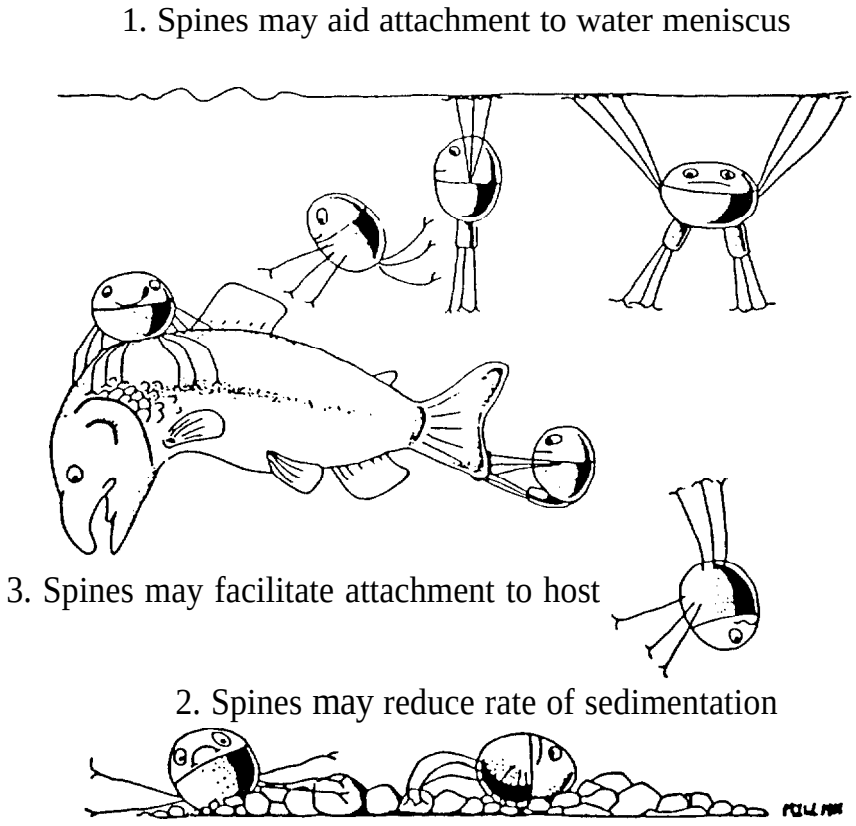


Figure 11. Cartoon summarizing the possible functions of the ornamented spines which characterize the secondary cysts of fish-lesion isolates. Adapted from a drawing devised by L.G. Willoughby and drawn by his son M.J. Willoughby.

Characterization of spore surfaces by lectins and MAbs

The zoospores of *Saprolegnia* isolates are both induced to encyst by the lectin concanavalin A (Con A), which binds to both the a-D-glucopyranosyl and to a-D-mannopyranosyl residues (Fig. 9a). The binding patterns of this lectin to zoospores and cysts of these two isolates has been summarised in Fig. 12. All spores bind fluorescein or gold conjugated Con A, although both the primary and secondary cysts appear to lose their Con A affinity with time with the exception of the bundles of spines on the secondary cysts of *S. parasitica,* which continue to be highlighted using this lectin (Fig. 12A). Sectioned spores stained with colloidal gold labelled lectin revealed that the Con A reactive material associated with a loose fibrillar material which coats the outer cyst wall and which is probably derived, at least in part, from the matrix of the encystment vesicles (in which the outer cyst coat and embedded boathook spines form).

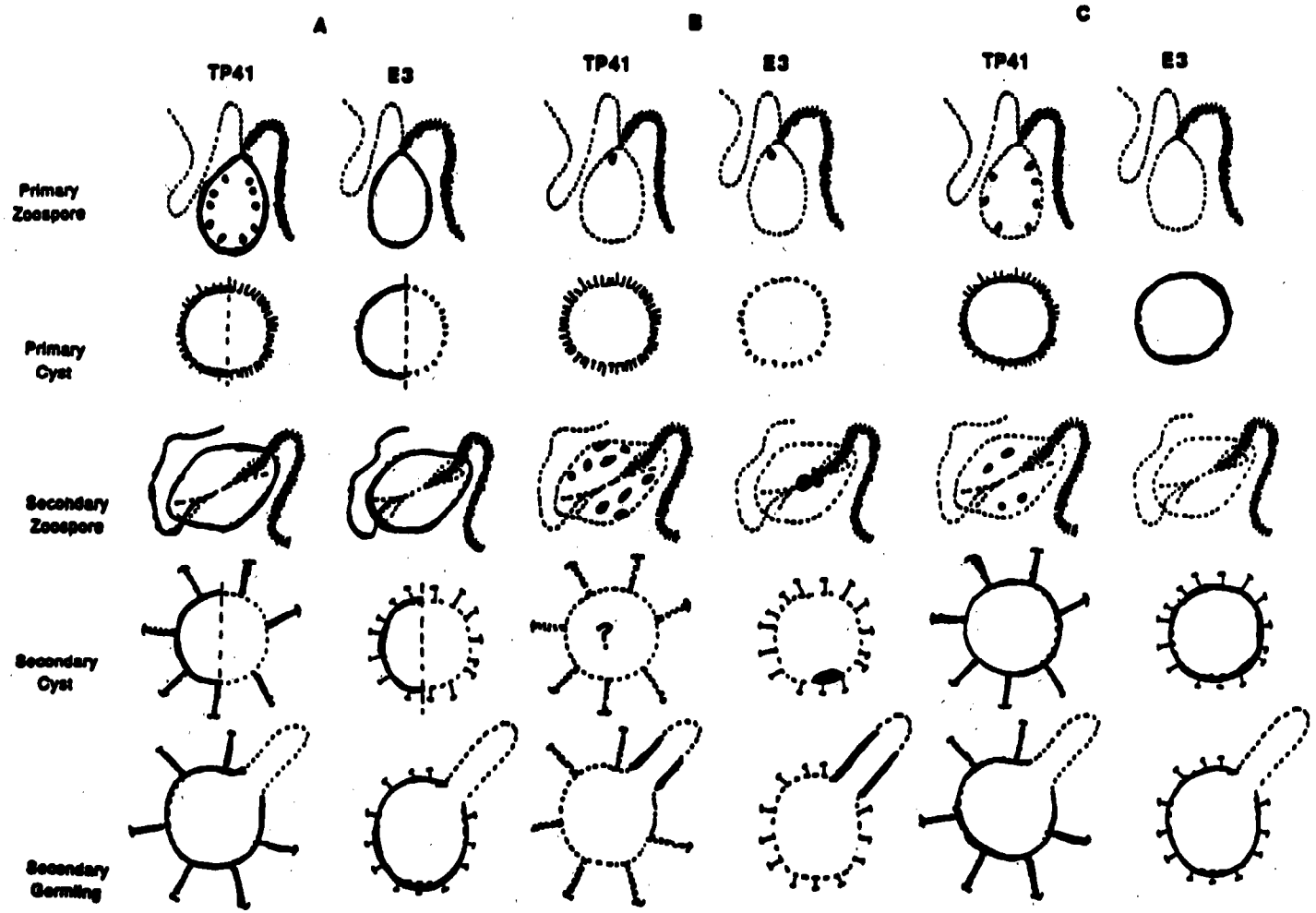


Figure 12. Schematic summaries of the binding patterns of fluorescein conjugated lectins concanavalin A (A), wheat germ agglutinin (B) and the monoclonal antibody HB5 (C) to zoospores and cysts of *S. parasitica TP41* and *S. diclina* E3. Binding of ConA in particular was very variable and the pattern illustrated is that shown by newly encysted spores. Older cysts loose reactivity, except for the spine bundles in TP41. From Burr (1992), Ph.D. Thesis Newcastle University.

In contrast wheat germ agglutinin (WGA), which binds to N-acetylglucosaminyl residues, induced encystment in *S. diclinu* but had no significant effect on zoospores of *S. purusitica* (Fig. 9b). In *S. ferax*, WGA has also been shown to bind to the adhesive material which is released into the ventral grove of the zoospore from the K-bodies to form a transient adhesive pad on the cyst (Lehnen and Powell, 1989). This difference is further supported by the binding pattern of this lectin to the zoospores and cysts (Fig. 12B). Whilst the secondary zoospore of *S. diclina* show a pad of WGA-binding material in the flagellar grove and on the secondary cyst surface as described in *S. ferax* (Lehnen and Powell, 1989), the zoospores of *S. parasitica* show much larger plaques over both ventral and dorsal surfaces. The active contact mediated discharge of K-bodies to form an adhesive pad which effects the initial attachment of an encysting zoospore to a suitable substrate (Lehnen and Powell, 1989), may therefore be normally be suppressed in the fish parasite. However, as in *S. diclinu* if encystment of TP41 spores is induced by the addition of pectin, K-body discharge was observed in the electron microscope. Different encystment stimuli may trigger different morphogenetic events in these organisms. In both species the initial germtube wall (<50 μm) also binds this lectin (Fig. 12B) and corresponds to the zone of the germ hypha which becomes firmly attached to solid surfaces, such as the Petri dish bottom. The lecitin soy bean agglutinin (SBA), which binds to α, ß- N-acetyl galactosaminyl residues, and binds to the ventral adhesive vesicles in *Phytophthora cinnamomi* (Hardham. 1989) has no effect on *Saprolegnia* zoospores and does not bind to any spore component (Fig. 9c).

MAbs raised against a mixed zoospore and cyst suspension of *S. parasitica* TP41 also induced encystment of zoosporcs. although less efficiently than Con A (Fig. 9d, e). They do however, show a broadly similar staining pattern to that produced by Con A (Fig. 12C) which suggests that these antibodies are reacting with some of the components of the immunodominant Con A-binding surface matrix material.

Adhesion of spores to an immulon test substrate

In order to compare the relative stickability of cysts of the two selected isolates, the effect of coating a model immunoabsorbent immulon test surface of various substances on the adhesion of cysts was tested (Burr, 1992). In general, the cysts of *S. parasitica* were found to bind more firmly (resisting removal in a constant water jet) to the test substrate than those of *S. diclinu*, particularly on surfaces coated in lectins (Con A, WGA), the MAb HB5 and general biological adhesives such as poly-L-lysine and amino-propyltriethyloxysilane (APES) (Fig. 13). Lectin-mediated adhesion was fairly specific since incubation of lectins with their inhibitory saccharides prior to adhesion reduced adhesion levels to the immulon to that of uncoated controls (100%). Interestingly when the immulon surface was coated with trout mucus the adhesiveness to the plastic was significantly reduced to the level of that of immulon coated with dimethyldichlorosilane (DDS) (Fig. 13). The latter reduces the stickiness of surfaces by increasing its negative

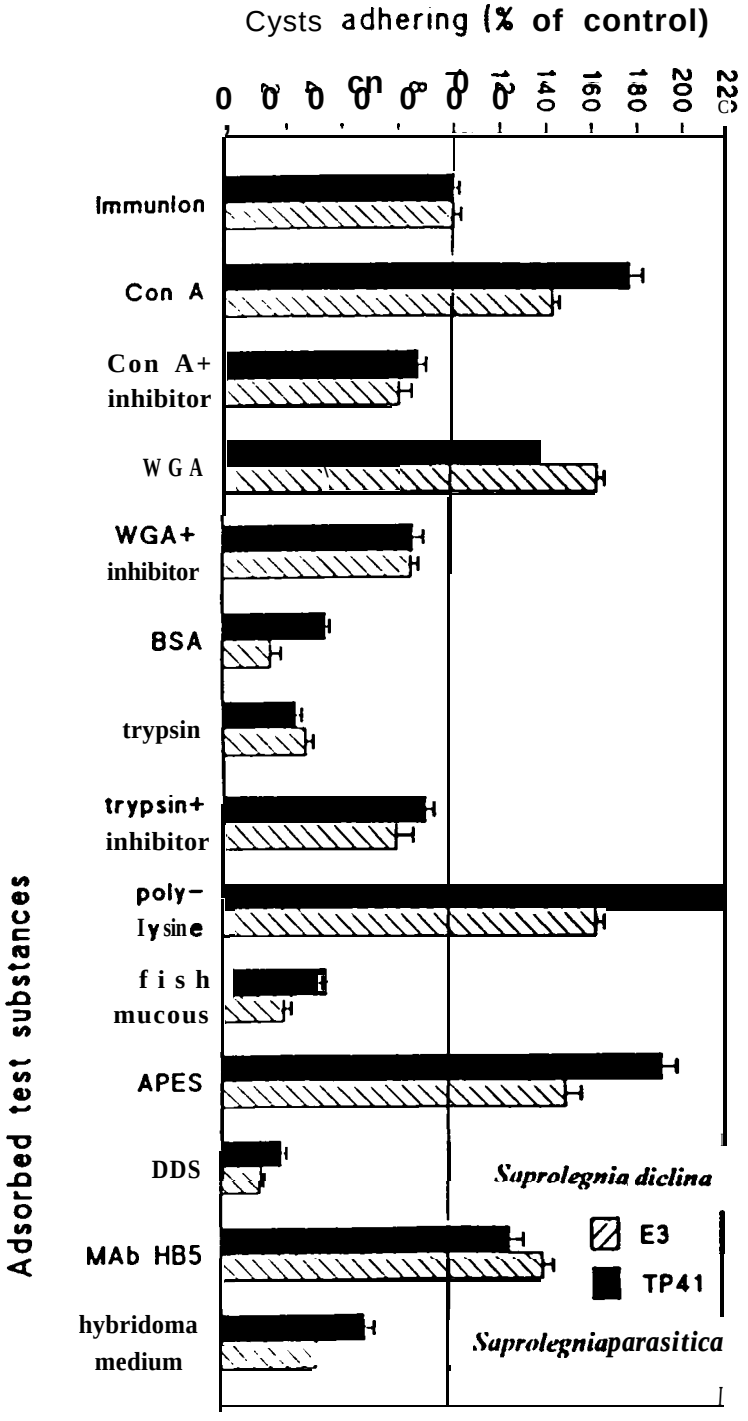


Figure 13. Histograms summarizing the adherance of cysts of *S. parasitica* TP41 (solid bars) and *S. diclina* E3 (hatched bars) to immulon plastic coated with various substrates. Each bar is based upon the mean of three independent replicate experiments. From Burr (1992), Ph.D. Thesis Newcastle University.

charge (Burr. 1991). It is possible that the mucus coat is reducing spore attachment by becoming detached from the immulon in an analogous way its sloughing from surface of the fish epidermis. When the immulon was coated by the proteolytic enzyme, typsin, cyst adhesion was also reduced again suggesting that surface (glyco) proteins play an important role in cyst adhesion.

Accumulation of spores on the fish surface

Coated immulon plastic surfaces are rather far removed from the surfaces of live salmon! There have been very few direct attempts at assessing the efficiency of fungal spore accumulation onto the surfaces of live fish. The only study which has examined localization of viable propagules on the epidermis was carried out after challenge with S. *parasirica* (Willoughby and Pickering, 1977). Brown trout were challenged by immersion in a spore bath containing 13 x 10^3 spores ml-1 for 10 min. followed by transfer to clean water for respectively 30 minutes and 24 h. The number of viable spores per cm^3 of epidermis on the body and fins were after 30 min. 2.91 (body), 3.96 (fins) and after 24h, 0.31 (body) and 0.48 (fins). Broadly similar spore levels were found in a duplicate experiment with char. This indicates that spores of the pathogen can successfully localize themselves on the fish epidermis and whilst the majority are removed or inactivated during quarantine in clean water, a small but significant number of viable fungal units still remain on the body surface after 24h (Willoughby and Pickering, 1977).

In an independent series of experiments clean brown trout were challenged by mixtures of spores of *S. parasitica* and *S. diclina.* Spores of both species showed a similar levels of accumulation in the mucus followed by a rapid decline (Wood et *al., 1987).* However. viable spores of *S. parasitica* did seem to remain localized on the fish surface for longer than those of the saprophyte since after 24 h only viable pathogen colonies were recovered from the mucus (Wood et al., 1987). In this study spore numbers in the mucus were assayed using the Polycell method. There was some evidence that mycelium which had been grown from propagules recovered from the mucus 12 h or more after the initial challenge exhibited abnormal morphogenesis (Wood *et al.,* 1987). Some of the hyphae even had fish cells attached to them suggesting an immunological response by the fish (Wood et *al.,* 1986). Subsequently. Willoughby (1989) has also examined hyphae from established crescent- shaped fungal lesions on gill netted char from Windermere and shown similar host cells attached to the hyphae. However, at present it is still unclear whether these cells are inhibiting the fungus and represent a genuine cellular defence mechanism. The potential for immunological control of fungal infections has not really been seriously pursued. Naturally acquired precipitating antibodies against fungal antigens have been detected in the serum of Atlantic salmon (Hodkinson and Hunter, 1970). However, when salmon sera from infected and clean fish were tested against culture filtrates of *S. parasitica* no detectable differences in precipitin responses were observed (Wilson, 1976). Neither was there any evidence of any precipitating antifungal

antibody or plasma factors in rainbow trout which had been sub-cutaneously challenged with an unidentified fish-lesion isolate of *Saprolegnia* although again inflammatory cells (neutrophils) were observed adhering to the hyphae (Sohnle and Chusid, 1983). Therefore, the case for an active immune response to *Saprolegnia* infection by salmonid fish is still not proven. However, there is some evidence that the presence of antagonistic bacteria *(Pseudomonas fluorescens)* in the mucus may confer resistance to *Saprolegnia* (Hatai and Willoughby, 1989).

CONCLUSIONS

Isolates of *Saprolegnia* isolated from epidermal lesions on live salmonids and other fish form a coherent and clearly recognizable group. This group of isolates are distinguished by their association with fish, the distinctive clusters of long-spines on their secondary cysts, fast growing septate cyst germ tubes and fast running esterase isozyme bands. I suggest that this group fulfils an updated species concept of *Saprolegnia parasitica* in function if not in taxonomic legality.

However, there is significant variation to be found within this group. Variations include differences in their apparent pathogenicity towards fish (Hatai, 1980), considerable variation in the fine-details of their cyst coat ornamentation (Beakes and Ford, 1983; Wood, 1988; Hatai et *al.,* 1990), sexuality (with many isolates apparently being exclusively asexual), growth rates, colony morphology (Wood, 1988) and esterase isozyme banding (Beakes and Ford, 1983; Wood, 1988). This group possibly represents a complex of different pathotypes or even species. However, the inclusion of the fish lesion isolates with the saprophytic species, *S. diclina* (Neish, 1976; Willoughby, 1978) detracts from the distinctiveness and importance of this group.

It should be stressed that isolates from fish must always be examined critically. It is clear that dead fish and eggs can be colonized by many different species of saprolegniaceae (Wood and Willoughby, 1986). Likewise severely damaged necrotic tissue or internal lesions will almost certainly yield many different water mould species (Hatai, 1980; Hatai et *al.,* 1990). Recently an outbreak of saprolegniasis in salmon fry has been reported to be caused by a Type 3 isolate of *Saprolegnia diclina (sensu stricta)* rather than the more usual "Type 1" isolate (Bruno and Stamps, 1987). Unfortunately, the cysts of this isolate were not examined by electron microscopy and it has subsequently been lost from culture. However it is possible that fry may exhibit a similar susceptibility to water moulds as eggs and dead animals.

Identifying potential fish pathogens is still far from easy and routine. Whilst experienced biologists can soon learn to recognise the spines on the cyst surface with the aid of a good phase contrast microscope, this still requires isolating the fungus and inducing it to sporulate. Recent studies on phytopathogenic *Phytophthoras* species have shown that it

is possible to raise species specific monoclonal antibodies, which could form the basis for the development of diagnostic ELISA kits (Hardham pers. comm.). The development of such diagnostic kit specific for *S. parasitica* would clearly make the diagnosis of potential fish pathogens much easier.

The basis for fish pathogenicity has not yet been established but there are a number of features of the fish parasites which could account for their effectiveness. There are many ways which the bundles of hairs on the secondary cyst coats might contribute to pathogenicity (Fig. 11). We have shown that both physical (vortexing) and chemical triggers (mucus, serum albumin) which readily induce zoospore encystment of S. *parasitica* probably ensures that, in river systems and densely stocked ponds the most likely infective spores which the fish will encounter **will be secondary/tertiary cysts or even germlings.** This is significant since if zoospores were the main infective agent then strategies which might prevent them encysting or cause these wall-less cells to lyse might provide the basis for an effective control protocol (Willoughby and Richards, 1992).

The apparent suppression or absence of K-body discharge and adhesive pad formation in encysting spores of the fish parasites is also consistent with the idea that cysts are the main infective spore. It is known that many saprophytic species of *Saprolegnia* are attracted to suitable substrates chemotactically (Ho, 1975). Subsequent contact induced discharge of the K- bodies would facilitate such attachment to solid substrates (Gubler et *al.)* 1989; Lehnen and Powell, 1989). However, in normal flowing water it is unlikely that zoospores of *Saprolegnia* could have any chance of "catching" an actively swimming fish and therefore its best strategy will be rely on chance contact with a suitable host and an effective passive attachment mechanism. The cyst coat spines may decrease the rate of spore sedimentation. keeping the spores suspended in the water column for longer that those from species with unornamented cysts (Willoughby and Richards, 1982, although this still needs to be tested experimentally), possibly trap the spores at the water-air interface (Hallett and Dick, 1986), as well as increasing the the contact area with the substrate, which may be significant in view of spines being coated in a glycoprotein (adhesive?) matrix, and aid physical entanglement with the fish surface (Fig. 11). Antibodies raised to specific spore surface components may reduce the stickiness of the spores and their attachment to the fish surface may be significantly impaired. Finally, the rapid growth of germ tubes of the fish pathogen within mucus may be advantageous in helping this isolate to successfully penetrate the reduced epidermal layers of stressed or damaged fish. These different attachment strategies have been summarized in the cartoon, Fig. 14. Although there are many questions still to be answered, the detailed knowledge accumulated over the last 20 years on the biology and structure of fish lesion *Saprolegnia* species, provides a sound platform for continuing studies into the basis of their pathogenicity which will, hopefully, ultimately lead to the development of more targeted and specific control measures.

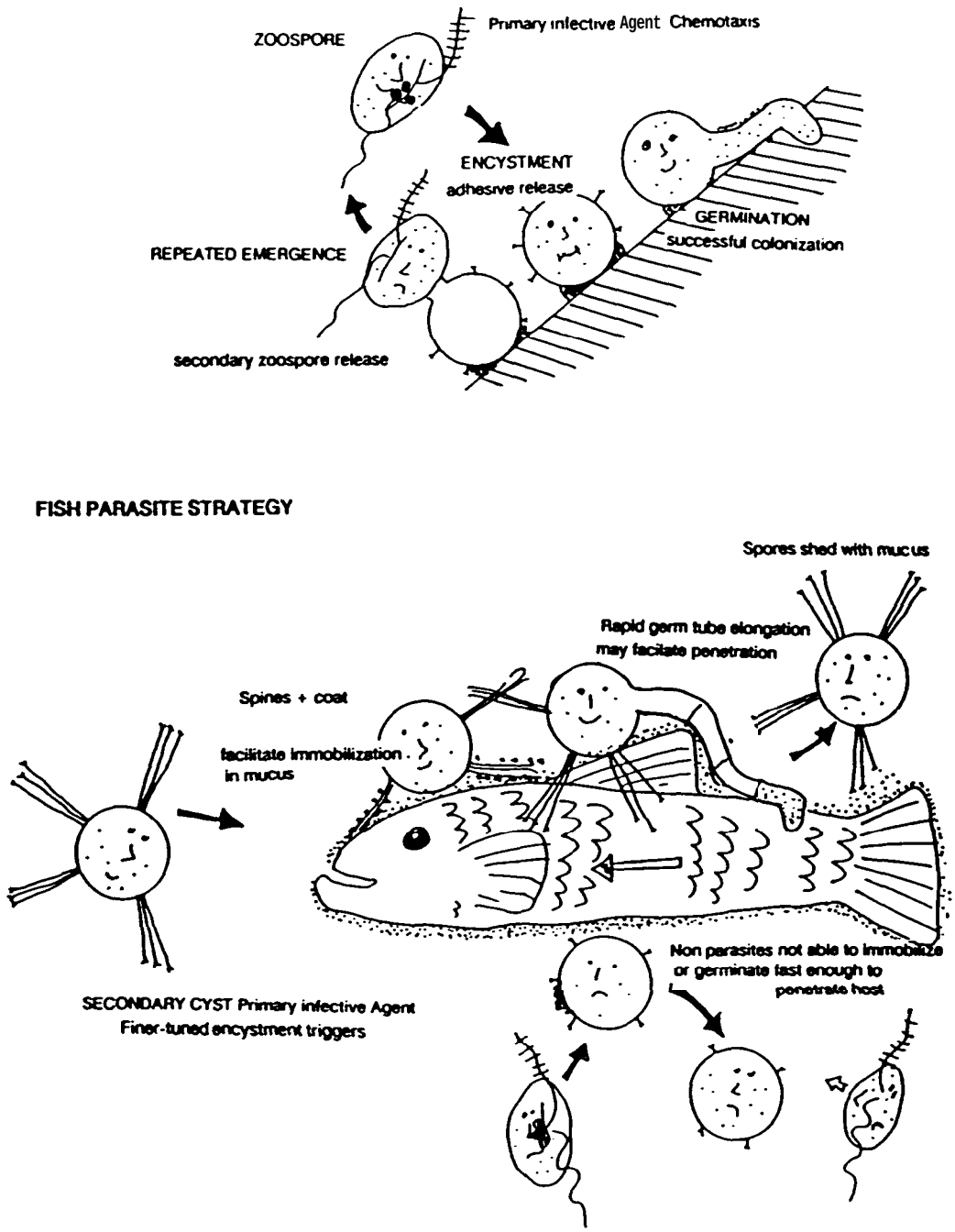


Figure 14. A schematic cartoon summarizing the comparative attachment strategies adopted by "saprophytic" and "fish-parasitic" isolates of *Saprolegnia*.

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chapter 3

FACTORS WHICH PREDISPOSE SALMONID FISH TO SAPROLEGNIASIS

A.D. Pickering1

ABSTRACT

Saprolegniasis of salmonid fish is normally associated with a group of closely-related strains of *Saprolegnia parasitica.* The defense systems of the fish to this fungus operate at several levels and this paper considers the various mechanisms by which a fish can protect itself. The role of the integument and associated mucus secretion is examined, together with some of the non-specific and specific internal defense systems. Particular attention is given to the role of environmental stress as a predisposing factor and to changes in susceptibility at different stages of the life cycle of the fish. It is concluded that activation of the hypothalamic-pituitary-interrenal (HPI) axis, as a result of external stress or of physiological changes within the fish, is a major factor responsible for the suppression of defense systems, and ultimately results in the outbreak of disease.

Key words: saprolegniasis, *Saprolegnia parasitica,* salmonids, stress, defense systems, mucus.

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INTRODUCTION

Saprolegniasis, the infection of freshwater fish (or their eggs) by aquatic fungi of the order Saprolegniales (class Oomycetes) has received less study than most of the other important diseases of freshwater fish. probably because fungal infections are generally considered to be the result of an opportunistic. often saprophytic. colonisation of already infected or damaged tissues. Whilst this may be true in some non-salmonid species (see, for example, Pickering and Willoughby, 1977; Bucke *et al.,* 1979) in the case of salmonid fish the infection is almost invariably caused by a single taxon, *Saprolegnia parasitica* Coker (Willoughby & Roberts. 1992; see also Beakes *et al.,* Chap. 2, this volume; Mueller and Whisler. Chap. 9. this volume) and not by other saprophytic species within this genus. *Saprolegnia parasitica* (previously *Saprolegnia* Type 1. *S. diclina* Type 1) does not always produce sexual structures when cultured *in vitro* and, therefore, it is impossible to apply the classical taxonomic approach to this species (see Seymour, 1970). However. several studies have shown that S. *parasitica* can be readily identified by the fine structure of the secondary zoospore cyst cases (Pickering *et al.,* 1979; Beakes *et al.*. Chap. 2, this volume) and a presumptive specific identification can be made using phase-contrast light microscopy (Willoughby, 1985). Evidence is now accumulating which shows that. whilst still able to act as an affective secondary colonist on diseased or damaged fish, *S. parasitica* is also capable of acting as a primary pathogen of salmonid fish (Neish, 1977). As such it merits further attention as an important fish disease, particularly in relation to the expanding aquaculture industry.

The present paper examines the effects of saprolegniasis on salmonid fish and outlines those periods of the life cycle, or those environmental circumstances, when the susceptibility of the fish is high. An attempt will be made to elucidate the defense mechanisms available to the fish to enable it to overcome this form of pathogenic challenge. Particular attention is paid to the effects of stress (either imposed by the environment or created as the result of internal physiological changes) and to methods of minimizing the stress response, thereby decreasing the probability of saprolegniasis under aquaculture conditions.

THE PATHOLOGY OF SAPROLEGNIASIS

On infected fish, *S. parasitica* takes the form of profusely-branched, non-septate mycelia which form cotton-wool-like tufts on the integument. The early lesions are grey-white in colour and often appear as circular or crescent-shaped colonies (Willoughby, 1989) growing by radial extension until adjacent lesions merge. The lesions are not randomly located (see below) but in severe cases, moribund fish may have as much as 80% of the body surface area covered by the fungus (Richards and Pickering, 1979). Penetration of the fungus is usually restricted to the integument and superficial musculature but in certain cases, penetration may be much deeper (G.J. Mueller, pers. comm.). Bruno and Stamps (1987) reported an outbreak of saprolegniasis in Atlantic salmon fry, *Salmo salar,*

in which the fungus attacked the oesophageal/gill region of the fish. However, this outbreak was unusual in that *Saprolegnia diclina*, and not *S. parasitica,* was identified as the causative organism.

Histopathological changes beneath the superficial mycelial mat include dermal necrosis and oedema during the early stages with deeper myofibrillar necrosis and extensive haemorrhage in the more progressive lesions. More detailed descriptions of the histopathological processes associated with *Saprolegnia* infections of salmonid fish have been provided by Neish (1977) and Pickering and Richards (1980). The tissue damage is probably caused by extracellular enzymes secreted by the advancing fungal hyphae. Thus, Peduzzi et al. (1976) and Peduzzi and Bizzozero (1977) demonstrated proteolytic, chymotrypsin-like enzyme activity in the fungus and more recently, Rand and Munden (1992) used a cytochemical approach to demonstrate both lipase and alkaline phosphatase activity around the hyphae of fungal-infected salmonid eggs.

It is generally accepted that the ultimate cause of death is the severe haemodilution caused by haemorrhage and by the destruction of the water-proofing properties of the fish's integument (Gardner, 1974; see also Hatai and Hoshai, Chap. 4, this volume). Figure 1 illustrates the inverse correlation of the serum osmotic pressure/sodium

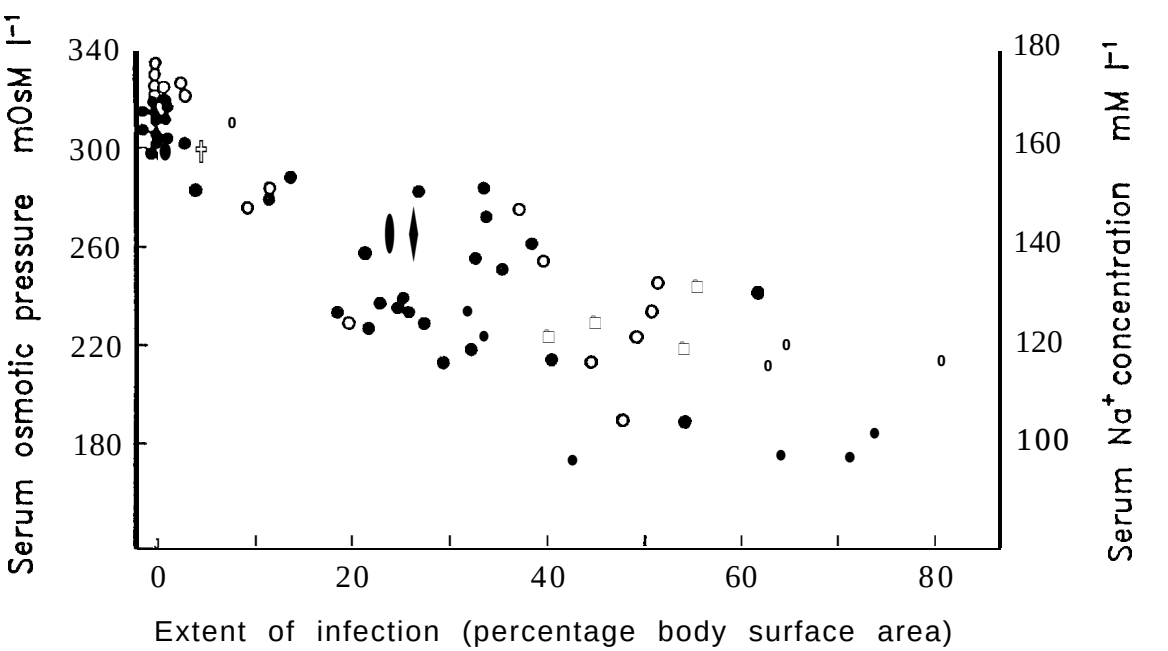

Figure 1. Relationship between the serum osmotic pressure (open circles), serum Na^{1+} concentration (closed circles) and the extent of the colonisation of the body surface of wild brown trout by *Saprolegnia parusiticu.* Original data in Richards and Pickering (1979).

concentration of *Saprolegnia*- infected wild and hatchery-reared brown trout, *Salmo trutta*, with the extent of the fungal infection. As would be expected, this haemodilution is also associated with hypoproteinemia although the changes in individual blood proteins suggest that this is more complex than a simple dilution process. Thus, Richards and Pickering (1979) found a significant reduction in the albumin/globulin ratio of Saprolegnia-infected brown trout. Moreover, Duran *et al.* (1987) reported marked increases in the serum levels of glutamate oxalacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) of Saprolegnia-infected brown trout but no changes in creatine phosphokinase (CPK). These findings are of particular interest because elevated levels of GOT and GPT in the serum are usually indicative of cellular damage to the liver whereas elevated levels of CPK, which might have been expected, usually indicate damage to the muscular tissue (see above). To date, there is no evidence to indicate that *S. parasitica* produces any toxin which could cause cellular damage at sites remote from the sites of hyphal invasion but the results of Duran et *al.* (1987) suggest that further studies in this area might be rewarding.

SUSCEPTIBILITY TO SAPROLEGNIASIS

There is no period during the freshwater stages of the salmonid life cycle when the fish are not susceptible to saprolegniasis but several studies have shown that the degree of susceptibility can increase quite markedly under certain circumstances. The fungus cannot survive in full-strength sea water and. therefore, saprolegniasis does not occur during the marine phase of migratory salmonids. However, Langvad (Chap. 10, this volume) reported that *Saprolegnia parasifica* could survive and grow at a salinity of 1.75% NaCl.

Sexual Maturation

Sexual maturation in both sexes of salmonid fish is associated with a marked increase in susceptibility to *S. parasifica* although male fish appear to be more vulnerable than females. This is as true for natural populations of fish as for those reared under aquaculture conditions. Thus, White (1975) described an annual outbreak of saprolegniasis in a naturalized population of brown trout in which 66% of the spawning population, examined over a period of five years, was infected by *S. parasitica.* During the same period, more than two hundred sexually immature brown trout from the same stream were examined and none showed visible signs of fungal-infected lesions. Similarly, Richards and Pickering (1978) showed that sexually mature wild brown trout and Arctic char, *Salvelinus alpinus,* were more frequently infected than sexually immature fish and that a similar increase in susceptibility occurred in maturing hatchery-reared fish of the same species. In the brown trout, this is also accompanied by an increased susceptibility to other common skin parasites *(Ichthyophthirius, Scyphidia, Gyrodactylus, Trichodina* -

Pickering and Christie, 1980). Robertson (1979) has shown a similar increase in the susceptibility of mature male rainbow trout. *Oncorhynchus mykiss,* to the protozoan flagellate, *Ichtyobodo* (= *Costia*). Thus, sexual maturation, particularly in the male fish, is associated with a general increase in susceptibility to skin pathogens, including S. *parasitica.*

Because salmonids are seasonal spawners, it could be argued that the apparent increase in susceptibility is simply an expression of a seasonal increase in the pathogenic challenge at a particular time of year (perhaps related to virulence of the pathogen at different water temperatures - see Willoughby and Copland (1984) for a consideration of the growth potential of *Saprolegnia* isolates at different temperatures). This would seem not to be the case because of the resistance of sexually immature fish subjected to the same challenge and also because of the observations of Pohl-Branscheid and Holtz (I 990) that the increase in susceptibility of male rainbow trout to saprolegniasis occurred in populations in which sexual maturation had been advanced or retarded by photoperiod manipulation. There is some evidence to suggest that the increase in susceptibility of male fish is related, at least partly, to elevated androgen levels. Cross and Willoughby (1989) reported that experimental administration of 11 -ketotestosterone increased the susceptibility of rainbow trout to fungal infection and Murphy (198 1) found a decrease in susceptibility of precocious Atlantic salmon parr, *Salmo salar,* when treated with methallibure and cyproterone acetate. drugs which have been shown to reduce plasma 1 l-ketotestosterone levels in this species (Murphy, 1980). Further elucidation of the mechanisms involved in these sex-related changes in susceptibility to fungal infections is given later in the section on defense systems.

The Egg

Salmonid eggs are normally laid in the gravels of spawning streams and are incubated at relatively low environmental temperatures for several months before hatching (see Crisp, 1988, 1990). Thus, the eggs may be subjected to challenge by fungal pathogens for prolonged periods. However, fungal infections of salmonid eggs are primarily associated with the aquaculture industry where large numbers of eggs, often of variable quality and viability, are incubated in relatively high densities. Any dead eggs which are not removed from the incubation system are readily colonized by *Saprolegnia,* although this probably represents saprophytic colonisation by a range of fungi from the family Saprolegniaceae. The fungal pathogen colonizing brook char, *Salvelinus fontinalis, eggs was* reported as S. *diclina* (Rand and Munden, 1992) whereas S. *diclina* and S. *ferax* was identified on rainbow trout eggs (Smith et *al.,* 1985). Careful removal of dead eggs or the use of prophylactic antifungal flushes (see Alderman, Chap. 6, this volume) will normally protect the living eggs under such circumstances. No data are available on the susceptibility of wild salmonid eggs in the spawning gravel. Kudo and Teshima (1991)

found that extracts of the fertilization envelope of fish eggs had antifungal properties but the importance of this as a protective mechanism is unknown.

Smoltification

Salmonids exhibit a spectrum of migratory types ranging from wholly freshwater species that are relatively sedentary in their habits to forms that invariably migrate between lakes and rivers, rivers and oceans or river, lakes and oceans. Smoltification, or smolt transformation, is the process by which the juvenile fish from a wide range of salmonid species undergo a series of physiological and behavioural changes which effectively transforms the fish from resident, freshwater forms to migratory fish fully equipped to cope with the demands of a marine environment (see Barron, 1986 for details of the endocrine control of smoltification). The timing and extent of this process is variable and has been well-described by Hoar (1976). Although few published data exist, feedback from experienced salmonid farmers indicates that the process of smoltification is associated with an increased susceptibility to disease. The problem is exacerbated if fully transformed smolts are held in fresh water beyond the time of their normal downstream migration and under such circumstances, *Saprolegnia* infections can become established. Thus, as with the eggs (see above), saprolegniasis during smoltification is a problem primarily associated with the aquaculture industry.

Environmental Stress

It has been argued that smoltitication (see above) results in environmental stress because the freshwater habitat becomes less hospitable than the marine environment (Langhome and Simpson, 1986). Irrespective of the validity of this argument, there can be little doubt that most forms of environmental stress (social, physical, chemical) can and do predispose salmonid fish to a wide variety of diseases (see Columbo et *al., 1990* for a full discussion of the concept of environmental stress and its relevance to aquaculture). However, direct evidence of stress-induced predisposition to saprolegniasis *per se* is limited.

In a study of an annual outbreak of saprolegniasis in brown trout, White (1975) reported that the infection zone was characterized by an increase in the large amount of organic debris as a result of a sharp decrease in flow rate because of no irrigation demand from an upstream dam during the fall and winter. Whether this change in the environment acted as a stress on the fish or whether it simply provided a suitable substratum for the saprophytic growth of potentially pathogenic strains of *Saprolegnia* was not resolved. Working with non-salmonids. Toor et *al.* (1983) also showed that high organic loading, in the form of farmyard or poultry manure, was capable of increasing the susceptibility of fish to S. *parasitica.* Using a more controlled experimental design, Carballo and Munoz

(1991) have recently demonstrated that 10 days exposure of rainbow trout to sublethal levels of ammonia and or nitrite increased their susceptibility to an experimental challenge of *S. parasitica* spores. Taking all these studies into consideration, it is reasonable to conclude that the decreased water quality from high organic loads is a predisposing factor to fungal infection.

Schaefer *et al*. (1981) reported on a major post-spawning mortality of Lake Superior rainbow smelt, *Osmerus mordax* (Family Osmeridae), and concluded that "the most probable cause of the die-off was temperature stress on spawning smelt in the spawning areas which increased the susceptibility of smelt to the fungus *Saprolegnia* sp. and may have promoted osmoregulatory imbalance. . . . the surface water temperature in the harbour *(en route* to the spawning grounds) was apparently 15°C, about 6°C warmer than the nearshore waters of the lake proper."

In a study of the effects of androgen treatment on the susceptibility of S. *parasifica,* Cross and Willoughby (1989) found that the social interaction resulting from the co-confinement of two or three fish markedly increased the susceptibility of one occupant of each tank (presumably the submissive fish - see Pottinger and Pickering, 1992).

DEFENSE SYSTEMS

This section will briefly describe the various defense systems available to salmonid fish to facilitate their resistance to challenge by pathogenic strains of *Saprolegnia* and, by reference to those periods of enhanced susceptibility (sexual maturation, smoltification, environmental stress - see above), will illustrate how each of these systems may be compromised under particular circumstances.

Epidermal Mucus

The external surface of salmonid fish is covered by a living epithelium capable of secreting copious amounts of mucus. In salmonids, the epidermal goblet cells are largely responsible for the production of this layer of slime, although secretions from other cell types (including the superficial Malpighian cells) may also contribute (see Whitear, 1986). It has been argued (Pickering and Willoughby, 1982) that the continuous replenishment of the mucous layer on the surface of a fish acts as an effective physical barrier to remove potential pathogens from the epithelium. Figure 2 compares the results of four independent studies illustrating the rate of disappearance of experimentally-applied, viable S. *parasitica* spores from the surface of rainbow trout and brown trout. Despite the methodological variations between the different studies, all four show a similar rate of disappearance of spores from the mucous layer. Pickering and Willoughby (1982) reported a similar rate of removal of inert particles (starch grains) and, therefore, it

is concluded that the physical removal of fungal spores by the continuous secretions of mucus is an effective defensive barrier. However, Figure 2 also shows that a small number of potentially pathogenic spores do remain in the mucous layer for periods in excess of 24h. This raises the questions of fungicidal or fungistatic properties of the secreted mucus as an additional defense system.

Certainly, fish mucus contains a wide range of different molecules (immunoglobulins - Bradshaw *et al.*, 1970; Itami *et al.*, 1988; lysozyme - Lindsay, 1986; chitinases - Lindsay, 1986; proteases - Braun *et al.*, 1990; haemagglutinins - Kamiya *et al.*, 1988, molecules which alone or in combination might inhibit spore germination and fungal growth. In practice, however, no convincing evidence has been published to

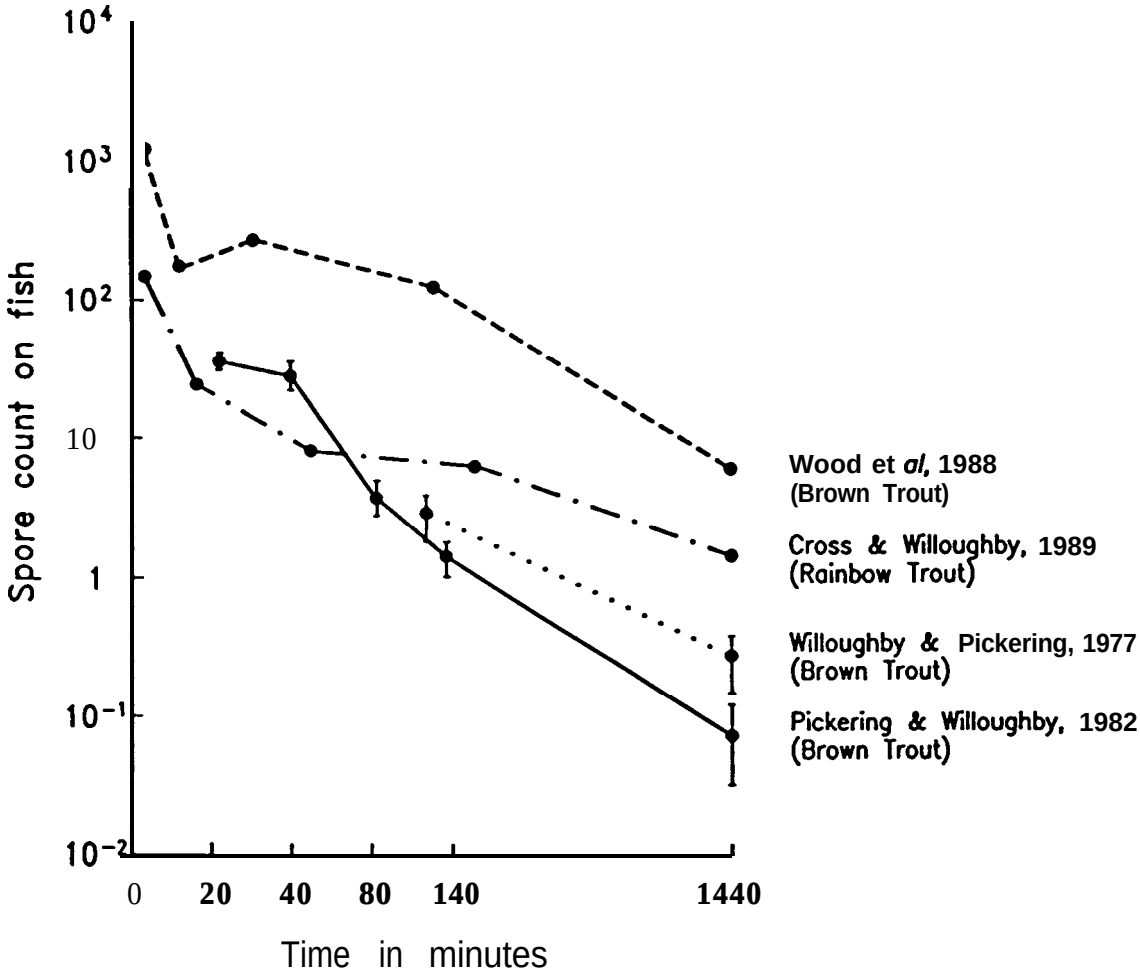

Figure 2. A comparison of the rates of disappearance of viable *Saprolegnia parasitica* spores from the epidermal mucous layer of salmonid fish. Data were obtained from the four separate studies listed on the diagram.

indicate that freshwater fungi are inhibited in this way. Indeed, isolated fish mucus has been shown to be an effective growth medium for pathogenic strains of S. *parasitica* (Willoughby *et al.*) 1983). Thus, the evidence to date indicates that the mucous layer acts primarily as a physical barrier to prevent colonisation.

The rate of secretion of mucus is dependent upon both the turnover rate of the epidermis and the secretion rate of individual cells (see Pickering, 1976). Direct measurement of the overall secretion rate of a fish has never been satisfactorily achieved but several studies have shown that the degree of mucification of the salmonid epidermis (i.e. the number of mucus-secreting goblet cells) is variable and sensitive to both internal and external stimuli. It is interesting, for example, that the enhanced susceptibility of sexually mature male salmonids described in the previous section coincides with a decrease in epidermal mucification (Pottinger and Pickering, 1985a). This reduction in the number of goblet cells in caused by elevated levels of the male steroid hormone, 1 l-ketotestosterone (Pottinger and Pickering, 1985b). Moreover, Richards and Pickering (1978) showed that fungal infection of hatchery-reared brown trout was more prevalent on the fins than on the rest of the body surface, corresponding to the reported decreased concentration of goblet cells on the fins (Pickering, 1974). This circumstantial evidence linking decreased mucification with increased susceptibility to fungal infection is consistent with the hypothesis that the mucous layer is an effective physical barrier.

Physical forms of stress, such as handling, have been shown to *increase* epidermal mucification in salmonid fish (Pickering and Macey, 1977) although the biological significance of this response is uncertain in view of the time delay (up to one week) between the stimulus and the peak expression of the response. Recently, Christiansen *et al.* (1991) have reported that sustained exercise significantly increases the degree of epidermal mucification in the Arctic char and Mikhaylenko (1990) found that training through sustained exercise improved the resistance of juvenile Atlantic salmon to saprolegniasis. However, whether these two papers should be linked together in this relatively simplistic way remains to be investigated.

Epidermal Integrity

Most experimental studies of the transmission of saprolegniasis have necessitated abrasion of the surface of the fish to achieve successful infection, thus indicating the importance to the fish of an intact epidermis in resisting colonisation by the fungus. Evidence from studies of sexually mature salmonid fish also emphasises the relationship between physical damage and saprolegniasis. White (1975) suggested that the annual outbreak of fungal infection in a population of wild brown trout followed wounds induced by spawning activities (female redd-digging and male territorial defense) and provided supporting evidence for this hypothesis from a study of the distribution of the fiutgal-infected lesions. This was confirmed by Richards and Pickering (1978) who

showed that large areas of the body of maturing, male brown trout were more frequently infected than the corresponding areas on the female fish. Clearly, in male fish it is difficult to distinguish the effect of androgen-induced demucification (see above) from that of damage caused by fighting during territorial defense as factors predisposing the fish to saprolegniasis. In the female fish. the situation is a little clearer because epidermal demucification does *not* occur. The caudal and anal fins of sexually mature female fish are more frequently infected than the male fish (Richards and Pickering, 1978) and it is precisely these areas that are used for redd-digging in the gravels of spawning streams. Abrasion to this area of the body is unavoidable. Hatai and Hoshai (Chap. 4, this volume) show that destruction of the epidermis overlying the adipose fin is an important predisposing factor for saprolegniasis.

The process of wound-healing has been described by Bullock *et al.* (1978) and attention has been drawn to the fact that epidermal migration, to close any minor breach in the integument. occurs almost immediately. However, Roubal and Bullock (1988) reported that some of the later stages of wound-healing, including fibrosis of the underlying dermis. are suppressed when plasma cortisol levels are experimentally elevated. Cortisol is a key hormone in the stress response of fish, and, therefore, any form of chronic stress could increase the susceptibility of fish to saprolegniasis by means of this suppressive effect on wound-healing. However, cortisol also has many damaging side-effects on the internal defense systems of salmonid fish (see below).

Internal Defences

Like higher vertebrates, teleost fish are equipped with a sophisticated immune system with both specific and non-specific components including phagocytic cells, a complement system, specific antibody in both serum and mucus, and cell-mediated immunity (see Manning and Tatner (1985) for further details). However, very little of the extensive literature on fish immunology deals specifically with the response to fungal infections. Early studies have demonstrated the occurrence of precipitating antibodies to *Saprolegnia* (Hodkinson and Hunter, 1970) but the widespread occurrence of "natural antibodies" which may react with a variety of microbial extracts (Ingram, 1980) complicates the picture. For example, Davies and Lawson (1985) describe a serum precipitin in Atlantic salmon capable of precipitating with extracts of S. *diclina.* This molecule bears no resemblance to any immunoglobulin, it reacts with a variety of carbohydrates (such as soluble starch and amylopectin) and its role in the defense systems of salmonid fish is not clear. Sohnle and Chusid (1983) examined the internal defences of rainbow trout against fungi of the family Saprolegniaceae but were unable to demonstrate any precipitating antifungal antibody or any plasma factor capable of inhibiting fungal growth. However, they did provide evidence of an inflammatory response to subcutaneous inoculation with fungal filaments, with a high proportion of neutrophils at the inoculation site. Moreover, inflammatory cells were found to adhere to the surface of fungal hypae removed from the

inoculation site after 24-72 h. Wood *et al.* (1986) noted a similar adhesion of cells to fungal colonies removed from infected brown trout. Interestingly, much of the mycelium showed signs of lysis in the vicinity of the attached inflammatory cells. It may be concluded from this rather limited information that non-specific defense systems, utilizing phagocytic inflammatory cells such as neutrophils and macrophages, are important factors protecting the fish against fungal infection but evidence of a specific immune response with elevated litres of circulating antibodies is equivocal. Clearly, this is an area of research which merits further attention.

Evidence was presented in the section on environmental stress to show that environmental stresses such as sublethal pollution, temperature shock and social interaction are all capable of predisposing freshwater fish to saprolegniasis. All three forms of stress are known to stimulate the hypothalamic-pituit-interrenal axis of salmonid fish, resulting in the elevation of circulating plasma cortisol levels. Cortisol, a steroid hormone, plays an important role in energy mobilization, respiration and osmoregulation when fish are faced with a stressful situation (Pickering, 1993) but it is also a known, potent immunosuppressant in salmonids (see Maule *et al.*, 1987; Kaattari and Tripp, 1987; Bennett and Wolke, 1987). Thus, it seems probable that elevation of plasma cortisol. acting via suppressive effects on both specific and non-specific components of the immune system, is largely responsible for the stress-induced increase in susceptibility to *Saprolegnia* infection reported above. This hypothesis is supported both by circumstantial and by direct evidence. Thus, during sexual maturation (a time at which salmonid fish of both sexes are particularly susceptible to fungal infection - see the section on sexual maturation) plasma cortisol levels are chronically elevated and blood lymphocyte counts are reduced (Pickering and Pottinger, 1987). Similarly, another period of enhanced susceptibility. smoltification, is characterized by prolonged plasma cortisol elevation (Specker and Schreck, 1982; Barton *et al.*, 1985; Langhorne and Simpson, 1986) together with a demonstrable immunosuppression (Maule *et al.*, 1987). Direct evidence of a cortisol-induced increase in the susceptibility of freshwater fish to fungal infections was first provided by Roth (1972), although the doses of hormone used were almost certainly pharmacological. However, Pickering and Duston (1983) showed that the oral administration of physiological doses of cortisol to brown trout markedly increased the susceptibility of the fish to *Saprolegnia* infection. More recently, Pickering and Pottinger (1989) demonstrated a clear dose-dependent relationship between experimentally-elevated plasma cortisol levels in brown trout and the mortality rate due to disease (saprolegniasis, fin-rot and furunculosis), with effects that were detectable at plasma cortisol levels well within the physiological range for the species. The weight of evidence, therefore, would suggest that any environmental stress capable of chronically elevating plasma cortisol levels in salmonid fish will also increase their susceptibility to fungal infection. Practical approaches to minimizing the magnitude and duration of the cortisol response under aquaculture conditions are outlined in Pickering (1992).

CONCLUSIONS

Saprolegnia parasitica is the principal pathogen involved in saprolegniasis of salmonid fish although other *Saprolegnia* species have occasionally been implicated. The fungus can act either as a primary pathogen or as a secondary colonist on damaged or diseased tissues. On larger fish, lesions are usually restricted to the integument and superficial musculature and tissue damage occurs from the action of lytic enzymes released by the fungus. The most probable cause of death is lethal haemodilution. Saprolegniasis is restricted to the freshwater phases of the salmonid life cycle and fish are particularly susceptible during smoltification and during the period of sexual maturation. FungaI infection of salmonid eggs generally originates as a saprophytic colonization of dead eggs and is not thought to be a problem in the wild.

Mucus secretion by the epidermis of the fish acts as an important physical barrier preventing colonization but minor injuries to the skin can act as foci for fungal infection. Internal defences depend heavily upon the presumably non-specific, phagocytic activity of inflammatory cells but evidence for a specific immune response is equivocal. Various forms of environmental stress can predispose salmonid fish to *Saprolegnia* infection and it is likely that this is mediated by means of a stress-induced elevation of the steroid hormone, cortisol. Cortisol is markedly immunosuppressive in salmonids, it can inhibit certain aspects of the wound-healing process and experimental administration of cortisol to otherwise unstressed fish increases their susceptibility to fungal infection. The periods of sexual maturation and smoltification are also characterized by chronically elevated plasma cortisol levels. Minimizing environmental stress, by maintaining the highest standards of fish husbandry, will do much to control saprolegniasis under aquaculture conditions.

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SECTION II

PATHOGENICITY

chapter 4

PATHOGENICITY OF *SAPROLEGNIA PARASITICA* COKER

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Gen-Ichi Hoshiai[2]

ABSTRACT

This paper describes the pathogenicity of *Saprolegnia parasitica* isolated from coho salmon, *Oncorhynchus kisutch* and the relationship between this pathogenicity and secondary zoospore cyst ornamentation. The changes in the number of mucous cells and in the epidermal thickness of the adipose fin after "ami-momi" treatment are discussed. Changes in levels of total protein, sodium, chloride, and osmotic pressure in serum after the treatment are presented. From these results, it is concluded that coho salmon die due to saprolegniasis because of the breakdown in osmotic balance when the tissue is destroyed by the penetration of the hyphae.

Key words: *Saprolegnia,* pathogenicity, coho salmon, zoospore cyst ornamentation, "ami-momi" treatment.

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INTRODUCTION

In Japan. saprolegniasis is the most problematic fungal disease affecting salmonid fish cultured in freshwater ponds (Hatai *et al.*. 1977a. 1977b; Hatai, 1980). One of the most popular of these fish. coho salmon *(Oncorhynchus kisutch* Walbaum) is reared in freshwater ponds until reaching weights of 150 to 200 g. The fish are moved to seawater, where they are kept in floating net pens for approximately 10 months until reaching a marketable weight of 1.5 Kg or more. Since 1985 the number of coho salmon cultured has increased steadily. reaching 17.000 metric tons in 1988. Over 90% of that production originates in Miyagi Prefecture in the north of Japan. Each year since 1985. epizootics of saprolegniasis have been identified in freshwater-cultured coho salmon in Miyagi Prefecture. Japan. The disease generally occurs in immature fish with body weights of 20-60 g in the period from May through July with mortality exceeding *50%*. *Saprofegnia parasitica* Cokcr is predominant in the lesions of coho salmon with saprolegniasis. S. *diclina* Humphrey (syn. *S. diclina* Type 3) has also been isolated in very rare cases (Hatai and Hoshiai. 1992).

This study describes the pathogenicity to coho salmon of two strains isolated from the lesions of coho salmon which have saprolegniasis. The relationship between pathogenicity and secondary zoospore cyst ornamentation, specifically the lengths of the hooked-hairs on the zoospore cyst is described. Changes that occur in the number of mucous cells and in the epidermal thickness of the adipose fin after the treatment are discussed. There is also a discussion of changes in the coho salmon's blood constitution after the "ami-momi" treatment, and the way in which saprolegniasis induces death.

MATERIALS AND METHODS

Strains Used In This Study

The predominant isolated strain. NJM 8604 (referred to *as* H2) of *Saprolegnia parasitica* Coker (syn. *S. diclina* Type 1) and the much rarer strain, NJM 0005 (referred to as H3) of *Saprolegnia diclina* Humphrey (syn. S. *diclina* Type 3) were used for both artificial infection experiments and to measure hooked-hair lengths. They were isolated on 9 July 1986 from infected sites on the body surface of coho salmon with saprolegniasis (Hatai and Hoshiai. 1992). These strains were used for two reasons: first. secondary zoospores of isolate H3 germinate directly in hemp seed dish water while those of isolate H2 germinate indirectly; and second, isolate H2 has long hooked-hairs on the secondary zoospore cyst, while isolate H3 does not (Hatai and Hoshiai, 1992). Some experiments used only isolate H2.

Fish Used In These Experiments

Coho salmon, *Oncorhynchus kisutch*, averaging 20-40 g (mean 70 g for blood examination) in body weight, grown in the Miyagi Prefectural Freshwater Experimental Station, were used in the experiments. These were carried out in an acrylic vat (30 x 60 x 36 cm, 27 cm deep) placed in a 2-ton FRP tank with water temperatures at a constant 12-13°C. The water was changed every three days for the first 10 or 12 days depending on the course of experiments.

Artificial Infection

To ensure lesions occurred on a variety of body surfaces, and to cause appropriate levels of internal stress, the "ami-momi" procedure was used as follows: The fish were not fed for a few days after which they were divided into groups of 10 fish each and shaken in the air in a fan-shaped scoop net (3 1.5 x 30.0 x 35.0 cm; tetron Russel; no knot; mesh size, 5 mm) for two minutes. This shaking process is the "ami-momi" treatment. After treatment, the fish were exposed to the zoospores of each isolate. Each vat held about 48 L with 2 x 10^5 zoospores/L. The water was changed every three days during the 10 day course of experiment. The zoospore suspensions of each isolate were prepared as follows. First H2 and H3 hyphae, cultured on GY agar (Hatai and Egusa, 1979) were cut. together with the agar, into pieces about 8 mm x 8 mm. Two pieces were cultured for six days at 15°C in 1,000 ml Erlenmeyer flasks containing 500 ml of the GY liquid medium (glucose, 1%; yeast extract, 0.25%). Zoospores were obtained by first washing the mycelia in two successive baths of sterilized well water then transferring them to a third bath of sterilized well water, where they were incubated for three days at 15°C. The zoospores were counted by using the Bürker-Türk hemocyte counting plate.

Measurement of Hook-Hair Lengths

This test was conducted to clarify the relationship between the pathogenicity to coho salmon of the isolates H2 and H3 and the lengths of the hooked-hairs on the secondary zoospore cyst. Each strain was grown in a GY broth (Hatai and Egusa, 1979) for three days at 15°C before zoospores were induced by transfer to tap water, sterilized by filtration through a 220-nm pore membrane filter. Before the transfer, the mycelia were washed in tap water three times. Several formvar-coated copper grids (3.0 mm, 200 mesh) were then placed in the bottom of each Petri dish under the sporulating colonies and left for three days. Before being removed the grids were checked for the presence of encysted spores. Excess water was carefully drawn off using hardened filter paper and the grids were dried. The grids were then examined with a JEM 100 CX-II electron microscope, and the length of hooked-hairs was measured.

Number of Mucous Cells and Thickness of Epidermis.

In batches of 10 the fish underwent "ami-momi" treatment. The fish were designated as either Group A or Group B. Five fish were taken from group A at following intervals: 0 and 30 minutes. 1. 2. 4. 8. 12. and 24 hours; 2. 3. 4. 7. and 10 days. The adipose fin of each fish was removed and fixed in 10% phosphate-buffered formalin solution. The fixed tissues were embedded in paraffin and sections cut 3 to 5 um in thickness. The sections were stained with haematoxylin and eosin (H & E). Grocott's stain, or Periodic-Acid Schiff stain (PAS) (Lennette et al.. 1985). Each adipose fin was observed at 100x magnification using a microscope equipped with an eyepiece micrometer (1 mm^2). The top of the field of vision was adjusted to the tip of the adipose fin. and all the PAS-stained mucous cells in the square were counted. The thickness of epidermis stained with H & E was measured at two predetermined points, and the mean was calculated. Group B was exposed to the zoospores of isolate H2 as described above and only used to observe the progress of the infection.

Measurement of Blood Constituents

Fish were divided into three groups: a control group (group C) which had undergone no treatment and two "ami-momi" treatment groups. Group D was exposed to isolate H2 zoospores after the treatment and group E underwent the treatment but was not exposed to the zoospores. Blood samples form groups C, D. and E were taken from between five and ten fish by syringe from a blood vessel in the caudal peduncle at intervals of 2, 4, 5, and 7 days; 2, 3. 4. and 5 days; and 1. 2. 3, 4. 5. and 7 days, respectively. The level of total protein was measured by using a protein meter (Atago Co.); electrolyte (sodium and chloride) by using an **EKTACHEM DT** 60 (Kodak Co.) and the osmotic pressure in serum was measured with a Vapor Pressure Osmometer 5500 (Wescor Co.).

RESULTS

Artificial Infection

The group exposed to strain H2 quickly exhibited external hyphal growths visible to the naked eye. They were observed in 20% of the experimental fish on day two and 100% after that. The cumulative mortality rate for strain H2 was 0% on day 4. 10% on day 5, 70% on day 7 and 100% by the 11 th day. The clinical signs of the fish infected with S. *parasitica* Coker (H2), which died. closely resembled those observed with naturally occurring saprolegniasis. The moribund fish infected by H2 had serious fungal infections characteristically in the head and caudal peduncle. In contrast, although the group exposed to the strain H3 also exhibited external hyphal growth visible to the naked eye, the hyphae observed tended to disappear with the passage of time and no dead fish were

found in the group. This experiment shows that the strain H2 is highly pathogenic to coho salmon. but H3 is not.

Measurement of Hooked-Hair Lengths

The lengths of the H2 hooked-hairs were clearly different form those of H3. The hooked-hairs on encysted spores ranged in length between 0.35 to 1.09 um for H3 and 0.65 to 4.52 um for H2. This suggests that the length of hooked-hairs is correlated with pathogenicity.

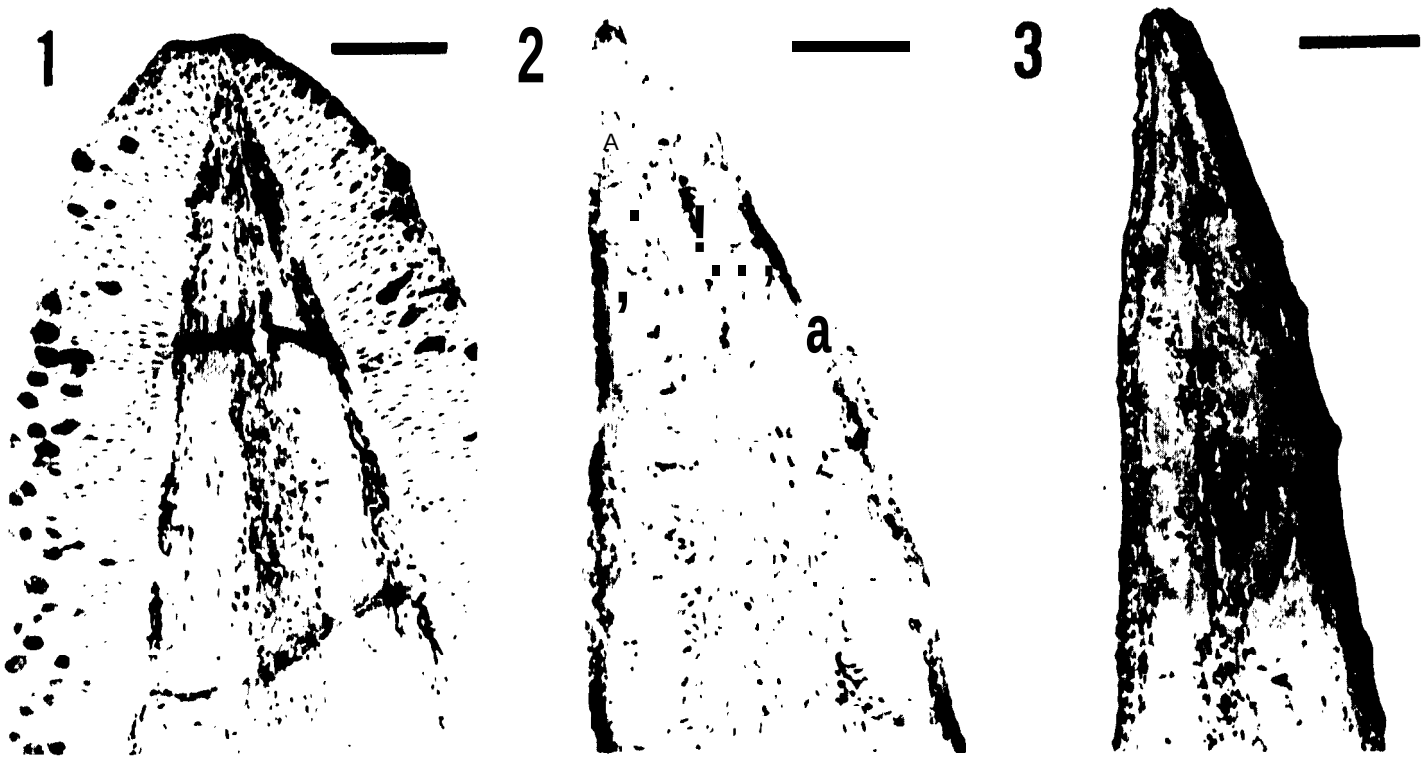
Number of Mucous Cells and Thickness of Epidermis

A normal adipose fin with many PAS stained mucous cells of the thick epidermal layer and before "ami-momi" treatment is shown in Fig. 1. After "ami-momi" treatment. the epidermal layer in the adipose fin has been removed (Fig. 2) and the dermis is exposed to water This condition is considered to be ulcerative. On day 4. a thin epidermal layer has grown back over the adipose fin and some of the mucous have been restored (Fig. 3). The number of mucous cells in the epidermis increases with time and after 10 days approaches that found before treatment (Fig. 4). Before the treatment the mean number of mucous cells from five fish was 56. but this dropped to zero just after the treatment. After that the number gradually increased and returned to 34.6 cells on the last day of the experiment.

The changes with time in the thickness of the adipose fin epidermis before and after "ami-momi" treatment are shown in Fig. 5. Before the treatment. the mean epidermal thickness of 10 fish was 83.6 μm. The epidermis was 0.0 μm thick just after the treatment. and grew back to 46.4 μm by day 11. Mortality rates of coho salmon in group B exposed to zoospores after "ami-momi" treatment were 100% when exposed immediately after treatment (0 h). 90% at 12 h. and 50% at 24 h. However, the rate decreased to 10% when exposed on day 4 and finally 0% by day 10 (Fig. 6). The number of mucous cells on day 4 of "ami-momi" treatment was 26.6 and the thickness of the epidermis was 32 μm. This data suggests that saprolegniasis could occur at anytime prior to 50% recovery of the epidermis. because on day 4 the number of mucous cells was 48% of the control and epidermal thickness was 38% of the control.

Measurement of Blood Constituents

The daily changes of the level of total protein in C. D. and E groups are shown in Fig. 7. In group C. which had undergone no treatment. the levels were 3.6 to 4.5 g/dl during the course of the experiments. However. in group D. which was exposed to zoospores. the levels gradually decreased. and reached 2.3 g/dl by day 6. Most fish died on day 5. In



Figures I-3. Adipose fin of coho salmon. PAS stain. 1. Normal adipose fin with many mucous cells in thick epidermis layer. 2. Adipose fin just after "ami-mom? treatment. The epidermis has been removed and the dermis is exposed. 3. Adipose fin on day 4. Scale bars: 1,2,3 = 100 μm.

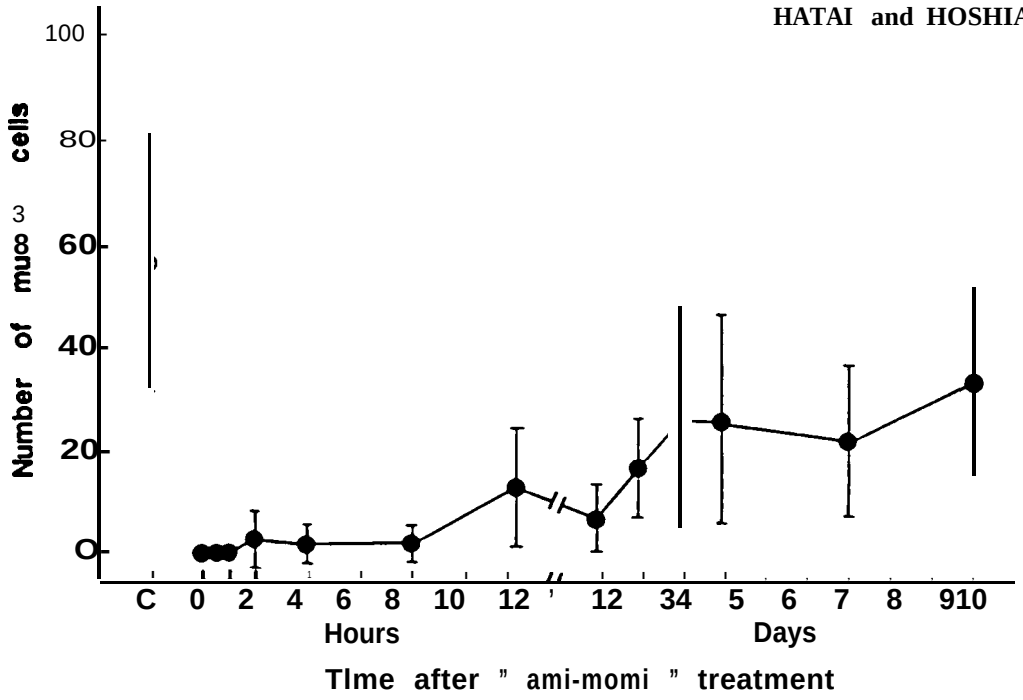
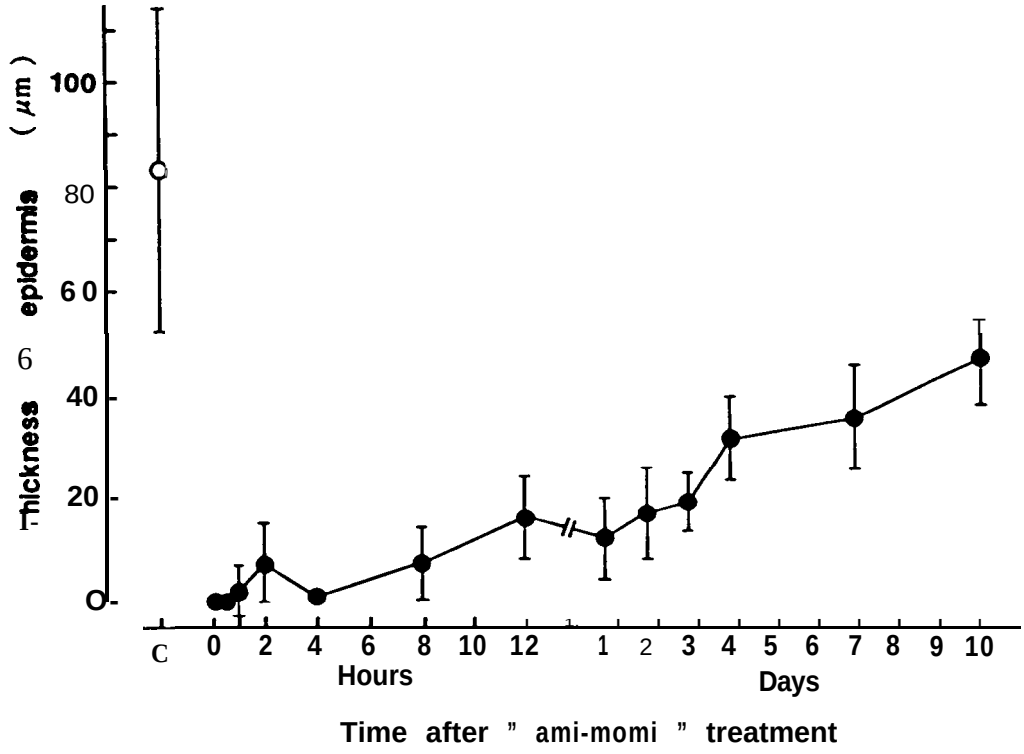


Figure 4. Changes in number of mucous cells in adipose fin of group A coho salmon at given periods after "ami-momi" treatment. C: control (before treatment). Vertical lines indicate mean ± standard error.

Figure 5. Changes in thickness of the adipose fin epidermis in group A coho salmon at given periods after "ami-momi" treatment. C: control (before treatment). Vertical lines indicate mean ± standard error.



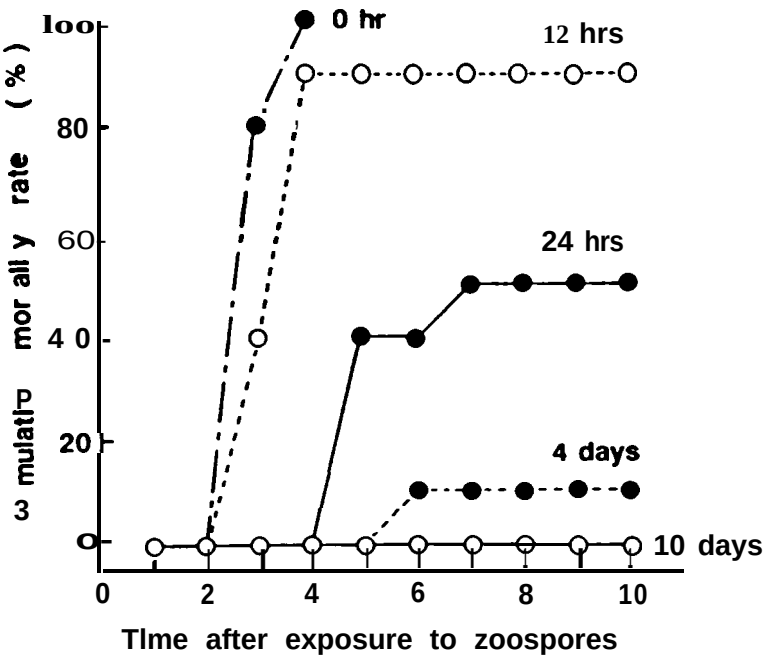
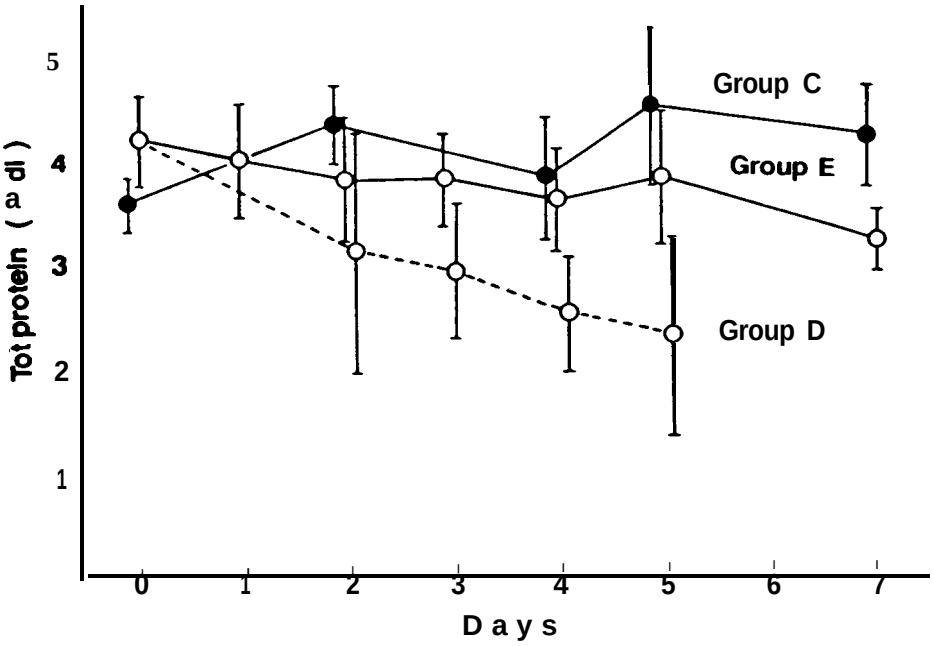


Figure 6. Changes in cumulative mortality rates of group B coho salmon exposed to water with 2 x 10^5 zoospores/L.

Figure 7. Changes in total serum protein levels of coho salmon in groups C, D and E. Group C: control group underwent no treatment. Group D and E: "ami-momi" treatment groups. Group D: exposed to zoospores. Group E: not exposed to zoospores. Vertical lines indicate mean ± standard division.



group E, which was not exposed to zoospores, the levels, changed very little by comparison with group C.

The level of serum sodium found in three groups appears in Fig. 8. Group C remained constant, between 146.8 to 15 1.8 mMol/L during the course of the experiments. In group E, the sodium levels decreased at first, began to increase on day 2: and eventually reached the level of group C. However, the level for group D rapidly decreased, and by day 4 dropped to between 110 and 119.4 mMol/L. Figure 9 shows that the changes in the levels of serum chloride were similar to the changes of serum sodium.

Changes to the levels of serum osmotic pressure are shown in Fig. 10. In group C the levels were 297 to 308 mosmol/Kg throughout the experiments. In group E, although the levels decreased at first, as they had with sodium and chloride, they began to recover on day two, but never reached those of group C. In contrast, the levels of group D rapidly decreased, and reached 258 mosmol/KG after four days, at which time the majority of the fish died.

Overall, the levels of total protein, sodium, chloride, and osmotic pressure in coho salmon artificially infected with *Suprolegnia parasitica* are significantly lower than those in the control fish. These results led us to conclude that the death of coho salmon with saprolegniasis was due to a breakdown in osmotic balance resulting from tissue destruction caused by the penetration of the hyphae.

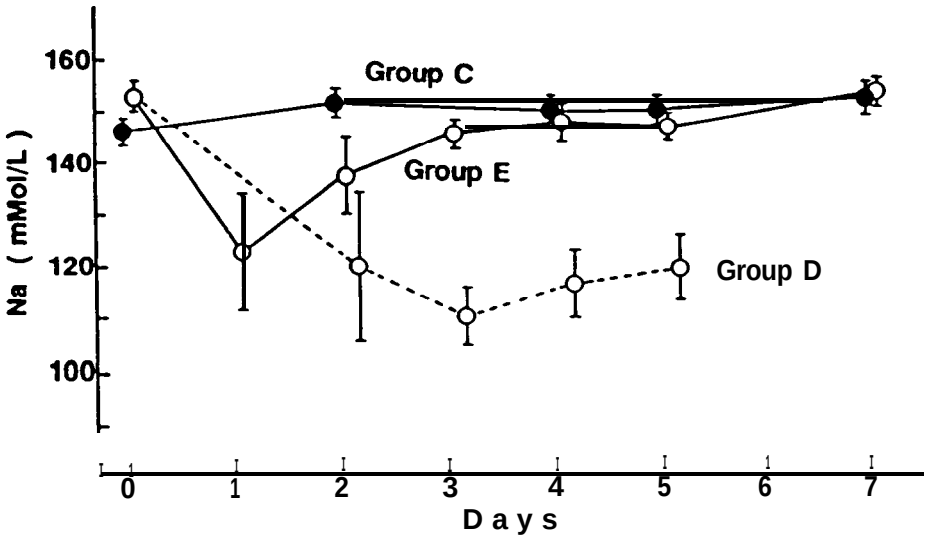


Figure 8. Changes in Na levels of coho salmon in groups C, D and E.

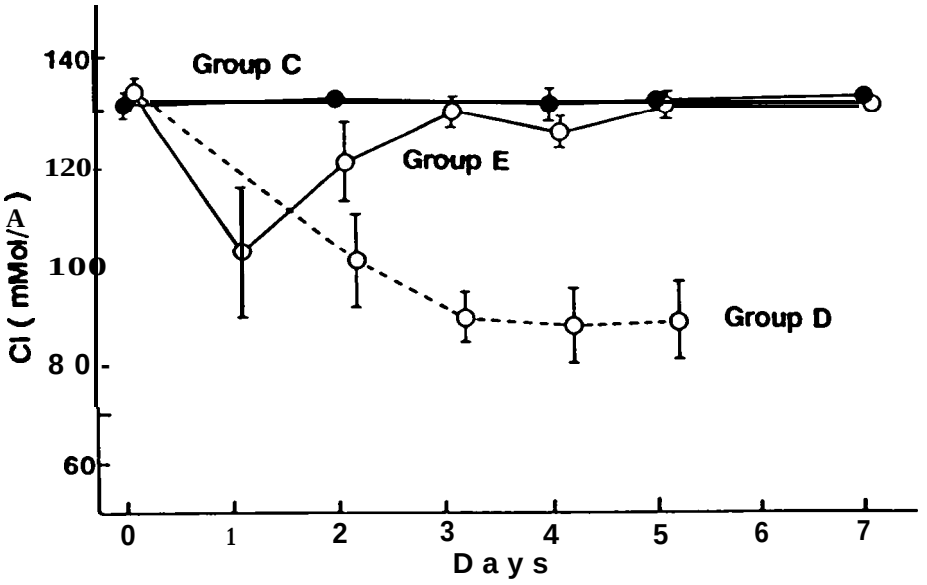
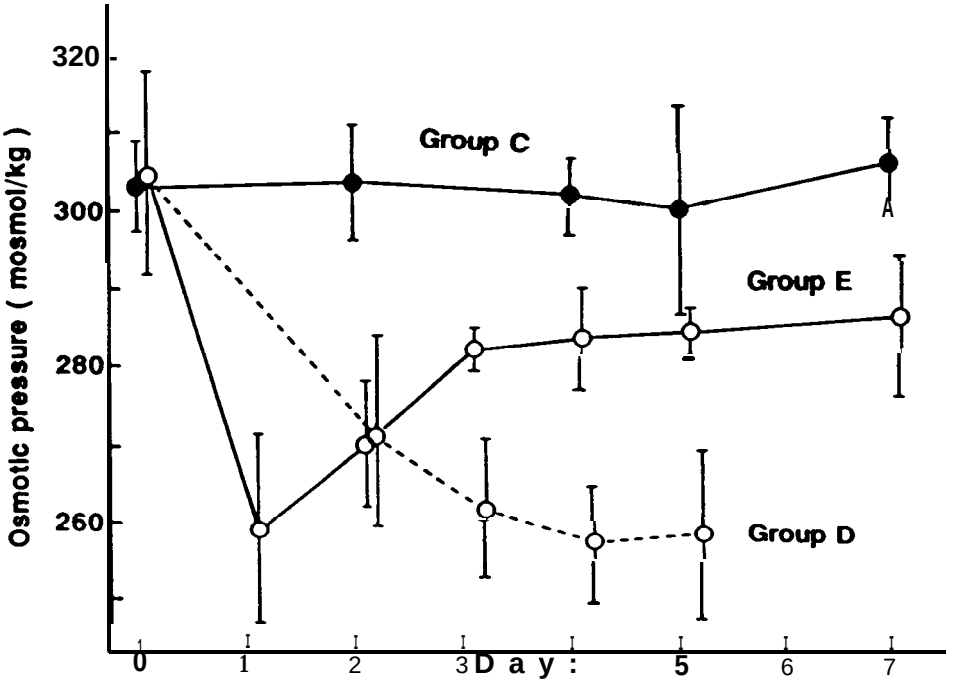


Figure 9. Changes in Cl levels of coho salmon in groups C, D and E.

Figure 10. Changes in osmotic pressure levels of coho salmon in groups C, D and E.



DISCUSSION

Strain H2 (long hooked-hairs) was pathogenic to coho salmon, while strain H3, which had short hooked-hairs, was not. This suggests that the length of hooked-hairs is correlated with pathogenicity. Pickering ef al. (1979) and Beakes (1983) based their conclusion that long hooked-hairs on the surface of encysted *Saproiegnia diclina* Type I spores were related to pathogenicity as a result of their experiments with river and cultured fish and without having access to any data relating to artificially induced infection. Our results, obtained from artificially infected fish, strongly suggest the idea that long hooked-hairs are important for pathogenicity as they enable the structure to float in water for a long time before attaching to a fish's surface. Our experiments demonstrate that "ami-momi" treatment is an excellent procedure for producing the pre-fungal stage (Willoughby, 1970) of artificial *Saprolegniu* infection. The procedure makes it easy to create an ulcerative condition on the surface of coho salmon and produce saprolegniasis. The results indicate a high probability of saprolegniasis if an ulcerative condition occurs on fish surfaces in cultured pond and pathogenic fungi like *Saprolegnia purasitica are* present in the water. This skin condition occasionally occurs, for example, after fish are handled. From our experimental results. it can be seen that fish management for two to four days after handling is of prime importance in preventing such problems. Although the serum total protein in coho salmon exposed to zoospores was less than in the control fish, the same condition, severe hypoproteinaemia, has been reported in brown trout (Richards and Pickering, 1979; Duran et al., 1987) and in Atlantic salmon (Mulcathy, 1969. 1971). The concentration of major ions. sodium and chloride, in the serum of infected fish were significantly lower than usual. Richards and Pickering (1979) also thought the cause of death was osmoregulatory breakdown resulting in a lethal haemodilution. This theory is borne out by the findings related to the reduction of serum osmotic pressure in infected fish demonstrated in this paper.

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chapter 5

ZOOSPORE MOTILITY, ITS LOSS AND RECOVERY, IN AN ISOLATE OF APHANOMYCES FROM A DISEASED FISH IN THAILAND

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RJ. Roberts2

ABSTRACT

Zoospores of *an Aphanomyces* ceased to be motile following physical shock, but motility was regained after 4.5 h. Similarly motile zoospores subjected to chemical shock (high Ca levels) lost and regained their motility after a time interval. These observations led to a study of dehiscence in *the Aphanomyces,* when high Ca backgrounds were present, and a general consideration of "dehiscence solutions" reported for biflagellate fungi.

Key words: *Aphanomyces, Channa,* Epizootic Ulcerative Syndrome (EUS), zoospore mobility.

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The isolate of *aphanomyces* was obtained from an ulcer on a diseased snakehead *(Channa* sp.) fish. which was suffering from the Epizootic Ulcerative Syndrome (EUS) in Thailand. So far this *Aphanomyces* has not produced a sexual state: therefore it cannot be named to species. The disease. which has a fatal outcome. has been widespread in South East Asia for several years and occurs on a variety of both wild and cultivated fish. Personal reports from North East India have suggested that the disease is least likely to occur in fish ponds which have high calcium levels and high pH levels in the water. This aspect is being studied in the UK and during the course of this work it came to light, unexpectedly. that zoospores motility could be arrested, and subsequently revived, following chemical shock. With the realization that these observations might have a more general applicability. to other biflagellate fungi such *as Phytophthora* and *Pythium,* the work was estended to investigate the effect of physical shock also. Many workers have noted that bitlagellate zoospores lose their motility. in zoospore suspensions, as the result of frequent contact with glass surfaces, violent mixing. agitation or forced aeration; e.g. see Ho and Hickman (1967) regarding *Phytophthora megasperma.* Hildebrand, Bimpong and Clerk (1970) and Tokunaga and Bartniki-Garcia (1971) regarding *Phytophthora palmivora.* Butler. Cerenius and Söderhäll (1984) regarding *Aphanomyces astaci,* Schikora. Willoughby and Roberts (1992) regarding *Saprolegnia parasitica* Coker. In addition, loss of zoospore motility induced merely by high dilution of the suspending medium has been reported by Gooding and Lucus (1959) for *Phytophthora parasitica* Dastur and by Ho and Hickman (1967) for *P. megasperma,* although this was not confirmed. by Bimpong and Clerk (1990) for *P. palmivora.* None of this previous work has ever hinted that zoospore motility loss, if it occurs. might be purely temporary, as reported here. The previous work has implied that zoospore encystment is induced. Encystment would involve loss of flagella. and definition of a rigid, possibly ornamented, outer cell wall (see Beakes, 1983).

The *Aphanomyces* isolate (TA l) from Thailand is maintained in serial transfer in glucose-peptone-yeast extract broth (GPY). it having been shown that sub-cultures often fail on solidified media. GPY has (g l^{-1}) glucose, 3; $MgSO_4 \cdot 7_2 0$. 0.13; KH_2PO_4, 0.014; pcptone. 1: yeast extract. 1; also trace micronutrients (mg l^{-1}) Ca. 8: Fe. 0.5: Mn, 0.5; Cu, 0. l : Zn. 0.1. Unless otherwise stated. all incubations are at 25°C, since this temperature is appropriate ecology. To obtain motile secondary zoospores, the mycelium is grown in 30 ml of GPY in a 100 ml flask for 96 h. then divided into six equal portions, each of which is given six consecutive washes in paper-filtered. autoclave-sterilized, Lake Winderrnere water (SL). see Table 1. In the context of this present contribution, Ca at 7.1 mg 1-t is the most relevant constituent of SL. The washed mycelium portions are each placed in 30 ml of SL. in polystyrene Petri dishes. and after 18 h, prolific dehiscence and release of motile zoospores has occurred. giving assays of about $l\ 0^4$ ml^{-1}. Motile zoospores are filtered out through double-thickness Whatman 541 paper and used. as suspensions in SL, in the esperiments. In order to minimize zoospore shock, in mixing. experimental solutions of chemicals arc made up in SL. rather than distilled water. Experiments are conducted in 25-30 ml volumes, in polystyrcnc Petri dishes and the results arc obtained from direct

microscopical observations on the whole dishes or additionally following removal of small aliquots from these.

TABLE 1 Major ions (mg l^{-1}) in Lake Windermere water (SL after autoclaving).

Na	K	Ca	Mg	Cl	NO_3	SO_4	PH
5.0	0.7	7.1	1.1	8.6	1.6	16.4	7.1

Experiment 1 (Table 2) investigated the effect of dilution on zoospore suspensions. The dilution mixtures were made gently and incubated at 22.5°C. During the first 6 h, observations were continuous, giving the records shown, and there was no disagreement with this pattern at 23 h. The results indicated that high dilution, with a suitable water, did not inhibit zoospore motility. Using paper-filtered but unsterilized Lake Windemere water, freshly collected, then the results (not reproduced) were essentially the same as those shown for SL. When A.R. grade pure water (B.D.H.) was the diluent, and it dominated the mixture, then zoospore motility was inhibited permantently (Table 2). This inhibition might be ascribed either to loss of ions from the zoospores or to an unfavorable back-ground pH.

TABLE 2. The effect of dilution on zoospore motility in *Aphanomyces;* + or – indicate motile zoospores observed or not, respectively.

	Zoospore suspension dilution factor				
	l/2	l/4	l/l0	l/100	l/1000
Motility-Diluent SL water	+	+	+	+	+
Motility-Diluent AR water	+	+	–	–	–
Final pH-Diluen SL water	6.5	6.0	6.0	7.0	7.0
Final pH-Diluen AR water	6.0	5.7	5.5	5.5	5.5

Experiment 2 (Fig. 1) investigated physical shock. Two 30 ml aliquots of motile zoospores were used, each in a 100 ml conical flask. Three glass microscope slides were broken into fragments and added to each of the flasks, one of which was agitated by hand for 3 minutes while the other (control) was left undisturbed. The flask contents were poured into polystyrene Petri dishes and observations began immediately. 0.1 ml aliquots were removed and placed on another polystyrene Petri dish. Using low magnification with the microscope. a working distance of 2 cm and dark-field illumination, counts were made of motile zoospores present in one field of view, area 4.5 x 10^3 sq μ. In making these counts the assumption held that if one motile zoospore left the field. then another would replace it. The results showed that zoospores from the flask which had been agitated lost their motility immediately, but it was largely regained after 4.5 h. Zoospores derived from the un-agitated flask retained their motility throughout the experiment. Examinations of the bottom surfaces of the experimental Petri dishes during the 4.5 h showed that some zoospores become encysted and germinated there in the control dish, derived from the un-agitated flask, but none encysted in the dish derived from the agitated flask.

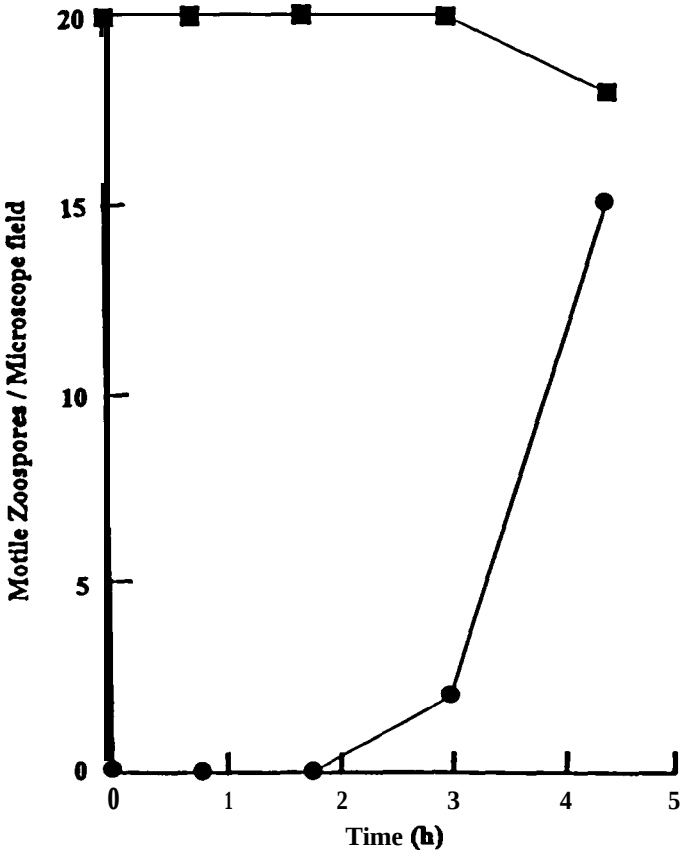

FIGURE 1. Revival of zoospore motility in *Aphanomyces,* following agitation with broken glass. ●, suspension from agitated flask; ■, suspension from un-agitated flask.

Experiment 3 (Table 3) investigated chemical shock. 25 ml aliquots of zoospore suspension were used. Whole Petri dish observations showed that if $CaCl_2$ had been added to SL as 100-200 mg l^{-1} Ca, then motility was lost initially but regained after 4 h. Using $CaCl_2$ at the 400 mg l^{-1} Ca level of addition, motility was lost and never regained.

TABLE 3. Revival of zoospore motility in *Aphanomyces,* using calcium chloride solutions. ++, +, -) indicate many, few, or no motile zoospores observed, respectively.

	Time (h) from start of experiment				
Calcium (mg l^{-1}) additions to SL	*0.33*	*0.8*	*2.0*	*4.0*	*5.5*
0	++	++	++	++	++
25	++	++	++	++	++
50	-l-	*i*	+	+	++
100	–	–	–	+	++
150	–	–	–	+	+
200	–	–	–	+	+
400	–	–	–	–	–

Experiment 4 (Table 4) also investigated chemical shock. 25 ml aliquots of motile zoospore suspension were used. Whole Petri dish observations showed that the calcium salts chloride, gluconate, lactate and succinate, added to SL at the 100 mg l^{-1} level, all inhibited motility initially, only for it to be regained after 4.5 h. Where Ca had been added at the 400 mg l^{-1} level, zoospore motility was not regained with chloride, but it was with the other calcium salts.

Experiment 5 (Table 5) investigated dehiscence. It had become of interest to determine whether or not dehiscence would occur, culminating in release of secondary motile zoospores, in the calcium solutions which had been used to inhibit zoospore motility. Accordingly washed mycelium, in equal amounts, was incubated in Petri dishes, in SL containing the different calcium salts, for 18 h. By this time dehiscences had

occurred and secondary motile zoospores were present in all the dishes, although it was obvious that there were numerical differences here. The zoospore suspensions in the dishes were predominantly of motile cells, although some individual, detached, primary zoospore cysts were also present. However, this complication was ignored in making assays in Sedgewick-Rafter chambers. These showed that when Ca was at 100 mg l^{-1}, chloride, gluconate and succinate were all conductive to good motile zoospore production, with gluconate particularly so. In contrast, lactate at 100 mg l^{-1} Ca gave poor production. At 400 mg l^{-1} Ca, only gluconate gave good motile zoospore production. Observations indicated that the development block for the poor producers was in the actual dehiscence process. Very few groups of encysted, primary zoospores, at the open ends of the empty sporangia, were seen in these particular dishes. On the basis of the results obtained, SL plus 100 mg l^{-1} calcium, as calcium gluconate, would appear to be a better "dehiscence solution" (see below) that SL alone, which had been used hitherto for the *Aphanomyces.*

TABLE 4 Revival of zoospore motility in *Aphunomyces*, using calcium salts. The numbers for calcium salts indicate mg-1 Ca added. +, -, indicate motile zoospores observed, or not, respectively.

Calcium additions to SL	Zoospore motility at 0.5h	Zoospore motility at 4.5h	Maximum linear growth of mycelium from germinated zoospores at 24h (um)	PH at 24h
none	+	+	167	7.0
Chloride 100	-	+	120	7.0
Chloride 400	-	-	33	7.5
Gluconate 100	-	+	140	7.5
Gluconate 400	-	+	113	7.5
Lactate 100	-	+	153	7.0
Lactate 400	-	+	100	7.8
Succinate 100	-	+	253	7.3
Succinate 400	-	+	320	8.0

TABLE 5 Dehiscence in *Aphanomyces*, using mycelium placed in various calcium salt solutions for 18h. The numbers for calcium salts indicate mg 1 -I Ca added. Ranges are numbers of motile zoospores per micro-litre.

Calcium additions to SL	Numbers of motile zoospores ul^{-1} Range	Mean ± S.D.	
None	19 - 35	27.0 ± 4.9	(10)
Chloride 100	18-28	23.4 ± 2.9	(9)
Gluconate 100	38 - 56	74.0 ± 6.0	(7)
Lactate 100	3- 13	7.7 ± 2.9	(9)
Succinate 100	21-37	29.0 ± 5.2	(9)
Chloride 400	O-5	1.0 ± 1.6	(15)
Gluconate 400	24-33	28.6 ± 2.9	(9)
Lactate 400	o - 4	1.3 ± 1.5	(10)
Succinate 400	o - 2	0.3 ± 0.6	(10)

This work has shown that high dilution of a zoospore suspension, with water of the same chemical nature as that already present, does not inhibit zoospore motility; i.e. contrary to some reports in the literature, the actual process of dilution is not deleterious. However, were this dilution to be done physically roughly, then the situation might well be one of physical shock, leading to temporary loss of zoospore motility. In this connection it is encouraging that in Experiment 3, zoospore motility was not arrested initially in the Petri dishes where the maximum Ca addition was only 50 mg l^{-1} (Table 3). This suggests that there was no deleterious physical shock when Experiment 3 was set up; the experiment related to the effect of chemical shock only.

Previous work with another member of the Saprolegniaceae, *S. parasitica*, showed that unless motile, secondary zoospores became encysted at a suitable surface they could not capitalize, by germination and growth, in a weak nutrient solution such as Sl (Willoughby, 1986). Thus when motile zoospores were placed in SL in a polystyrene Petri dish, some soon encysted, germinated and grew well, at the dish bottom: whereas if motile zoospores were placed in SL in a glass Petri dish, then encystment and germination did not occur. These findings can be considered in relation to this current work on *Aphanomyces*. Generally speaking the *Aphanomyces* zoospores, in contrast to

those of *S. parasitica*. have not encysted and germinated well on polystyrene surfaces and in the low nutrient solutions such as SL used here. However, where germination did occur. subsequent growth was not inconsiderable; it was even shown that some of the calcium compound ions, such as succinate. could be used to enhance this growth (Table 4). Considering zoospore encystment of the *Aphanomyces* further, in the context of this current work. it was of great interest that zoospores with their motility temporarily destroyed by agitation. did not encyst at the polystyrene dish bottom at all, during the course of Experiment 2. Evidently a potentially receptive surface must be approached by the motile zoospore (not an inert one) if successful encystment and attachment is to occur. The observations made provide strong evidence that restoration of motility to individual zoospores actually occurred in this experiment; the restoration was not the result of repeated emergence from zoospores which had first been induced to encyst. Similar observations were made in the calcium addition experiments. leading to the same conclusion. that the restoration of motility observed was a valid, direct event.

From the preceding discussion. it seems a reasonable deduction that zoospore motility is an essential first part of the infection cycle for the biflagellate fungi which are pathogenic. Therefore conditions conducive to this motility are of interest. In work on *P. megasperma,* Ho and Hickman (1967) reported that zoospores could remain motile for 24 h or more when placed in synthetic buffer solutions with high ionic contents (0.1 Molar), although they made no mention of any initial shock reaction, such as that reported here for *Aphanomyces* In work on *P. palmivora,* Bimpong and Clerk (1990) reported that zoospores maintained good motility in concentrated calcium chloride solutions (40 mg l^{-1} - 300 mg l^{-1} Ca) or even in 2% w/v glucose.

Other work on biflagellate aquatic fungi featured “dehiscence solutions” aimed at evoking motile zoospores from mycelium. and these solutions were often of high ionic content also. e.g. that used by Mitchell and Yang (1966) for A. *euteiches* Drechsler, which contained KCI. $MgSO_4$ and $CaCl_2$ (with Ca at 200 mg l^{-1}). However, they did take this work further and show that the additional presence of Na, as sodium bicarbonate, prevented the good production of zoospores. In this current contribution it was shown that the best “dehiscence solution” devised for the *Aphanomyces* contained only 100 mg l-1 Ca. but it was also shown that Ca levels as high as 400 mg l^{-1} Ca did not inhibit zoospore motility (Table 5).

Both from this current work and from the literature it therefore appears that the motile zoospores of biflagellate aquatic fungi are expremely tolerant of high ion, and presumably osmotically active. solutions, even if motility is lost temporarily. In this current work, not reported above. physically shocked. non-motile, secondary zoospores of the *Aphanomyces* regained their motility in full-strength GYP broth after 4 h. The stimulus to resume swimming is apparently so strong here that it over-rides the nutrient stimulus to germinate. in the absence of a stage, (the motile zoospore) in the life-cycle which is ameanable to the encystment trigger. This wide tolerance to chemical solutions

on the part of the motile zoospores is apparently paralleled by tolerance to physical shock. This current contribution has shown that physical shock inhibited motility, but this inhibition was only temporary, under the experimental conditions used. Philosophically it does not seem reasonable that biflagellate fungi should easily lose the advantage of location and selection of their potential substrata and have to rely on repeated re-emergence from cysts, with the consequent loss of energy and biomass which this would inevitably entail, to re-establish the motile phase. In summary it appears to be mistaken to regard motile, biflagellate zoospores as physically delicate and physiologically vulnerable. On the contrary, they appear to be surprisingly resilient. However, limits to their tolerance evidently exist; this is shown in this current contribution by their motility failure, and lack of any recovery, when A.R. water was their background (Table 2).

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SECTION III

CONTROL MEASURES

chapter 6

CONTROL OF OOMYCETE PATHOGENS IN AQUACULTURE

D.J. Alderman [1]

ABSTRACT

Members of ***the*** Oomycetes such ***as Saprolegnia*** and ***Aphanomyces*** have been a major problem in aquaculture since its inception. Whilst ***Saprolegnia*** remains largely opportunistic, ***the Aphanomyces*** species involved tend to be specialised and 'aggressive pathogens. They are diffkult and yet easy to control. Easy in that malachite green, a triarylmethane dye is a powerful and effective fungicide which has been used without any recognisable problems for 60 years. Difftcult, in that, in the modem legislative arena, that same product is an unlicensed medicine with environmental and consumer health problems raised against its continuing use. The problem of malachite green and the search for alternatives is discussed. Does malachite green's primary mode of action as an inhibitor of respiratory enzymes present a problem and is there really any evidence that it is a carcinogen or mutagen? Of the possible alternatives, are any as effective and is there any reason why these will not present even greater registration problems?

Key words: Malachite Green, ***Saprolegnia, Aphanomyces,*** salmonid culture.

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INTRODUCTION

Oomycete fungi present a continuing challenge to aquaculture. In salmonid culture the hatchery is particularly vulnerable where non viable eggs quickly become overgrown by *Saprolegnia* which then spreads to smother and kill viable eggs unless the dead eggs are removed by hand or fungal growth is controlled chemically. Adult fish are generally not vulnerable. except males in the breeding season. However, this general statement is not true for all circumstances and species, thus the European brown trout *Salmo trutta* is far more susceptible to Oomycete infections than the rainbow trout *Oncorhynchus mykiss.* Certain types of water bodies and water sources routinely appear to produce much higher frequencies of Oomycete infections in fish held in them. The prime example of such waters is that of drinking water reservoirs where fish in cages in the reservoir and in tanks and ponds fed from the reservoir show high prevalences of Oomycete infection.

In warmer waters recent years have led to the recognition of the involvement of Oomycetes presumed to be members of the genus *Aphanomyces* in a range of very similar ulcerative and granulomatous infections in fresh water and estuarine fish from areas such as southeast USA, Japan, Australia and the Philippines. However the best known and classic *Aphanomyces* infection is crayfish plague caused by *A. astaci* which has spread and continues to spread through Europe since 1865.

In the marine environment, Oomycetes are significant pathogens of crustaceans in culture including lobsters *Homarus* spp. The fungi involved include *Lagenidium callinectes, L. chthamalophilum,* and *Haliphthoros milfordensis.*

Control of such pathogens is difftcult in that in most cases the pathogens are widely, if not universally, distributed and there is a considerable degree of opportunism in infection because most are not obligate pathogens. Therefore the primary means of prevention, the prevention of the introduction of the pathogen into the aquaculture environment is not possible. The next line of defence, good husbandry is less effective against the largely opportunist fungi than it is against other pathogens. This leaves the aquaculturist forced to consider chemotherapy or chemoprophyllaxis as the only effective means of control.

MALACHITE GREEN

For nearly sixty years the compound of choice for the control of Oomycete infections in aquaculture has been malachite green (Foster and Woodbury, 1936), a triarylmethane dye (Fig. 1) belonging to the same group as crystal violet and basic fuchsin. Malachite green has over the last twenty of those sixty years been increasingly regarded with suspicion and has been suggested to be a potential teratogen or carcinogen. Perhaps the major problem with malachite green as with a number of other traditional aquaculture medicines has been that. in an increasingly regulated environment, it is an old chemical lacking the

patent protection which would encourage a company to carry out the work necessary to determine whether it is possible to satisfy regulatory requirements (Alderman, 1985).

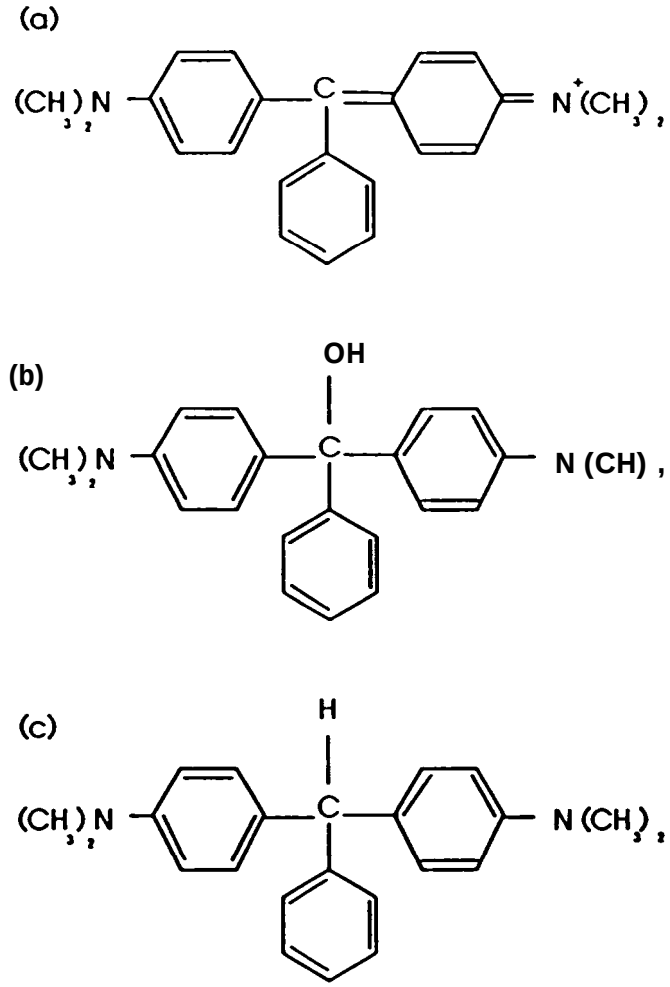

FIGURE 1. Dye ion (a), pseudobase or carbinol (b) and leucobase (c) of malachite green.

Mode Of Action

Malachite green is a respiratory enzyme poison which decouples oxidative phosphorylation (Werth and Boiteaux, 1967), producing an endogenous oxygen deficiency at the subcellular level. Fish subject to excessive exposure to malachite green show clear evidence of respiratory distress which cannot be alleviated by increasing oxygenation of the water since the respiratory distress is at the subcellular level resulting from damage to mitochondria. The differential of toxicity between host and pathogen is evidently largely a reflection of a greater tolerance to this respiratory damage in the larger organism rather than a differential absorption of the dye by fish cells in comparison with fungal cells.

Certainly malachite green is equally as toxic to protozoan ectoparasites as it is to fungal parasites.

Chemistry

Although. in aquaculture the most frequently used salt of malachite green is the oxalate, other salts are available. The hydrochloride is often avoided since it is normally produced as the double zinc salt and zinc-free malachite is normally specified for fish use. In a production batch of malachite green, the hydrochloride salt is also likely to contain more impurities than the oxalate and this rather than the zinc content may the cause of increased fish toxicity encountered with malachite green hydrochloride. It has become increasingly common to use a 50% liquid product rather than a dry malachite green. This product is not an oxalate, but is instead a combination of acetic and hydrochloride, consisting of 36% malachite green. 6.5% acetic acid, 4.0% hydrochloric acid and 53.5% water. This is a dark green viscous fluid from which solid malachite green tends to precipitate. The 36% dye in the 50% liquid malachite is lower than in the equivalent dry dyes e.g. 82% dye in malachite green oxalate, the remaining 18% being the oxalic acid.

The triarylmethane dyes are one the few chemical groups which. although they exist in a number of ionic forms, do not form true bases, but form “pseudobases” or carbinols. The crystalline green dye is the salt of the cation which exists only in aqueous solution. The chromophore which is responsible for the colour is the quinoid benzene ring. In aqueous solution at pH 4.0 all of the malachite green molecules are in the form of the dye cation and the double bonded quinoid ring is present. At higher pH molecules are able to combine *covalently* with available OH- ions to form the carbinol or carbinol base which is a pseudobase since it is a non ionized substance. The carbinol is colourless since it lacks the quinoid ring. Although this change is an ionic reaction, it is a covalent combination so that, relative to most ionic reactions it is slow and complete ionic equilibrium takes in excess of 8 h to become established. The equilibrium ionization constant (expressed as pK_a^{Ek}) of the reaction is pH 6.9 when 50% is in the form of the dye ion and 50% in the form of the carbinol. As pH falls below 4.0. an increasing number of malachite green molecules gain a second positive charge, which produces a molecule with two quinoid benzene rings. This “double charged” malachite green cation is a pale straw to orange in colour. Thus the most intense green colour of malachite green is at pH 4.0.

The solubilities in water of the dye ion and the carbinol base differ considerably, >7g/l for the dye ion and <0.5g/l for the liposoluble carbinol. The dye : carbinol equilibrium is a factor which complicates the determination of the exposure dose. Whilst the treatment dilution for a static bath treatment may be prepared in advance so that the dye : carbinol equilibrium is established before the exposure, with a flush treatment, as frequently used in hatcheries, not only are the concentrations of malachite green changing

as the flush treatment progresses, but the dye : carbinol equilibrium is effectively "chasing" the continuously changing concentration of dye in the water as additional OH- ions become available with the incoming water.

Toxicity

It must be borne in mind that fisheries use of malachite green is small and malachite green is initially produced for use as commercial dye where chemical purity is not of primary importance, certainly not to the standards required for a veterinary medicine. What is of importance is uniformity of performance as a dye. Nominal 100% dyes are often "diluted" by the addition of bulking agents (e.g. sucrose) to give a final concentration equivalent to the poorest quality of product synthesised and such quality may be very poor indeed. The yield of malachite green from an initial commercial manufacturing synthesis is approximately 65%. Such inevitable variations of concentration and of chemical purity present obvious toxic hazards which cannot be quantified in advance so long as dye grade product is used. The variety of commercial synonyms and trade names that have been used over the more than 100 years availability of malachite green is such that there is a significant possibility that a commercial source may not be malachite green, but a related dye with similar dyeing properties. The Colour Index (1982) lists known synonyms and allocates to each dye a unique index number and name. Malachite green is Cl42000, Cl basic green 4.

A major difficulty in discussing published information on the exposure of fish to malachite green is the considerable range of exposure concentrations, times and temperatures at which fish have been exposed by different investigators. As indicated above the method of exposure. primarily flush treatment or a static bath treatment also complicate definition of exposure. To reduce this, Alderman and Clifton-Hadley (1988) introduced the term "ppm minutes". This consists of the summation of the concentration to which animals are exposed during each minute of exposure, thus 0.5 ppm malachite green for 100 minutes would represent a "50 ppm minute exposure". By integrating the area under the curve of a flush treatment, this too can be converted into a "ppm minute" figure (Alderman, 1992). Temperature also plays a major role in the toxicity of malachite green (Alderman, 1985), its uptake and elimination (Alderman and Clifton-Hadley, unpublished). Relatively few of the studies discussed below have taken these factors fully into account. Neither, in the case of intended "long term" exposure to low doses in bath treatments have the investigators concerned considered that, with the tendency of malachite green to accumulate in biological materials because of its high octanol/water partition coefficient, the low exposure dose will have been removed from the water, either into the fish or into organic materials within the tank in a very short time. For these reasons alone, many studies of malachite green have limited value.

Werth and Boiteaux (1967) demonstrated that malachite green was a respiratory enzyme poison whose effect could be reversed by cytochrome C. Key1 and Werth (1959) had previously described developmental disturbances and chromosome derangements, including inhibition of salivary gland development, in chironomid larvae exposed to malachite green for 7 h whilst Pfeiffer (1961) found similar disturbances in *Drosophila melanogaster* injected with the dye. Whether these abnormalities were attributable to direct effects on chromosomes or indirectly to its cytotoxic effects as a respiratory enzyme poison was not established. Werth (1959) published the results of extensive studies which may be summarised as follows : malachite green shows genetic toxicity towards rat reproductive cells. inducing malformations and tumours in the progeny. However this toxicity could be suppressed by the simulatneous application of cytochrome C. This study is flawed in that the ages of the progeny were not taken into account in the study in which the progeny of the malachite green treated rats survived longer and therefore had a greater possiblity of expressing spontaneous turnours. Also, although malachite green is a flat basophilic molecule which could act directly by intercalation between DNA base pairs, with the possibility of thus affecting replication, but cytochrome C would not be expected to interfere with such action, but only on malachite green's direct effects on respiration.

In rainbow trout, *Oncorhynchus mykiss*, studies by Steffens *et al.* (1961) showed mitotic effects, predominantly chromosome breaks, in eggs treated with malachite green, and more recently, Meyer and Jorgenson (1983) demonstrated spinal, head, fin and tail abnormalities in trout fry hatched from eggs which had been exposed to standard, repetitive malachite green treatments. In that study all lots of eggs treated with malachite green experienced a delay in hatching as compared to the control eggs and an increased frequency (20 to 34% against 7%) of abnormalities such as head or jaw deformities, spinal curvature, missing fins or bob-tail condition. The Meyer and Jorgenson (1983) study also examined the effect of malachite green dosing on pregnant New Zealand white rabbits. Reduced feed intake was noted and at all three dose levels (5, 10 and 20mg/kg body weight daily by oral gavage on days 6 to 18 of gestation) there were significant increases in pre-implantation losses and in the ratio of dead to total implants. The incidence of observed developmental anomalies in rabbits failed to show a dose related response. As Meyer and Jorgenson (1983) commented, the doses used in their study far exceeded those used in aquaculture. These authors interpreted their results as indicating teratogenic activity, but an alternative interpretation of embryo toxicity is also possible.

FIGURE 2a and 2b. Electron micrographs showing S. *parasitica* hyphae after 5 minutes exposure to 5 ppm malachite green. Note increasing area of electron lucent material (L) at the centre of mitochondria and, in some, a swelling and breakdown of cristae (arrow).


2b
1.0 μm
2a
1.0 μm

In studies on the target organism. *Saprolegnia.* exposed to malachite green Alderman (unpublished) found that the most evident and rapid changes were to be seen in the mitochondria. At 5ppm. within 5 minutes mitochondrial cristae began to swell and disintegrate and the mitochondrial matrix became more electron lucent (Fig. 2). This disintegration proceeded rapidly and within a short period the mitochondria became unrecognizable.

In a recent study by Get-undo *et* al. (1992). rainbow trout were exposed to malachite green at normal therapeutic levels weekly for seven weeks. using an 80 ppm minute exposure at 16°C. No histological lesions were observed at the light microscopy level in livers after the first two malachite green exposures. but at the ultrastructural level there was already some evidence of mitochondrial damage even that early stage of treatment. This included dilation of the rough endoplasmic reticulum (RER) (Fig. 3a) which was interpreted as resulting from functional derangement of ATP dependent membrane transport mechanisms of the cell. perhaps resulting in accumulation of water in the RER (Slauson and Cooper. 1982). Presence of autophagic vesicles suggested an increased turnover or destruction of organelles. Decreased hepatocyte glycogen and lipid content was observed under the electron microscope in some treated fish which may have related indirectly to malachite green impairing oxidative phosphorylation. resulting in compensatory anaerobic glycolysis with concomitant glycogen depletion. A diminution of enzyme activity in the stomach and small intestine together with anorexia has been reported in rainbow trout after malachite green treatment (Reichenbach-Klinke. 1972; Reichenbach-Klinke and Ollenschlauer. 1975).

Get-undo *et* al. (1992) suggested that temporary interruptions in feeding and impaired digestive efficiency contributed to the decrease in stored products noted in the livers of their experimental fish. After the third exposure. the histological appearance of livers of these fish showed a fairly consistent pattern of increasing pathological changes. The principal alteration was sinusoidal congestion. but focal coagulative necrosis, diffuse degenerative changes and cytoplasmic vacuolation also increased with increasing exposure. Similar tissue changes have been reported in fish exposed to other toxins particularly pesticides (John. 1975: Mattheisen and Roberts. 1982) and therefore the changes obsened by Get-undo *et* al. (1992) may perhaps be regarded as generalised structural responses to toxic cell damage. These authors found consistent mitochondrial damage to be evident at the ultrastructural level with swelling and disruption of cristae.

FIGURE 3. (a) Mitochondrial damage and swelling of endoplasmic reticulum in rainbow trout hepatocyte after two malachite green exposures. (b) Bizarre rainbow trout hepatocyte nucleus and distortion of ER seen after repeated malachite green exposures.


2 µm
3b
2 µm
3a

Nuclear alterations were also evident in hepatocytes of treated fish, becoming more frequent after the fifth week of exposure. Particularly evident was an increasing irregularity of the nuclear margin resulting from marked indentation of the nuclear membrane. At the seventh week of exposure. clumping of chromatin and formation of non-membrane bound. granular bodies of various size near the nucleolus were reported. determined. but, although there were some similarities to inclusions observed in animal These nuclear alterations increased in frequency and severity in the later periods of exposure (Fig. 3b). The nature of the nuclear inclusions reported could not be determined, but. although there were some similarities to inclusions observed in animal and plant cell nuclei subject to viral infections. Gerundo *et* al. (1992) considered that there was no reason to attribute these inclusions to anything other than to the toxic effects of malachite green.

In the gills of fish treated with malachite green Get-undo *et al.* (1992) observed separation of the epithelial lining from both lamellae and interlamellar zones (Fig. 4) which may have been due in part to an influence of the hyperosmotic external medium through physiologically impaired epithelial cells (Rombough and Garside, 1977). Similar lesions have been reported from gills of fish exposed to a range of different toxicants (Mallat, 1985). Lamellar cell necrosis and leucocyte infiltration were also observed as previously reported by Waluga (1975). The latter reported destruction of gill peripheral respiratory epithelium. accompanied by haemorrhages and haemolytic alteration of the gill capillaries after chronic exposures of trout to 0.2 mg ml^{-1} malachite green for 10 days. Since the gills are the most exposed and vulnerable organ in fish with regard to risks of exposure to and damage from aquatic. toxicants (Roberts. 1978) the effects observed were not surprising.

In the Get-undo *et al.* (1992) study. the morphological alterations that developed in both the livers and gills of trout exposed to repeated sublethal levels of malachite green suggested at least some impairment of normal function of these organs as the malachite green exposures progressed. However. the hepatic changes were apparently not so severe as to be reflected in the serum protein values measured during the course of the experiment. This suggested that any malachite green induced liver impairment might be localized and could be compensated for by other areas of less affected tissue. This correlated with the histological picture in the experimental animals, where hepatic tissue changes were focal and varied in intensity from one area to another within the same specimen. The gill lesions. on the other hand, were apparently sufficient to hinder urea excretion. This was reflected in the general increase in serum urea values over the experimental period as compared with the control values.

The series of seven malachite green treatments applied to the trout in the trials reported by Get-undo *et* al. (1992) were equivalent to the most severe treatment regimes applied to trout in the therapeutic trials against PKD reported by Alderman and Clifton Hadley (1988).

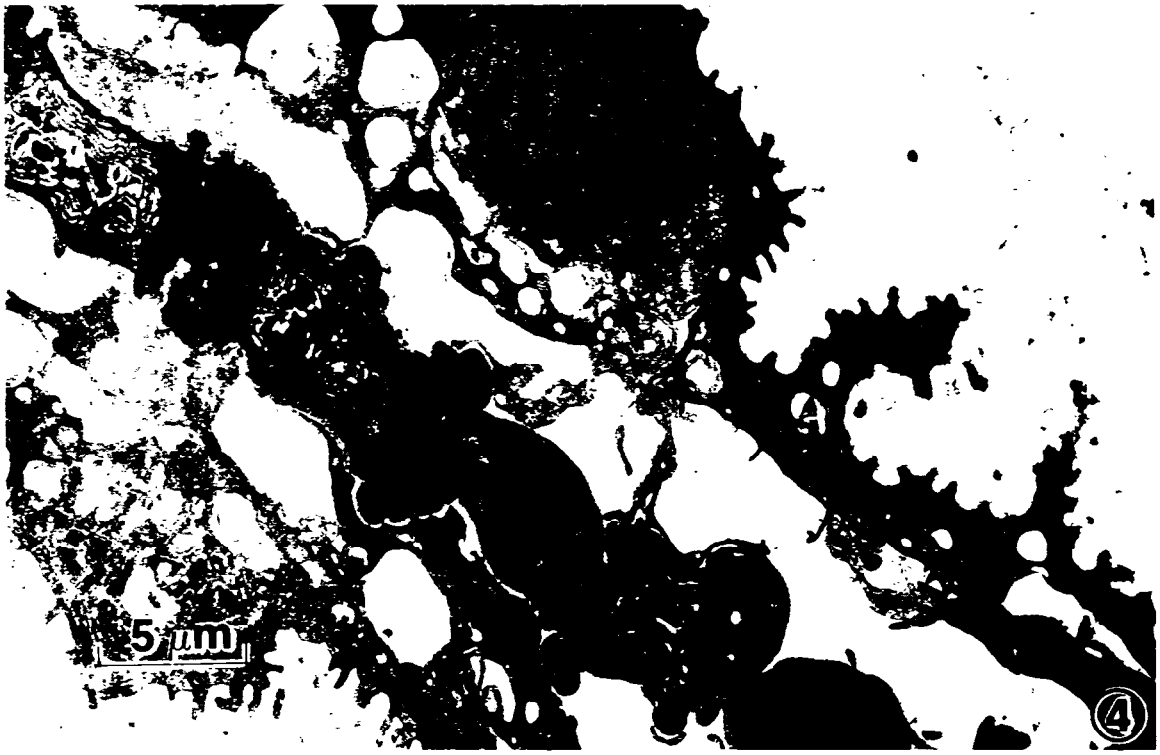

FIGURE 4. Rainbow trout gill after repeated exposures to malachite green showing detachment of gill epithelium.

As Meyer and Jorgenson (1983) commented, in most studies of malachite green toxicity and adverse effects reported in the literature. the dose levels used grossly exceed those used in aquaculture. In addition to this, a problem which has not often been considered is the nature of the product used in many of the tests. The work of Clemmensen *et* al. (1984) is an example. These authors determined the LD_{50} of malachite green oxalate to rats and mice and reported positive results for mutagenicity in the *Salmonella*/microsome test after metabolic activation, but found no clastogenic activity when tested at maximum tolerated levels in mice with the micronucleus test. The product tested was a sample of malachite green obtained from a fish feed supplier, so that in many ways it was representative of supplies of malachite green available to the aquaculture market. However such product. a commercial dye. is unlikely to be chemically pure malachite green. If the object is to determine whether or not malachite green (as opposed to crude commercial dye) is teratogenic or mutagenic, then tests of probably impure substance have very limited if any value. The importance of this point is emphasised by studies on related dyes by Bonin *et* al. (198 1) investigating the mutagenic potential of 22 arylmethane dyes using the *Salmonella/* mammalian microsome mutagenicity test, who

commented that since conflicting results were obtained using dyes from different sources, minor dye components might have been responsible for their mutagenicity. These authors suggested a need to improve knowledge about the impurities and the toxicological role of such impurities present in arylmethane dyes. particularly those still used in food.

The above comments on the toxicology of malachite green are not intended to indicate that malachite green is not a problem chemical, which it clearly is, but do make it clear that the evidence currently available is scientifically flawed and that further major studies are needed to determine the real hazards involved.

Residues

The original use of malachite green was as a topical disinfectant fungicide and parasiticide. thus for long there was little consideration paid to the possibility that such topically applied therapeutants might also be absorbed systemically and produce significant internal effects, despite the evident respiratory distress of exposed fish. The evidence of Steffens *et* al. (196 1). Werth and Boiteaux (1967) and Meyer and Jorgenson (1983) made it clear that the dye would enter cells and could result in adverse effects, and indeed, the lipophilic nature of the carbinol means that this form of malachite green enters cells and tissues readily. The high octanol/water partition coefficient of the carbinol form of malachite green means that bioaccumulation is inevitable.

Since the pH of most fish tissues is above pH 7.0. any malachite green within such tissues will remain as carbinol. Therefore the presence of malachite green residues are not immediately evident in fish directly after exposure since the carbinol in the tissues is colourless. Only brain. spinal cord, gall bladder and lateral line are sufftciently acid to show the presence of malachite green as the dye ion. “Freezer bum” which caused a shift in tissue pH producing a green staining on the surface of catfish steaks from animals treated soon before they were killed led Poe and Wilson (1983) to recognise that malachite green is absorbed by fish tissues during treatment. Edelhauser and Klein (1986) and Klein and Edelhauser (1988) also reported the detection of malachite green residues in muscle of deep-frozen farmed rainbow trout.

Only brief studies into the uptake and excretion of malachite green have been published (Keitzmann *et al.*. 1990) and these workers used low concentration long exposures which are not relevant to normal patterns and methods of use. plus an HPLC residue detection method with a limit of detection (LOD) of 0.02 mg/kg. Such treatments are difficult to relate to normal fish farm usage of malachite green and the authors did not specify the water temperatures at which the work was carried out. Nevertheless their work confirmed that significant uptake of malachite green into fish tissues would occur even at low levels of exposure and that extended withdrawal periods post treatment are necessary. Clifton-Hadley and Alderman (1987) working on malachite green therapy of proliferative

kidney disease, used a simple extraction with pentan- l-01 followed by spectrophotometry at 624 nm to quantify the levels of malachite green in the fish under treatment.

A fuller study has been completed (Alderman and Clifton-Hadley in preparation), which shows that uptake, distribution, peak tissue concentration and elimination of malachite green in all tissues are all strongly temperature-dependant (Fig. 5). In serum a best fit was achieved with a three-compartment first-order model giving a ß half-life of 0.625 days at 16°C and of 4.78 days at 8°C. Since uptake was also found to be temperature-dependent, fish exposed at lower water temperatures entered the elimination phase at lower initial concentrations than animals exposed at higher temperatures and thus malachite green could in practice, actually be eliminated more rapidly at lower temperatures than at higher. Whilst not recommending a post malachite green withdrawal period, these studies found that at least 600 degree days would be necessary.

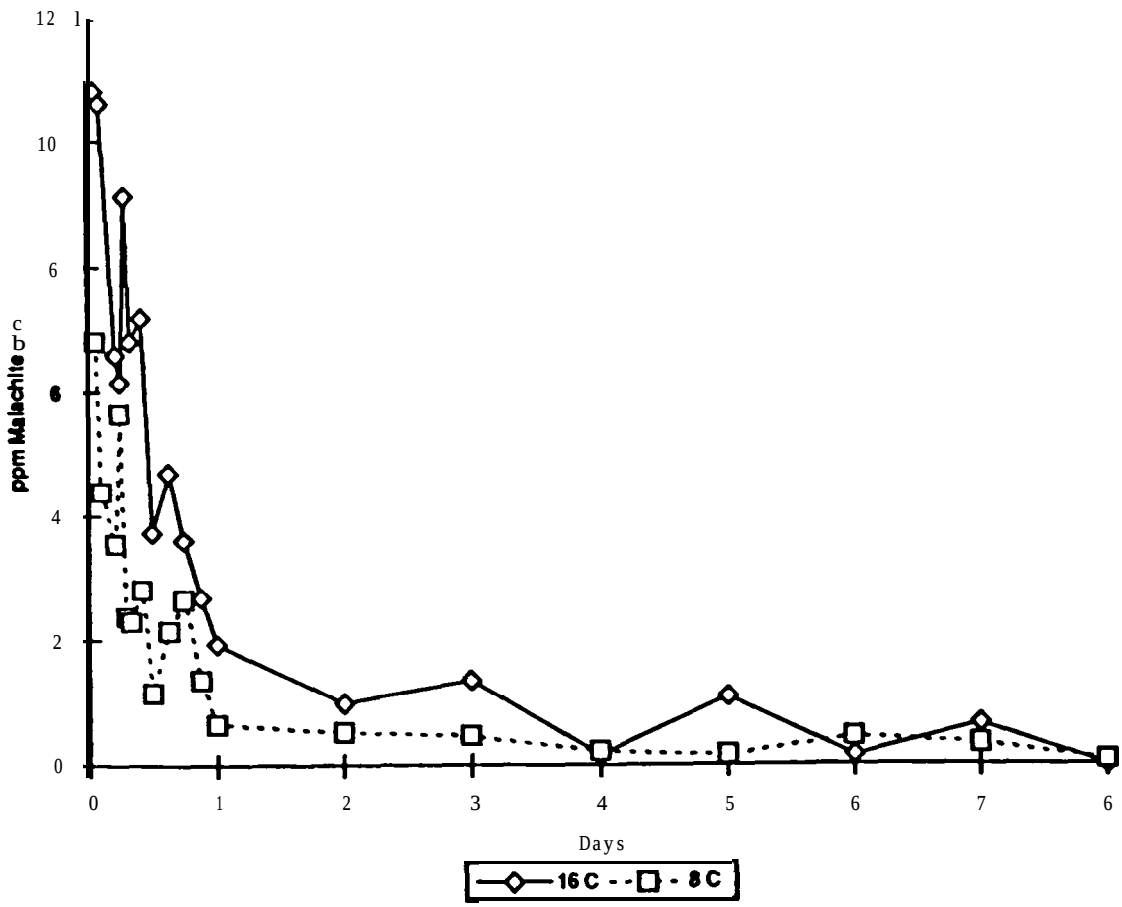

FIGURE 5. Kinetics of elimination of malachite green from muscle of rainbow trout at **8°C** and **16°C**. At 16°C muscle levels of malachite green are much higher at the end of an 80 ppm minute exposure than at **8°C**, but the shorter elimination half life at **16°C** causes a more rapid elimination.

ALTERNATIVE FUNGICIDES

The search for alternative methods and compounds for the control of oomycete pathogens in aquaculture has not arisen as a result of any failure of therapeutic efficacy of malachite green or for that matter as a result of suspect (or proven adverse) reaction reports (SARs) from treated stocks, farm staff or consumers. It has instead arisen from the increasingly tight regulatory environment. New regulations are being introduced in most countries that require that all compounds used as medicines receive official approval in the form of a licence or registration before their use is permitted. Because of its small. but rapidly growing importance, aquaculture initially missed or was derogated from much of the initial phase of such legislation. In the last ten years in many countries the protection provided from any such derogations has steadily been removed, so that aquaculture medicines have now to meet the full regulatory requirements despite the relatively limited size of the market that supports such medicines. As described previously, malachite green is an old product (> 100 years), lacking in any modem data to support such registration and a member of a chemical group, the arylmethanes which has had a number of significant safety questions raised against it. It should also be recalled that malachite green is not used solely as a fungicide in aquaculture, but also as a very effective antiprotozoal agent, both against topical and against systemic infections, so that, although here only a replacement fungicide is considered, replacements for all of its range of activity are needed.

For marine crustacean culture, there exists a largely suitable fungicide, trifuralin which will effectively control oomycetes such as *Lugenidium* and *Halipthoros* infections (Bell and Lightner, 1992). Trifluralin is however not an antiprotozoal agent, nor is it effective against freshwater oomycetes such *as Suprolegniu* and *Aphunomyces.* Formalin has some antifungal effect and indeed has synergistic activity in conjunction with malachite green (the Leteux-Meyer mix, Leteux and Meyer, 1972) and is licensed in some countries for fisheries use (e.g., USA). Fornalin is however in practice, not very satisfactory and presents operator safety hazards, particularly in enclosed situations.

Both in the USA and the UK considerable programmes to develop *in vitro* test methods were started as the regulatory environment began to tighten in the late 1970s. In the UK well over 800 candidate compounds from a wide range of chemical groups were tested using three *in* vitro screening methods (Alderman, 1982; Alderman, unpublished). Very similar methods were developed in the American programme and inevitably many similar chemicals were tested (Bailey, 1983a,b; Griffin, 1987; Schnick, 1988).

Results from the UK *in vitro* testing programme identified two possible candidate fungicides, the bisphenols and the 8-quinolinols. Of the bisphenols, dichlorophen was found the easiest to handle in the laboratory, but was toxic to fish at concentrations below therapeutic. Similar results were obtained in the USA (Schnick, 1988) where dichlorophen was found to control protozoan ectoparasites at 0.5-0.7mg/l and was not

toxic to fathead minnows or golden shiners in 24 h static tests, but could not control fungi on rainbow trout eggs at 10 mg/l daily for 4 d and was highly toxic to rainbow trout and fathead minnows at that concentration after 15 min. A related bisphenol, bithionol was found in the UK to be effective against *Saprolegnia in vitro* and relatively non-toxic to fish, but when tested *in vivo* only a slight reduction in *Suprolegniu* infection of brown trout was achieved and the product was not pursued further.

The 8-quinolinols (the 8-hydroxyquinolines) are a long established group of antimicrobial agents, some of which are licensed for use in veterinary medicine, including a commercially available mixture of dichloro salts of 8 quinolinol used to control scour in pigs. A wide range of 8-quinolinols were tested, some of which were effective *in vitro* to control *Suprolegniu,* but were unable to control infestations of rainbow trout eggs (Alderman, unpublished). A feature of the 8-quinolinols is that they are not biocidal until they form a chelate within cells with a metal. and the availability of metals in sufficient quantity to form one to one or one to two chelates is essential for activity (Albert, 1979). The 8-quinolinols were also tested in the American fungicidal programme (Schnick. 1988). where the sulphite controlled egg infestations at doses of 70 mg/l for 15 min, but the fry died shortly after hatching

A side aspect of this investigation of the antifungal potential of the 8-quinolinols was the subsequent inclusion of these products into a study of antibacterial agents for fish use. They were found to be particularly effective against *Vibrio* spp, particularly 5,7-dichloro-8-hydroxyquinoline (Austin *et al.*. 1982) but no commercial interest in progressing this finding has followed.

After testing all possible candidate fungicides available at the time, the UK malachite green testing programme was halted. since it was felt that there was little point in continuing unless new suitable products could be found. The following paper (Marking, Chap. 7, this volume) describes the progress of the American programme.

CONCLUSION

We therefore have a situation in which malachite green remains the **only** effective fungicide for use in freshwater aquaculture and is moreover a very effective antiprotozoal for both topical and systemic protozoan infections. Malachite green is no longer licensed for use as an aquaculture medicine in any country, but is still readily available and used in many. There may well be good grounds for regarding it as a potentially hazardous chemical to use in the aquatic environment and yet the case against it fails to be proved because of the lack of adequate studies. The value of malachite green and the need for an effective fisheries fungicide is evident from the efforts already expended on it in the USA and the UK, but we still seem to be no nearer a solution to the problem than we were in

the mid 1970s when the problem of malachite green as an important fisheries chemical with major problems first became evident.

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chapter 7

SEARCH FOR ANTIFUNGAL AGENTS IN FISH CULTURE

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ABSTRACT

The special permit to use malachite green as a fungicide in culture of endangered and restoration species of fish was canceled on August 27, 1991; the need for a replacement fungicide has intensified. Selected candidate chemicals were tested on cultured fungus for growth inhibition and on infected eggs of rainbow trout to evaluate their antifungal activity. Candidate chemicals were Amorolfine, Clotrimazole, Fenpropidin, Fenpropimorph, glutaraldehyde, iodine, hydrogen peroxide, sodium chloride, potassium permanganate, and VigorOx and reference fungicides were malachite green and formalin. Since infection rates in these tests were high at 20% or greater, higher concentrations of malachite green (5.0 ppm) and formalin (1,667 ppm) were required to demonstrate fungicidal activity. The most effective compounds other than reference chemicals were sodium chloride at 30,000 ppm, glutaraldehyde at 200 ppm, and VigorOx at 200 ppm. Iodine and potassium permanganate at 100 ppm were fungistatic only at this high level of infection. Registrations for Amorolfine, glutaraldehyde, and VigorOx are nonexistent as therapeutants; their development would require further studies and negotiations with regulatory agents. Results of these studies suggest that formalin at 1,667 ppm and sodium chloride at 30,000 ppm are superior to the other compounds tested.

Key words: antifungal, salmonid eggs, malachite green, formalin.

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INTRODUCTION

Aquatic fungi (Saprolegniales) are ubiquitous in natural water supplies of fish hatcheries often causing serious disease problems. Malachite green is effective in control of fungus on fish and fish eggs, but due to suspected teratogenicity (Meyer and Jorgenson 1984) its use was limited to the treatment of non-food fish (i.e., eggs or adult salmon held for spawning) under an Investigational New Animal Drug Application (MAD) held by the U.S. Fish and Wildlife Service. That INAD was cancelled on August 27, 1991 and uses were to be discontinued within 45 days. Heretofore special exemptions are required by the FDA for any uses. Presently, there is one registered aquatic fungicide, formalin, but it is not completely satisfactory in control of fungus on fish or their eggs. Consequently, the search for safe and effective aquatic fungicides must continue.

Bailey (1984) and Bailey and Jeffrey (1989) reported results of tests with over 200 compounds that were chosen for fungicidal activity. More than half were found to be unsuitable as aquatic fungicides in preliminary tests because of their lack of activity against fungi, toxicity to fish or their eggs, insolubility in water, or potential carcinogenicity. However, several of the better candidates showed potential for control of fungus on fish eggs and were chosen for further evaluation.

This study is a continuation of "Research to Identify Effective Antifungal Agents" sponsored by Bonneville Power Administration (Schreck et al 1990 and Schreck et al 199 1). The objectives of the present study were to select and evaluate up to 10 candidate fungicides. Evaluations involve laboratory studies on efficacy of candidate compounds on cultured Saprolegniales, and on eggs of rainbow trout that were previously infected with the fungus. Candidates that are demonstrated to be effective for control of fungus on eggs will be tested further on adult spring chinook salmon.

MATERIALS AND METHODS

In vitro Tests

Pure strains of aquatic fungi were obtained from the American Type Culture Collection (ATCC). *Saprolegnia hypogyna* (ATCC 28275) was used for both range finding and minimum inhibitory concentration tests. Test procedures used were those developed by Bailey (1983). The method involved an *in vitro* screening technique modified from that of Golden and Oster (1947) and a minimum inhibitory concentration determination based on the percent inhibition of growth in diameter of colonies (Bailey and Jeffrey 1989).

Chemicals in solid or liquid form were weighed or measured and dissolved in aqueous solutions; concentrations were corrected for purity but not for specific gravity. For example, malachite green was prepared from a 50% active solution; twice the volume of stock was added to the dilution media to account for purity. Solid samples included

Amorolfine, Clotrimazole, sodium chloride, and potassium permanganate. For continuity. concentrations are reported in ppm for solid and liquid chemicals in these tests.

Range Finding

Standard petri dishes were filled with 20 mL of corn meal agar and inoculated with agar plugs augmented with fungi (5 mm in diameter). The fungi was allowed to incubate at 20°C for approximately 96 hours. Agar plugs were removed from the edge of the colonies with a #l cork borer. Stock solutions of chemicals were prepared to achieve final concentrations of 1) 10, and 100 ppm. The depressions of Coors porcelain spot plates were filled with three replicates of each of the candidate chemicals and the solvent, positive. and negative controls. Agar plugs were then added to the depressions of the spot plates for exposures of 15 and 60 min. Agar plugs were removed from the spot plates, rinsed three times with sterile distilled water, and placed on n-i-petri dishes containing 30 mL of corn meal agar. Cultures were incubated in continuous light inside an environmental control chamber maintained at 20°C (± 2°C). The plates were examined for mycelial growth after 48,96, and 168 hours of incubation.

Minimum Inhibitory Concentration

Inoculations, incubation, and stock solution preparation were as stated above. Agar plugs were removed from the edge of the fungal colonies as previously described and exposed in triplicate to live delineative concentrations between 0.1 and 1 .O ppm. 1 .O and 10.0 ppm, or 10.0 and 100.0 ppm, or at higher levels. depending on the activity observed in the range-finding test.

The agar plugs were exposed to the test chemicals in triplicate for 15 or 60 min. They were rinsed with sterile distilled water and placed on standard petri dishes containing 10 mL of corn meal agar. Cultures were incubated in a lighted environmental control chamber at 20°C for 48 hours, and the colony diameters were measured with a vernier caliper.

In vivo Tests

Green eggs from Trout Lodge (Sumner, Washington) were placed in Heath incubating trays (500 eggs per tray) with the use of a modified egg counting board. Characteristics of the well water used for incubation was a total hardness of 138 ppm as $CaCO_3$, alkalinity of 105 ppm as $CaCO_3$, pH of 8.0, and temperature of 12°C. Concentrations of dissolved oxygen remained at 9.0 ppm or above during the exposures. Water flow was about 1 L/min during incubation and treatments. During infection water flow was discontinued

for 2 h periods in morning and afternoon to promote infection of the eggs. The eggs were confined within a 6-inch diameter acrylic ring that was 1 inch in height and attached with silicone to the screen of each incubator tray. Two trays of 500 eggs each were used as replicates for each treatment level. Eggs were inoculated with 12 S. ferax infected hemp seeds suspended by a tea ball in the upper tray of each replicate treatment for exposures of approximately 7 days or until the initial infection rate was about 20% or greater. Prior to exposure infection rates were equalized by exchanging infected eggs in trays with a high infection rate with uninfected eggs in trays with a low infection rate. Eggs were exposed to the fungicides for 15 or 60 min.

The chemicals were delivered to the water inflow of a separate mixing tray above the egg hatching trays with the use of a peristaltic pump to achieve specific desired concentrations. The mixing tray contained a maze of baffles to ensure complete mixing. Concentrations were calculated on the basis of amount of material added to a specific volume of water flow. Treatments were administered three times weekly for a period of 2 weeks or until eggs began hatching. Mortality and fungal infection were assessed prior to the first treatment (pretreatment), after the last treatment (posttreatment), and after the eggs hatched (post-hatch). Infection rates (% increase) were calculated by subtracting pretreatment infection rates from post-treatment rates. The percent hatch was corrected for initial mortality by subtracting the pretreatment mortality from the total number hatched according to the following formula.

$$\text{Percent hatch} = \frac{\text{number hatched}}{\text{total - initial morts}} \times 100$$

Toxicity Testing

Toxicity of the candidate fungicides was performed in the egg incubators simultaneously with the efficacy treatments. The setup was the same as stated above for the ***in vivo*** testing; however, the eggs were uninfected. The dilution series was generally based on a use pattern of 1, 3. and 5X. The 1X concentration was the concentration we felt would be effective for control of fungus. Mortality observations were taken daily and egg hatching success was recorded at the end of each test. Margins of safety for each chemical were established by dividing the toxic concentration by the effective concentration for respective exposure times.

Concurrent Exposure

Past results have shown seasonal variability in the infection rates and effectiveness of some antifungal agents. During a three month period concurrent exposures of the better candidates were undertaken to minimize these effects. These exposures were for 60

minutes and the antifungal candidates were tested on the same group of eggs. Each month 2,000 eggs were exposed in each treatment with two replicates of 1,000. The candidates chosen for these exposures were Amorolfine. glutaraldehyde. iodine. potassium permanganate, sodium chloride. and VigorOx along with formalin and malachite green as reference compounds.

RESULTS

Ten chemicals were evaluated for antifungal activity on cultured fungus (S. ***hypogyna)*** on the basis of their minimum effective concentration and safety (Table 1). All of the compounds were antifungal, especially at the 60 minute exposure period, but high levels were required of some of the candidates. Malachite green was used as a reference chemical because of its known antifungal activity. However, we found that 5.0 ppm ***in vivo*** were required to give satisfactory results. The higher treatment rate for malachite green is perhaps related to higher infection rates (usually 20% or greater) in the infected eggs whereas they are treated at lower infection rates in hatchery situations.

Fenpropidin and Fenpropimorph

Fenpropidin and Fenpropimorph are structurally similar to the morpholine derivative called Amorolfine which demonstrated antifungal activity previously. These chemicals possess remarkable antifungal activity against a broad spectrum of fungi that are pathogenic to plants, animals, and humans. Fenpropidin was somewhat effective at 300 ppm in 60 minutes exposure; the infection rate decreased but the hatch rate did not show improvement (Table 2). Toxicity was noticeable at 300 ppm and evident at 500 ppm in the 60 minute exposures. Fenpropimorph was evaluated on rainbow trout eggs that were highly resistant to infection; exposure had to be started with low infection rates (Table 3). The effective exposure was at 300 ppm for 60 minutes and toxicity was not apparent at 500 ppm. Formulations of these materials were not soluble in water and precipitation occurred even when ethanol was used as a carrier. Neither of these analogs was as effective as Amoroltine and will not be tested further.

Glutaraldehyde

Glutaraldehyde, a five-carbon dialdehyde with some commercial and clinical uses, demonstrated antifungal activity at levels much lower than formaldehyde. Concentrations of 50, 100, and 200 ppm were effective for decreasing infection rates and increasing hatching rates (Table 4). Antifungal activity was especially noticeable in the 60-minute exposures. Toxicity was apparent in the 60-minute exposures, moderately at 300 ppm and

TABLE 1. Effectiveness @pm) of candidate fungicides on cultured ***fungus (Saprolegnia hypogyna)*** and on eyed eggs of rainbow trout infected with fungus (S. ***ferax)*** **in terms** of MIC (minimum inhibitory concentration), MEC (minimum effective concentration), MSC (minimum safe concentration), and safety index.

Chemical name	Exposure time (min)	*In vitro* Range	*In vitro* MIC	*In vivo* MEC	Toxicity *(MSC)* eyed eggs	Safety index eyed eggs
Amorolfine	15	>lOO	<300	300	>500	1.7
	60	>lO<lOO	<50	100	>500	5.0
Clotrimazole	15	>lO<lOO	> 10<30	--	--	--
	60	>10<100	-10	--	--	--
Fenpropidin	15	>lOO	>300<500	300	>500	>1.7
	60	>lOO	>200<300	100	<300	<3.0
Fenpropimorph	15	>lOO	-500	500	>500	>l.O
	60	>lOO	100	300	>500	>1.7
Formalin	15	>lOO	<300	1,667	5,000	3.0
	60	>lOO	<300	1,000	1,667	1.7
Glutaraldehyde	15	>lO<lOO	>50<75	200	>500	>2.5
	60	>10<100	>25<50	50	<300	<6.0
Iodine	15	>10<100	>70<100	50	<500	40.0
	60	>10<100	>50<70	50	<300	<6.0
Malachite Green	15	>l<lO	>I<3	1.0	_5	-5.0
	60	>l<lO	>1<3	1.0	_5	_5.0
Hydrogen Peroxide	15	>10<100	>lOO	--	--	--
	60	>10<100	>lOO	--	--	--
Sodium Chloride	15	> 1,000	>50,000	50,000	>50,000	>1.0
	60	> 1,000	>30,000	30,000	>50,000	>1.7
$KMnO_4$	15	>10<100	>50<100	50	>250	>5.0
	60	>10<100	>50	25	<150	<6.0
VigorOx	15	>10<100	>20<60	100	>250	>2.5
	60	>10<100	<20	100	<250	<2.5

TABLE 2. Effectiveness of Fenpropidin on infected eggs of rainbow trout and toxicity of 1,3, and 5X concentrations to uninfected eggs at 12°C.

Treatment (ppm)	Exposure (min.)	Infection (%)		Hatch (%)
		Initial	Increase	
Efficacy				
(-) Control	--	**0.0**	59.6	27.9
(+) Control	--	15.6	53.5	30.1
100	15	39.7	36.0	32.6
100	60	22.6	48.3	50.9
300	15	25.9	43.9	56.8
300	60	33.5	10.5	33.8
500	15	34.8	31.7	43.5
500	60	28.3	14.0	9.1
Toxicity				
(-) Control	--	--	--	83.9
(-) Control	--	--	--	89.1
100	15	--	--	86.3
100	60	--	--	78.6
300	15	--	--	85.2
300	60	--	--	63.7
500	15	--	--	87.4
500	60	--		5.0

TABLE 3. Effectiveness of Fenpropimorph on infected eggs of rainbow trout and **toxicity** of 1, 3, and 5X concentrations to uninfected eggs at 12°C.

Treatment (ppm)	Exposure (min.)	Infection (%)		Hatch (%)
		Initial	Increase	
Efficacy				
(-) Control	--	0.5	3.3	92.2
(+) Control	--	0.0	1.1	94.0
100	15	13.2	3.7	85.6
100	60	14.6	3.5	91.4
300	15	16.7	3.4	89.6
300	60	19.1	1.0	90.3
500	15	14.8	2.0	91.2
500	60	13.4	0.5	91.9
Toxicity				
(-) Control	--	--	--	98.0
(-) Control	--	--	--	97.5
100	15	--	--	97.5
100	60	--	--	95.5
300	15	--	--	98.4
300	60	--	--	95.5
500	15	--	--	97.0
500	60	--	--	96.3

TABLE 4. Effectitiveness of Glutaraldehyde on infected eggs of rainbow trout and toxicity of 1. 3. and 5X concentrations to uninfected eggs at 12°C.

Treatment (ppm)	Exposure (min.)	Infection (%)		Hatch (%)
		Initial	Increase	
Efficacy				
(-) Control	--	1.2	20.9	85.3
(+) Control	- -	18.8	44.0	54.7
50	15	18.7	34.2	72.9
50	60	18.1	4.4	75.9
100	15	28.5	33.3	68.6
100	60	23.5	17.0	78.9
200	15	20.9	23.1	79.4
200	60	23.7	3.7	75.8
Toxicity				
(-) Control	--	- -	- -	94.8
(-) Control	- -	--	- -	95.4
100	15	- -	- -	94.5
100	60	- -	- -	93.6
300	15	- -	- -	94.2
300	60	- -	- -	59.5
500	15	--	- -	93.1
500	60		- -	8.6

extensively at 500 ppm. In addition to mortalities. the treatments at these high levels caused a delay in hatching for up to one week Glutaraldehyde demonstrated desirable antifungal activity and further testing and evaluations are warranted.

Potassium Permanganate

Potassium permanganate has been reported to be effective for control of fungal infections on trout eggs. The EPA has allowed its use as an oxidant and a detoxifier in water treatment processes. Treatment levels of 2 ppm and effluent levels of 0.05 ppm were considered safe. Exposure concentrations of 25 to 100 ppm did not effectively control the fungal infections and the hatch rates were not improved (Table 5). Toxicity was apparent in 60 minute exposures at 150 and 250 ppm. The higher levels of potassium permanganate caused heavy staining on the eggs which complicated mortality evaluations. Also. the hatching was delayed by a few days at the higher treatment rates. The use of potassium permanganate as a therapeutant in fish culture would be regulated by the FDA and some kind of registration would be required.

VigorOx

VigorOx is an effective sanitizing agent that is formulated to contain 5% peracetic acid and 20% hydrogen peroxide. It is approved by the EPA and accepted as safe by the FDA as a sanitizer for food surfaces. This non-corrosive. non-staining. oxidant provides advantages over other oxidants. Treatments of rainbow trout eggs for 60 minutes of exposure at 100 ppm was effective for decreasing infection rate and improving the hatch rate (Table 6). Toxicity was apparent at 250 ppm in the 60 minute exposure. Additional testing is warranted to further evaluate the potential of VigorOx for antifungal activity.

Concurrent Exposures

Candidate antifungal agents were chosen from those tested the past two years for further evaluation. They were Amorolfine, glutaraldehyde, iodine, potassium permanganate, sodium chloride, and VigorOx in addition to reference compounds formalin and malachite green (Table 7). In the first trial, the 1.667 ppm concentration of formalin was considerably more effective than 250 ppm for decreasing the infection rate (-3.6%) and improving the hatch rate (87% vs 5 1 %). In fact. the high treatment of formalin produced a better hatch rate than any of the candidate antifungal agents or malachite green. The next most effective treatment was the 30.000 ppm of sodium chloride. The increase in fungal infection was only 1.8% and the hatch was 78.2%. All other candidates were less effective at rates tested. but there were some positive effects on infection and hatching rates.

TABLE 5. Effectiveness of Potassium Permanganate on infected eggs of rainbow trout and toxicity of 1 _ 3. and 5X concentrations to uninfected eggs at 12°C.

Treatment (ppm)	Exposure (min.)	Infection (%)		Hatch (%)
		Initial	Increase	
Efficacy				
(-) Control	--	2.9	25.8	81.6
(+) Control	--	21.8	23.5	71.4
25	15	22.3	20.7	64.8
25	60	21.6	19.7	74.7
50	15	24.3	14.9	71.0
50	60	21.7	17.7	69.0
100	15	23.6	13.1	68.3
100	60	21.9	12.0	67.6
Toxicity				
(-) Control	--	--	--	96.8
(-) Control	--	--	--	96.9
50	15	--	--	95.0
50	60	--	--	96.4
150	15	--	--	96.0
150	60	--	--	75.9
250	15	--	--	91.5
250	60	--	--	5.9

TABLE 6. Effectiveness of VigorOx - 5% Peracetic Acid on infected eggs of rainbow trout and toxicity of 1. 3, and 5X concentrations to uninfected eggs at 12°C.

Treatment (ppm)	Exposure (min.)	Infection (%)		Hatch (%)
		Initial	Increase	
Efficacy				
(-) Control	--	2.4	24.7	71.8
(+) Control	- -	21.6	24.2	60.7
20	15	18.8	33.5	47.8
20	60	20.3	20.0	70.9
50	15	20.7	36.1	56.9
50	60	21.1	28.3	71.0
100	15	19.3	30.4	64.2
100	60	20.1	11.2	79.5
Toxicity				
(-) Control	- -	- -	--	97.5
(-) Control	--	- -	- -	97.1
50	15	- -	--	97.4
50	60	- -	- -	97.9
150	15	- -	--	97.1
150	60	- -	- -	97.5
250	15	--	--	97.3
250	60	--		60.9

TABLE 7. Effectiveness of reference and candidate fungicides on infected eggs of rainbow trout at 12°C for an exposure time of 60 minutes.

Chemical Name	Treatment (ppm)	Mean (N=2) infection (%)		Mean hatch (%)
		Initial	Increase	
(-) Control	-	0.3	46.9	56.0
(+) Control	--	27.2	44.9	19.3
Malachite Green	1.0	25.9	22.3	63.5
Formalin	250	27.5	39.9	50.9
Formalin	1,667	27.2	-3.6	87.0
Amorolfine	100	29.0	24.1	67.2
Glutaraldehyde	50	28.4	23.0	50.3
Iodine	100	28.8	24.4	44.2
Potassium Permanganate	100	27.8	23.8	26.0
Sodium Chloride	30.000	28.2	1.8	78.2
VigorOx	100	26.8	38.8	31.0

Iodine exposures of 100 ppm for 60 minutes were extremely high and regulatory agents would not allow those levels without verification of safety. The FDA has concluded that povidone iodine compounds were low in regulatory priority when used at 100 ppm for 10 minutes as egg disinfectants after water hardening. Consequently. these iodine compounds can be used after water hardening but there is no formal approval for those uses and exposures. Iodine will not be evaluated further in these ***in*** vivo exposures. but it should be evaluated for efficacy at hatcheries.

Potassium permanganate demonstrated poor hatch at the 100 ppm exposure for 60 minutes. That high level again stained the eggs and caused a delay in hatch. Potassium permanganate will not be evaluated further in this program.

The 1 .O ppm of malachite green also was not as effective as formalin or sodium chloride. Additional evaluations were done at higher treatment levels.

Subsequent exposures were done at identical concentrations for two monthly trials. The results of duplicated exposures for the two separate trials are reported as the mean values (Table 8).

The reference fungicide, malachite green, was tested at 5.0 ppm. This exposure level was necessary to be effective at the high infection rate of 25%. This treatment was effective for decreasing the infection on infected eggs and increased the hatch rate substantially over the negative control group of eggs. Although the prescribed treatment rate for malachite green in fish culture is 1.0 ppm, that level would not be effective for treating high levels of fungal infection.

Formalin at 1.667 ppm was the most effective treatment on infected eggs. Infection rates were negative values which suggests that some of the infected eggs survived to develop and hatch. Hatch rates were highest among any of the candidates and malachite green. The low treatment level of 250 ppm was ineffective for treating this high rate of fungal infection.

Amorolfine was the most effective antifungal agent among three morpholine derivatives. In these trials it was ineffective for decreasing infection rates and the resulting hatch rates were not impressive even though they were better than the negative control. The manufacturer of Amorolfine has indicated it is no longer interested in the development of that material as an antifungal agent; the manufacturer’s policy is that drugs for human use are not used for animal or crop care. Therefore. this material wil1 not be evaluated further.

TABLE 8. Effectiveness of reference and candidate fungicides on infected eggs of rainbow trout at 12°C for an exposure time of 60 minutes.

Chemical Name	Treatment (ppm)	Mean (N=2) infection (%)		Mean hatch (%)
		Initial	Increase	
(-) Control	--	3.5 4	62.9	27.8
(+) Control	--	24.2	60.0	13.6
Malachite Green	5.0	23.3	0.1	66.6
Formalin	250	23.2	39.0	32.3
Formalin	1.667	24.1	-6.0	73.3
Amorolfine	100	23.2	35.4	42.8
Glutaraldehyde	200	23.0	16.2	36.3
Sodium Chloride	30.000	23.3	10.2	59.5
VigorOx	200	23.2	37.6	39.8

Glutaraldehyde at 200 ppm provided effective antifungal activity: the increase in infection was only about 16% and the hatch rate about 36%. The literature suggests that high concentrations of glutaraldehyde may be mutagenic; additional information on safety is required to evaluate the potential of this material. Inquiries to FDA agents on registration requirements have confirmed the need to develop additional toxicology and teratology information. Glutaraldehyde continues to be a candidate antifungal compound for treatment of eggs.

Sodium chloride or mixtures of sodium and calcium chloride have been suggested and reported as a safe. efficacious, and economical treatment for saprolegniasis in salmonid incubation and rearing. Taylor and Bailey (1979) reported that daily treatments of 2-3 hours sea water were effective for control of *Suprolegnia diclina* on eggs of pink salmon *(Oncorhynchus gorbuscha).* In our studies 30,000 ppm or 3% were effective for

decreasing infection rates and increasing hatch rates (Table 8). Salt remains a candidate fungicide. however. the large quantities required complicate the logistics of use and the disposal of effluent.

VigorOx at 200 ppm was not particularlv effective for decreasing infection rate or improving the hatch rate. Additional testing of the components of peracetic acid and hydrogen peroxide are necessary to fully evaluate this osidant as an antifungal agent.

DISCCUSSIOIN

Willoughby. and Roberts (1992) reported that 0.25 ppm of malachite green osalate killed zoospores and zoospore cysts of *Saprolegnia* parasitica in the water column and that exposure for 15 minutes should control fungal growth and protect the fish. In reality most fish culturists treat with malachite green at 1 ppm or higher for exposures of up to 1 hour. However. control of fungus on infectcd eggs requires higher concentrations. especially when infection rates are at 20% or greater as in our esperiments. The 5.0 ppm applications for I hour was fungicidal: whereas. the 1 .O ppm treatment is probably fungistatic. The higher levels required for antifungal activity on eggs are perhaps too excessive for use on fish.

Results of in vivo testing suggested that Amorolfine. glutaraldehyde. iodine. potassium pcrmanganate. sodium chloride. and VigorOx showed potential for control of fungus on infected eggs. In the first concurrent esposure of infected eggs to antifungal agents (Table 7) the concentrations of malachite green. Amorolfine. glutaraldehyde. and VigorOx were inadequate to demonstrate desirable antifungal activity The concentrations of iodine and potassium permanganate were as high as the eggs would tolerate yet neither compound produced satisfactory antifungal activity. However. both compounds have been reported to be useful for treating or preventing fungal infections in cultured eggs. Perhaps either compound would be useful for treating eggs that had a lower infection rate and lower concentrations may be effective. Iodine treatment of salmoid egg surfaces has been recognized as an effective means to prevent the spread of disease (Piper. et.al. 1982). The 100 ppm iodine for up to 60 minutes exposure was demonstrated to be safe and effective for water hardening salmonid eggs (Chapman and Rogers 1992). however. they reported significant losses of the iodine concentration in treatment solutions to the eggs. Formalin at 1.667 ppm and sodium chloride at 30.000 ppm were the most effective compounds for control of fungal infection and increasing the hatch rate.

The remaining candidate antifungal agents. two levels of formalin. and 5.0 ppm of malachite green were tested simultaneously for two months. Results of these exposures (Table 8) suggest that the high level of malachite green was antifungal for infection rates of about 23%. Formalin at 1.667 ppm was the most effective treatment: the infection decreased and the hatch rate was 73%. Sodium chloride was more antifungal than the

remaining candidates and those treatments resulted in a better hatch. Amorolfine. glutaraldehyde. and VigorOx treatments produced a better hatch rate than the negative control groups. but improvement was only moderate.

Earlier studies by Cline and Post (1972) reported malachite green to be superior to seven other candidates for control of *Saprolegnia* on trout eggs. Their candidate glutaraldehyde did not inhibit fungus in concentrations as high as 2.000 ppm *in vitro,* but 200 ppm produced some antifungal effect *in vivo.* In these same studies formalin did not inhibit fungus at 3.800 ppm *in vitro.* but showed antifungal activity *in vivo* at 150 and 300 ppm. Cline and Post's effective concentrations differed from our results. especially the *in virro* exposures. and they reported malachite green superior to other compounds. Our data suggest that formalin or sodium chloride treatments may be as effective as malachite green. Differences are probably due to procedures used. especially those that involve infection. Embryo susceptibility to infection and toxicity changes seasonaly and with the condition of the eggs. Variations in our results were minimized by exposing eggs from the same group to all of the antifungal agents concurrently in replicated tests for efficacy.

Registration requirements are rigid. expensive, and time consuming for compounds that have no other registered uses or for those that have not been tested for mammalian safety. Amorolfine. glutaraldehye and VigorOx have no existing registration by FDA for therapeutic uses. Their development would require further negotiations with regulatory agents. At this time. none seem to compare with formalin or sodium chloride as an antifungal agent on salmonid eggs. Sodium chloride has been recognized as a safe treatment on fish and their eggs for disease and stress. Therefore, sodium chloride must be considered as an effective antifungal agent. However, the high concentrations required are logistically difficult for hatchery managers. If sodium chloride is to be used in fish culture. procedures will be required to implement administration and effluent treatment.

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chapter 8

SCREENING FOR BACTERIA ANTAGONISTIC TO *SAPROLEGNIA PARASITICA* WITH BASF PLURONIC POLYOL F-127

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ABSTRACT

BASF pluronic polyol F-127 is a low temperature liquifying polyol which solidifies upon warming. When BASF polyol F-127 is used for screening, microorganisms or other targets are not subjected to a heat shock as when they are embedded in molten agar. Bacteria colonies do not spread when overlayered with BASF pluronic polyol and thus as many as 2000 colonies per plate can be screened for antagonist activity. Bacteria from a variety of ecological habitats including the mucus of healthy and diseased fish were screened for antagonists to *Saprolegnia parasitica* Coker. Bacterial antagonists to *S. parasiticu* were present in the mucus of the majority of healthy fish tested but not from fish infected with *S. parasitica*. The use of *S. parasitica* as a target organism yields isolates which show broad antagonist activity against a variety of *Pythium* and *Phytophthora* species. The biological activity spectrum of the antagonists varies with the ecological habitat examined. The importance of control of water quality, nutrient levels and the use of antibiotics on the composition of the microflora of fish mucus is discussed.

Key words: *Saprolegnia,* fish, pathogen, bacteria, antagonist.

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INTRODUCTION

Fungi belonging to the Saprolegniaceae are often described as pathogens on freshwater fish (Vishniac and Nigrelli, 1957; Srivastava and Srivastava, 1978). *S. parasitica* is the most frequently described fungus attacking freshwater fish and eggs. A mucus layer protects fish from infection by physical and immunological factors (Wood, Willoughby and Beakes, 1988; Jorgensen, 1990) but in the spawning season endocrine changes take place reducing the defence system of the fish and mature males are at special risk (Pickering and Christie, 1980). Stress (Helle. 1990) and physical damage to the fish during the mating season increases their susceptibility to infection (Richards and Pickering. 1978).

Saprolegnia parasitica is effectively controlled by malachite green (Oláh and Farkas. 1978; Sati, 1983). Bauer. Bangschat. Hnöppler and Neudegger (1988) showed that malachite green is absorbed and accumulated in the muscle tissue of fish and Steffens, Leider, Nehring and Hattop (1961) showed that malachite green is carcinogenic and thus its use for the control of fungal diseases of fish has been prohibited in many countries. The effect of several other compounds on *Suprolegnia* spp. has been investigated (Alderman and Polglase. 1984; Bailey, 1984). but an ecologically acceptable method for the control of *S. parasitica* has yet to be found.

The purpose of this study was to screen the bacterial flora of soil, fish and plant materials, including the mucus of healthy and *S. parasitica* infected fish to determine: 1. whether bacterial antagonists to *S. parasitica are* common, 2. the biological activity spectrum of bacterial antagonists to *S. purusitica* against plant pathogenic members of the Oomycetes and other common plant pathogens, and 3. whether microorganisms in the mucus might contribute to the protection of fish and if their presence/absence can be used as an indicator of the “health” of the fish.

MATERIALS AND METHODS

BASF pluronic polyol F-l 27 (BASF Wyandotte. 100 Cherry Hill Road, Parsippany, New Jersey 07054, U.S.A.) is a block copolymer of propylene oxide and ethylene oxide which is a liquid (a sol) at 4°C and solidifies to form a solid (a gel) upon warming (Gardener and Jones. 1984; Choi and Ross. 1988). The temperature at which the medium solidifies is determined by the percent (w:v) of the compound used. An 18% solution gels at ca 32°C while a 50% solution solidifies at ca 10°C. In the work described here a 30% solution was used which gels at ca 18°C.

For the selection of microbial antagonists from soil, 5-10 g soil was mixed with 10 ml of sterile dilute salts solution (DS) (Dill and Fuller, 1971). For the selection of microbial antagonists from potato leaves, tubers or roots the material was washed in 10 ml of sterile DS. Samples of the mucus layer of healthy and *S. parasitica* infected fish

were taken by streaking a sterile cotton swab over the surface of the fish. The cotton swab was then transferred to a culture tube containing 1 ml sterile DS. A dilution series of each sample was prepared. The samples (0.1 ml of each dilution) were inoculated into cold (4°C) BASF pluronic polyol F-127 (30%) YPG nutrient medium (1% Difco yeast extract, 2% Difco peptone and 2% glucose). The BASF-F-127 nutrient medium was autoclaved and allowed to liquify at 4°C for at least 24 hrs prior to use. (The BASF F-127 nutrient medium can be liquifred and solidified repeatedly without loss of "gelling" activity).

We have found it convenient to use commercial plastic drinking cups for mixing the samples with the cold (4°C) BASF F-127 nutrient medium. Because the BASF F-127 polyol solidifies at room temperature, all glassware which has been in contact with the polyol must be washed in cold running water. This either means that the staff who washes the glassware must be trained to wash this glassware separately in cold running water or the user washes their own glassware!

The samples were poured into sterile Petri dishes (9 cm diam) and placed on a levelling table to solidify. Plates were incubated for 4 days at 20-23°C and plates containing 200-2000 colonies/plate were suitable for screening. The plates were overlayered with cold (4°C) BASF F- 127 nutrient medium inoculated with zoospores of *S. parasitica.* (Stock cultures of *S. purasitica* were maintained on potato carrot agar (Van der Plaats-Niterink, 1981). Zoospores were produced by placing autoclaved hemp seeds at the margin of the culture and after 24 hrs transferring l-2 hemp seeds to 10 ml sterile DS solution. Zoospores could be harvested ($1\ 0^4$ to 10^5 /ml) after incubating the culture at 23°C for 24-36 hrs.

After l-2 days at 23°C microorganisms antagonistic to *S. parasitica* in the bottom layer were seen at the centre of clear zones in the overlayer. These microbial antagonists were streaked for single colonies on YPG nutrient agar (Olson and Lange. 1989). Morphologically different bacteria were isolated for further screening for anti microbial activity against *S. parasitica.*

For the isolation of secondary metabolites from these bacterial antagonists, the isolates were inoculated into 2X YPG broth (i.e. double the normal concentration of YPG) (50 ml/100 ml Erlenmeyer flask) and incubated on a rotary shaker (180 rpm) at 23°C for 4-12 days. The broth from these cultures was centrifuged at 12,000 rpm in an Eppendorf centrifuge for 15 min and the cleared supematant or a methanol extract of the pellet were then tested against a variety of organisms using a "Biological Activity Spectrum" test (BAS).

For the BAS test, inoculum of *Pythium* and *Phytophthora* isolates was produced as follows: the fungi were maintained on sterile grass leaves in sterile DS in culture tubes at 4°C; hyphal fragments of these fungi were produced by a brief vortex mixing of the culture. Hyphal fragments or spores of the other target fungi *(Botrytis cinerea, Alternaria*

brassicae. Rhizoctonia solani, Fusarium oxysporum, Colletotrichum dermatium) were used to inoculate cold (4°C) BASF F-127 nutrient medium. Oospores of *Pythium ultimum, P. torulosum. P. aphanidermatum, P. irregulare* and *Phytophthora* sp. 186 were produced by inoculating Petri dishes containing 20 ml of potato carrot broth (Van der Plaats-Niterink. 1981) with 5-10 1 mm^3 agar blocks. After incubation at 23°C for 7 days the mycelial mat was removed. washed in sterile DS and allowed to dry on a stack of sterile Whatman #1 filter papers (Rosendahl and Olson, 1992). The dried mycelium was briefly rehydrated in l-2 ml sterile DS and ground in a sterile mortar. The oospore suspension was used to inoculate the cold (4°C) BASF F-127 liquified nutrient medium.

In the BAS test, 30 µl aliquots of the centrifuged culture broth or methanol extract were applied to sterile Whatmann AA filter discs (6 mm diam). The inhibition zone formed around the filter disc was scored as follows: 8 mm diam = l/2 , 10 mm diam = 1, 15 mm diam = 2.20 mm diam = 3.25 mm diam = 4 and 30 mm diam = 5.

RESULTS

In Table 1 are shown the results of the BAS test of bacteria selected for antagonist activity against *S. parasitica* (isolates 8 18-828 were obtained from roots of plants from very dry soil from Virginia, U.S.A.; isolates 829-841 were from potato tubers; isolates 868-887 were from the mucus of rainbow trout *(Salmo gairdnei* Richardson) and isolates 895-916 were from bean roots). Although all the bacteria isolates analyzed in the BAS test (Table 1) were pre-selected for anti *S. parasitica* activity in the primary screening, their activity against other oomycetous fungi and a variety of common plant pathogens varies greatly (marked fields in Table 1).

When secondary metabolites from some of the isolates listed in Table 1 were tested for their ability to inhibit oospore germination a very different pattern of inhibition was observed (Table 2). Secondary metabolites from isolates 829-841 show no activity against hyphal development of *Pythium torulosum* but are extremely active in inhibiting oospore germination of this species.

Table 1. Biological activity spectrum (BAS test) of bacteria selected for antagonist activity against *Saprolegnia parasitica* (isolates 8 18-828 from roots of plants from dry soil, 829-841 from potato tubers, 868-887 from the mucus of rainbow trout, 895-916 from bean roots).

Isolate	anti Sp	P207	P12	Pu	Pt	Pa	Pi	Po	Ph186	Ph360	B	A	R	F	C
LWO															
818	2	0	0	1/2	2	0	0	0	0	0	0	0	0	0	0
819	2	0	0	1/2	0	0	0	0	0	0	0	0	0	0	0
821	3	0	0	2	0	0	0	0	0	0	0	0	0	0	0
822	3	0	0	2	0	2	2	0	2	0	0	0	0	0	0
824	3	1/2	2	2	0	3	2	0	2	0	0	0	0	0	0
825	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
826	2	1/2	2	2	3	3	3	0	0	0	0	0	0	0	0
828	2	0	0	2	3	2	2	0	0	0	0	0	0	0	0
829	1	0	0	0	0	1/2	0	0	0	0	0	0	1	0	0
832	2	1	0	1	0	1	0	0	0	0	2	0	2	0	0
833	2	2	0	1	0	1/2	0	0	0	0	2	0	2	0	0
836	3	0	1/2	2	0	1/2	2	0	0	0	0	4	1	0	0
837	2	3	0	1	0	1/2	3	0	2	0	1	4	3	0	0
839	2	1	0	1/2	0	0	0	0	0	0	1	0	1	0	0
840	2	1	1	1/2	0	0	0	0	0	0	2	0	3	0	0
841	2	1	0	1/2	0	1	0	0	0	0	1	4	1	0	0
868	2	0	1	0	2	1	2	3	0	0	0	0	0	0	0
869	1	1	3	0	3	3	3	3	0	2	0	0	0	0	0
870	1	0	3	0	2	2	3	3	0	1	0	0	0	0	0
871	1/2	0	0	1/2	2	3	3	2	0	0	0	0	0	0	0
873	3	2	3	2	3	3	3	3	1	2	0	0	0	0	0
874	2	0	3	1/2	2	3	3	3	0	2	0	0	0	0	0
875	3	2	0	1/2	2	2	0	0	0	1	0	0	0	0	0
879	2	0	1/2	0	1	1/2	2	1	2	0	0	3	3	0	0
884	1/2	2	0	1	3	0	0	0	2	0	0	0	0	0	0
885	1/2	2	1	2	3	2	2	0	3	0	0	0	0	0	0
886	1/2	3	0	1/2	3	2	2	0	4	1/2	0	0	0	0	0
887	1	0	0	0	1	1/2	1/2	0	0	1/2	0	3	4	0	0
895	1	0	0	0	1/2	0	0	1	0	0	0	0	0	0	0
896	1	1/2	0	1/2	1	1/2	1/2	1	0	0	0	0	0	0	0
898	1	1/2	1	1/2	1	2	3	0	2	1	0	0	0	0	0
899	1	1/2	2	1/2	1	3	3	0	2	0	0	0	0	0	0
900	1/2	0	0	0	1/2	0	0	0	0	0	0	0	0	0	0
901	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
902	2	1	1	1/2	1	2	3	0	2	1	0	0	0	0	0
903	1	0	0	0	1/2	0	0	0	0	0	0	0	0	0	0
912	1	0	0	0	1/2	1	2	0	1/2	0	0	0	0	0	0
913	2	2	2	3	2	2	3	0	3	1	0	0	0	0	0
914	2	1	1/2	2	1	1	3	0	1	1	0	0	0	0	0
916	1/2	0	1	1/2	1	1	1	0	3	0	0	0	0	0	0

Sp = Saprolegnia parasitica
P207 = Pythium sp. 207 (F type)
P12 = Pythium sp. 12 (F type
Pu = Pythium ultimum
Pt = Pythium torulosum
Pa = Pythium aphanidermatum
Pi = Pythium irregulare
Po = Pythium oligandrum
Ph186 = Phytophthora sp. 186
Ph360 = Phytophthora sp. 360
B = Botrytis cinerea
A = Alternaria brassicae
R = Rhizoctonia solani
R = Fusarium oxysporum
C = Colletotrichum dermatium

Zone of inhibition - Use Whatmann AA filter disc, 6 mm diam. Use 30 µl/filter disc. 1/2 = 8mm diam; 1 = 10 mm diam; 2 = 15 mm diam; 3 = 20 mm diam; 4 = 25 mm diam; 5 = 30 mm diam.

Table 2. Inhibition of oospore germination by secondary metabolites. Compare to Table 1. For zones of inhibition see Table 1.

Isolate	Pu	Pt	Pa	Pi	Ph186
818	0	0	0	0	1
819	0	0	0	0	**I/2**
821	0	0	0	0	2
822	0	0	0	0	I
824	0	0	0	0	1
825	0	0	0	0	0
826	0	0	0	0	1
828	0	0	0	0	2
829	**0**	**2**	**0**	**0**	**1/2**
832	**0**	**1**	**0**	**0**	3
833	**0**	**3**	**0**	0	2
836	**0**	**3**	**0**	0	4
837	**0**	**3**	**0**	0	5
839	**0**	**3**	**0**	0	0
840	**0**	**3**	**0**	1	3
841	**0**	**4**	**0**	0	4

Pu = Pythium ultimum
Pt = Pythium torulosum
Pa = Pythium aphanidermatum
Pi = Pythium irregulare
Phl86 = Phytophthora sp. 186

For zones of inhibition, see Table 1

The activity of secondary metabolites from bacteria from fish mucus against a *variety* of *Pythium* species (Table 1) suggested that these bacteria could be of interest for our screening program for antagonists to *Pythium* and *Phytophthoa* species. In Table 3 is shown the number and species of fish collected at 7 different Danish localities and the number of fish from each locality with mucus containing bacteria which showed anti *S. parasitica* activity in the primary screening.

Table 3. Number of fish from each locality with mucus containing bacteria antagonistic to *Saprolegnia parasitica.*

Locality	Number of fish	Fish with antagonists	Saprolegnia infected fish
Locality 1	7	7	0
Locality 2	5	0	5
Locality 3	7	5	0
Locality 4	6	6	0
Locality 5	2	2	0
Locality 6	3	2	0
Locality 7	3	3	0
Total number	33	25	5

Locality 1: 7 roaches *(Scardinius erthropthamul* L.) from pond in The Botanical Garden of Copenhagen.

Locality 2: 5 rainbow trout *(Salmo guirdneri* Richarson) infested *with Saprolegnia parasitica* from a fish farm near Silkeborg.

Locality 3: 6 young brown trout *(Salmo trutta* L.), 1 eel *(Anguilla anguilla* L.) from a stream near Vedbaek

Locality 4: 6 rainbow trout *(Salmo guirdneri* Richardson) collected from a combined electric power station/fish farm in Stignaes.

Locality 5: 2 goldfish *(Curassius aurutus* **L.)** from pond in the greenhouse of the Botanical Garden of Copenhagen.

Locality 6: 3 rainbow trout *(Salmo guirdneri* Richardson) from Voldbjerg Mole1 fish farm, Voldbjerg.

Locality 7: 3 rainbow trout (ISalmo gairdneri *Richardson) from* Hededambruget (fish farm) Spjarup.

Table 4. Time of expression of inhibitory secondary metabolities from day 4-12. For zones of inhibition see Table 1.

Isolate	Day 4	Day 6	Day 9	Day I 1	Day 12
28	2	3	3	1/2	1
29	1	I	2	1/2	1
30	2	1	1	1/2	2
31	0	1	I	2	I
32	I	0	0	0	0
33	I	0	0	0	0
34	0	0	1	1	1
35	2	1	1	1	I
36	1	1	0	1	0
37	1	1	0	I	0
38	0	0	0	0	0
39	0	0	0	0	0
40	0	0	0	0	0
41	0	0	0	0	0
43	1	0	0	0	0
44	1	0	0	0	0
45	1	0	0	0	0
46	0	2	1	1	1
47	0	1	1	1	1

The secondary metabolites tested in Table 1 were obtained after 5 or 7 days incubation and the best of the two incubation times was used. In Table 4 is shown the inhibitory activity of secondary metabolites of bacteria from fish mucus (Table 3) after incubation for 4- 12 days. These results strongly suggest that there is an extreme variation in when anti S. *parasitica* activity is first expressed. The disappearance of anti S. *parasitica* activity followed by its reappearance in later samples suggests that more than one secondary metabolite with anti S. *parasitica* activity is being expressed and the stability of the metabolites can vary.

DISCUSSION

In classical screening of bacteria for antagonist activity. a sample is spread on a nutrient agar plate and after incubation overlayered with the target organism to be selected against. This type of screening has several inherent disadvantages: 1. bacterial colonies tend to spread dramatically when overlayered with agar and form diffuse and/or smeared colonies when the agar overlayer sets; 2. bacteria embedded in agar are difficult to recover and 3. the target organism as well as the organisms to be screened are subjected to a severe heat shock when embedded in molten agar. cooled to 45°C. The selection of microbial antagonists from the north temperate to arctic regions of the world requires that the samples not be subjected to elevated temperatures and zoospores of many of the oomycetous fungi which we are interested in working with cannot survive even a brief exposure to molten agar. Because BASF pluronic polyol F- 127 forms a liquid at 4°C and solidifies at room temperature. this compound is useful when working with organisms which are or are suspected to be heat sensitive (Olson and Lange. 1989). Because the compound forms a gel and not a true solid. it is particularly easy to recover colonies after being overlayered with the'target to be selected against. Bacteria colonies do not spread when overlayered with cold (-1°C) liquid BASF pluronic polyol F-127 nutrient medium and thus a large number of colonies per plate can be screened for antagonist activity.

The results presented in Table 1 suggest that bacterial antagonists to *S. parasifica* are common and occur in widely different ecological habitats. The biological activity spectrum of these bacteria against a variety of *Pythium* and *Phytophthora* species and other common plant pathogens *(Botrytis, Alternaria* and *Rhizoctonia)* varies with the ecological habitat examined. The relatively broad antagonist activity of some of these isolates suggests that they should be tested *in vivo* in soil known to cause damping off. Such isolates might be used singly or in various combinations as seed dressing for the control of fungi causing damping off.

Table 2 shows that there is no apparent correlation between inhibition of hyphal growth and inhibition of oospore germination. For the biological control of soil borne *Pythium* and *Phytophthora* species it would obviously be an advantage to obtain isolates which inhibit both hyphal growth and oospore germination.

In the present study a considerable number of morphologically different antagonists to *S. parasitica* were found in the mucus of healthy fish from different localities (data not shown). This suggests that microorganisms in the mucus might contribute to the protection of the fish against infection by *S. parasitica.* In contrast to the results of Hatai and Willoughby (1988) no antagonists were found on S. parasifica infected fish (Table 3) even though a large number of bacteria from the mucus were screened for anti S. *parasitica* activity. Admittedly the number of fish and localities sampled for the presence of bacteria antagonistic to *S. parasitica* is extremely limited. However, the total absence of bacteria antagonistic to *S. parasitica* in mucus of fish infected with the fungus suggests

that a systematic screening of the bacteria present in fish mucus of healthy and diseased fish should be undertaken.

Hatai and Willoughby (1988) isolated two strains of *Pseudomonas fluorescens* from *S. parasitica* lesions on two out of three fish examined. A Petri dish assay showed that both strains were able to inhibit mycelial growth of *S. parasitica*. Inoculation experiments with gold fish *(Carassius auratus* L.) showed that the most active of the strains was not a pathogen and the authors suggested that strains of *P. flurorescens* could be used as a biological control agent against *S. parasitica.* As noted by Håstein (1990) *P. jluorescens* can function as a secondary fish parasite so care must be taken in the use of this bacteria for the control of *S. parasitica*.

The idea of inoculating bacteria antagonistic to *S. parasitica* into the water used in fish farms does appear interesting but the problems in selecting the correct isolate are daunting. Such an isolate must be non pathogenic to fish, plants and man; it must be able to readily establish itself in the mucus of fish and must be resistant to the chemical treatments used in commercial fish farms. The antibiotics and other chemical treatments used in commercial fish farms could be important factors in determining the composition of the mucus microflora and hereby affect the vulnerability of the fish to infection by *S. parasitica.* Because of the extensive use of antibiotics and other chemicals in commercial fish farming, the use of bacteria antagonistic to *S. parasitica* is probably not practical.

The isolation of secondary metabolites from bacteria antagonistic to *S. parasitica* offers an alternative approach but also one fraught with problems. Table 4 illustrates how important it is to select the precise time at which to harvest the culture broth for the maximum anti *S. parasitica* activity. The appearance, disappearance and reappearance of anti *S. parasitica* activity in the culture broth (Table 4) suggests that we are studying a cocktail of different secondary metabolites active against *S. parasitica* produced at different times during incubation and which differ in stability.

We find that the presence of antagonistic bacteria in mucus of healthy fish is interesting and the significance of their number and diversity for the vulnerability of fish to infection by *S. parasitica* should be studied further. One approach could be to compare the composition of the microflora in mucus of fish living in natural localities and commercial fish farms. This could reveal if the presence of antibiotics, chemicals and/or high levels of nutrients in the water is of importance to the composition of the mucus microflora. Biological control of *S. parasitica* might not require a direct inoculation of bacterial antagonists or metabolites derived from these organisms into the water; control of the fungus could be strongly influenced by the composition of the mucus microflora. Controlling water quality, nutrients and exposure time to antibiotics may improve the natural microflora present in the fish mucus and thus lead to greater protection against infection by *S. parasitica.*

This preliminary study shows that it is easy to select for bacteria with anti microbial activity against *S. parasitica* from a variety of ecological habitats including the mucus of healthy fish. Whether these bacteria or their metabolites can be exploited in the control of *S. parasitica* on fish and/or the control of *Pythium - Phytophthora* species in pre and post emergence damping off in plants will require extensive and long term studies of the toxicity of these bacteria and their metabolites in the ecosystems to which they are to be introduced.

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SECTION V

POSTSCRIPT

chapter 9

FUNGAL PARASITES OF SALMON FROM THE COLUMBIA RIVER WATERSHED

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ABSTRACT

Suprolegnia sp. and a variety of other fungi were isolated from salmonids. predominantly chinook salmon, at 20 hatchery sites in Washington and Oregon. The isolates, taken from all life-stages of the host, were examined for morphology, growth rate and gelatin liquidification. One hundred and eighty lesions were sub-sampled for intra-lesion variation. Our observations suggest the great majority of isolates fit within the currently proposed boundaries of *Saprolegnia parasitica* but physiological differences between isolates are detectable. The hyperparasite, *Woronina polycystis,* was recovered from 3 hatchery sites. It has been isolated into monoxenic culture and studies on its development and potential for biological initiated.

Key words: *Saprolegnia parasitica, Woronina polycystis,* salmon, saprolegniasis, Columbia River.

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INTRODUCTION

Saprolegniasis has been recognized as a disease of fish for almost 250 years. The disease is world-wide in distribution (Neish and Hughes. 1980: Post. 1987; Hughes et al., 1993. this volume. chap. 13) and occurs in most populations of cultured salmonids where it infests all developmental stages: eggs. fry smolts. and adults. Epizootics are rare, but the chronic nature of the infection usually results in a small but steady mortality. Adult Chinook salmon are particularly labile to infection and spring returning stocks must be treated to survive for spawning in the fall. Up until the present time serious outbreaks of saprolegniasis and valuable infected stocks have been treated with Malachite Green. However, the suspected teratogenicity and toxicity of Malachite Green (Meyer and Jorgenson. 1983; Alderman. 1985). has limited its use. Fish stocks have dwindled in recent years making each fish more important in maintaining the stocks. Formalin, which has replaced Malachite Green in treatment. is fungistatic. and is only partly effective as a curative fungicide.

Identification and knowledge of the fungi that attack spring and summer returning adult Chinook salmon is needed for the proper assessment of new control measures. This 18 month study assesses directly the identification and distribution of the *Saprolegnia* species infecting Columbia River and nearby hatchery stocks. Growth rate and gelatin liquefaction rate were chosen as physiological measures having the potential to be tied directly to pathogenesis. Fungi. other than *Saprolegnia* spp. also were isolated from juvenile and adult fish.

MATERIALS AND METHODS

Returning adult Chinook salmon were sampled extensively for saprolegniasis in six hatcheries in Washington and Oregon State (Table 1. Fig. 1). Early returning adult Chinook salmon at Cowlitz Hatchery (Washington State) were sampled four times on arrival (before holding and treatment). twice during the summer and again in the fall at spawning time. One group of late returning adult Chinook salmon (27 August 1991) was also sampled. Twelve additional sites (Table 1. Fig. 1) were visited, but because of the valuable nature of the material (eggs, fry, smolts or brood stock) smaller samples were obtained. A number of single samples obtained from water sources also were examined (Table 1. Fig. 1). Surface lesions were scraped and the recovered material examined under a low power stereoscope. Six relatively clean individual hyphae were plated individually near the margin and under the surface of the agar plate forcing the hyphae to grow up through the agar (Seymour and Fuller. 1987). The hyphae were selected to include the range of morphological variation (e.g., gemmae, zoosporangia, vegetative hyphae) found on the host animals. After 24-48 hours three of the six colonies were subsampled by hyphal tip transfer. The choice of the colonies sub-sampled reflected differences in growth rate. colony appearance, or morphological variation as indicated above. When hyphae were not apparent, excised fish tissue from surface wounds, muscle

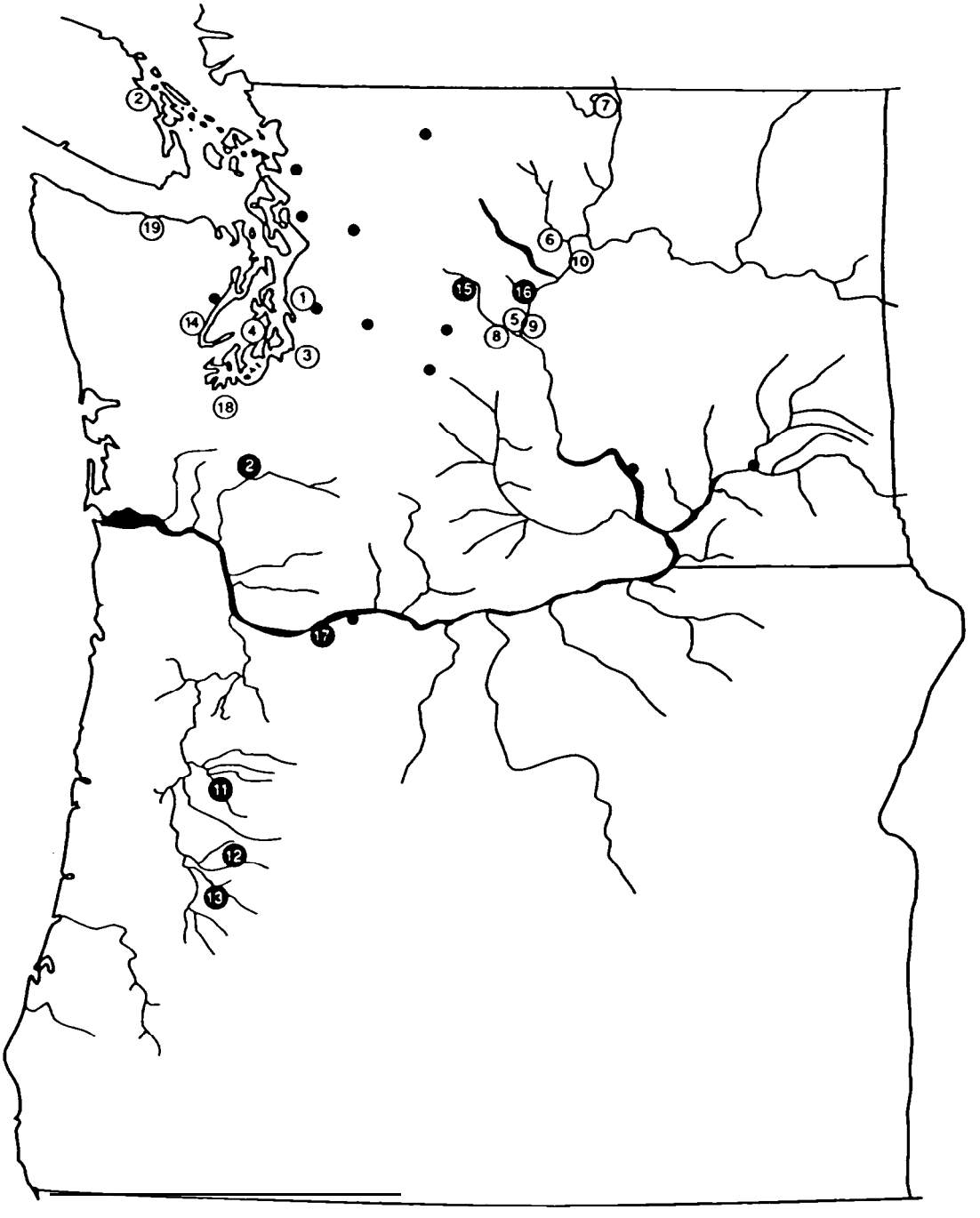


Figure 1: Location of sample sites in Washington, Oregon, and British Columbia. Dark circles represent sites with extensive or unusual collections. Open circles represent sites with small collections. Dots represent single samples. Key to site numbering is contained in Table 1.

TABLE 1. SAMPLE SITES

	LOCATION	DATE			SAMPLE
1	Univ. of Wash. Hatchery	16	Jan.	1991	Rainbow - adult
2	Cowlitz Hatchery	6	Feb.	1991	Chinook - eggs, yearling Coho - adult, eggs
		28	May	1991	Chinook - adult
		2	July	1991	Chinook - adult
		5	Aug.	1991	Chinook - adult
		27	Aug.	1991	Chinook - adult
		3	Sept.	1991	Chinook - adult
		3	Apr.	1992	Chinook - eggs
		4	March	1992	Chinook - yearling
3	Green River Hatchery	6	Feb.	1991	Coho - yearling; Chinook - eggs
4	Hupp Springs Rearing Pond	15	March	1991	Chinook - fingerling
5	Turtle Island Rearing Pond	18	Apr.	1991	Steelhead - yearling; Coho - yearling
6	Methow Rearing Pond	19	Apr.	1991	Chinook - yearling
7	Similkameen Rearing Pond	19	Apr.	1991	Chinook - yearling
8	Eastbank Hatchery	19	Apr.	1991	Chinook - yearling, eggs
		10	June	1991	Chinook - adult
9	Dryden Rearing Pond	19	Apr.	1991	Chinook - yearling
10	Wells Hatchery	10	June	1991	Chinook - yearling
11	South Santiam Hatchery	25	June	1991	Chinook - adult
12	Leeburg Hatchery	26	June	1991	Rainbow - adult
13	Dexter Holding Pond	26	June	1991	Chinook - adult
14	Hoodsport Hatchery	23	July	1991	Chinook - yearling
15	Leavenworth Hatchery	25	Aug.	1991	Chinook - adult
16	Entiat Hatchery	31	Aug.	1991	Chinook - adult
17	Bonneville Hatchery	15	Nov.	1991	Chinook - adult
		23	Apr.	1991	Chinook - fingerling
18	Global Aqua Rearing Pond	27	Dec.	1991	Atlantic - yearling
19	Lower Elwha Hatchery	6	May	1992	Coho - yearling; Chinook - fingerling
20	Seaspring Salmon Farm	25	June	1992	Atlantic - yearling; Sockeye - yearling

tissue. or internal organs were placed directly on the agar. The fungi were sampled when hyphae appeared. Approximately half the lesions were screened for fungi other than *Saprolegnia*. chimps of hyphae and other material from the lesion were placed directly on the agar. Corn meal agar with 1 gm of penicillin G and 1 gm of streptomycin sulfate per liter was used initially for isolation. This did not prove to be an effective bactericidal combination (Barr. 1987) and later isolation used corn meal agar plus 1 gm of ampicillin and 40 mg of tetracycline per liter.

Linear growth rates were determined on all isolates using Ryan or race tubes (Ryan et al.. 1943: Mandels. 1965). Rates were determined at 20°C with a photoperiod of 10 h light and 14 h dark (Szaniszlo. 1965). Tubes 30 cm long and 12 mm in diameter were filled half full with 15 ml of chemically defined media designed for *Suprolegnia parasitica* Coker (Powell et al., 1972). Each isolate was transferred to defined media from corn meal agar and cultured for three days after which time, hyphal tips were inoculated into each end of a Ryan tube. The duplicate colonies. one originating at each end of the race tube were allowed to grow towards the center of the tube. The colonies were allowed to grow for 72 h or longer until the hyphal tip front completely filled the agar across the diameter of the tube. The leading edge of the hyphal tips of both colonies were marked at 24 hour intervals for a minimum of four days, depending on the experiment in progress. The daily growth rate of each colony was measured to the nearest mm. examined for linear growth and the resultant data from both colonies averaged to yield a daily growth rate (mm/day). Duplicates that differed by more than 1 mm per day were tested additional times in the same manner. Isolates from the same locality were tested as a group and grown simultaneously. Two isolates were maintained on the chemically defined medium and their growth rate was measured with each group of isolates to determine reproducibility from group to group. These two isolates and others. 15 tubes or 30 replicates per test. were used to determine within group variability.

Gelatin liquefaction rate was used as a measure of proteolytic activity (Haley and Callaway. 1975; Hanken and Anagostakis. 1975; Peduzzi and Bizzozero, 1977; Espinel-Ingroff. 1988: Alberts et al. 1989). at least one measurement was made on all isolates. Slants were prepared from 8 ml of 12% gelatin. Tubes were inoculated from corn meal agar onto the center of the slant. The slants were incubated for 120 h at 20°C with a 10 h light 14 h dark photoperiod. The tubes containing the slant, the colony, and the liquefied gelatin were weighted at 120 h. After weighing the tubes were inverted, and the liquefied gelatin allowed to drain for 2 hours. when the tubes were again weighed. The difference between the initial weight and the drained weight was expressed as a daily rate (g/day liquefied). Reproducibility was determined in a manner similar to that determined for growth rate.

The morphology of all isolates was determined using colonies grown on hemp seeds in dilute pond water contained in a crystallizing dish (Emerson, 1958). The following morphological states were recorded for each isolate: (1) the type of zoospore

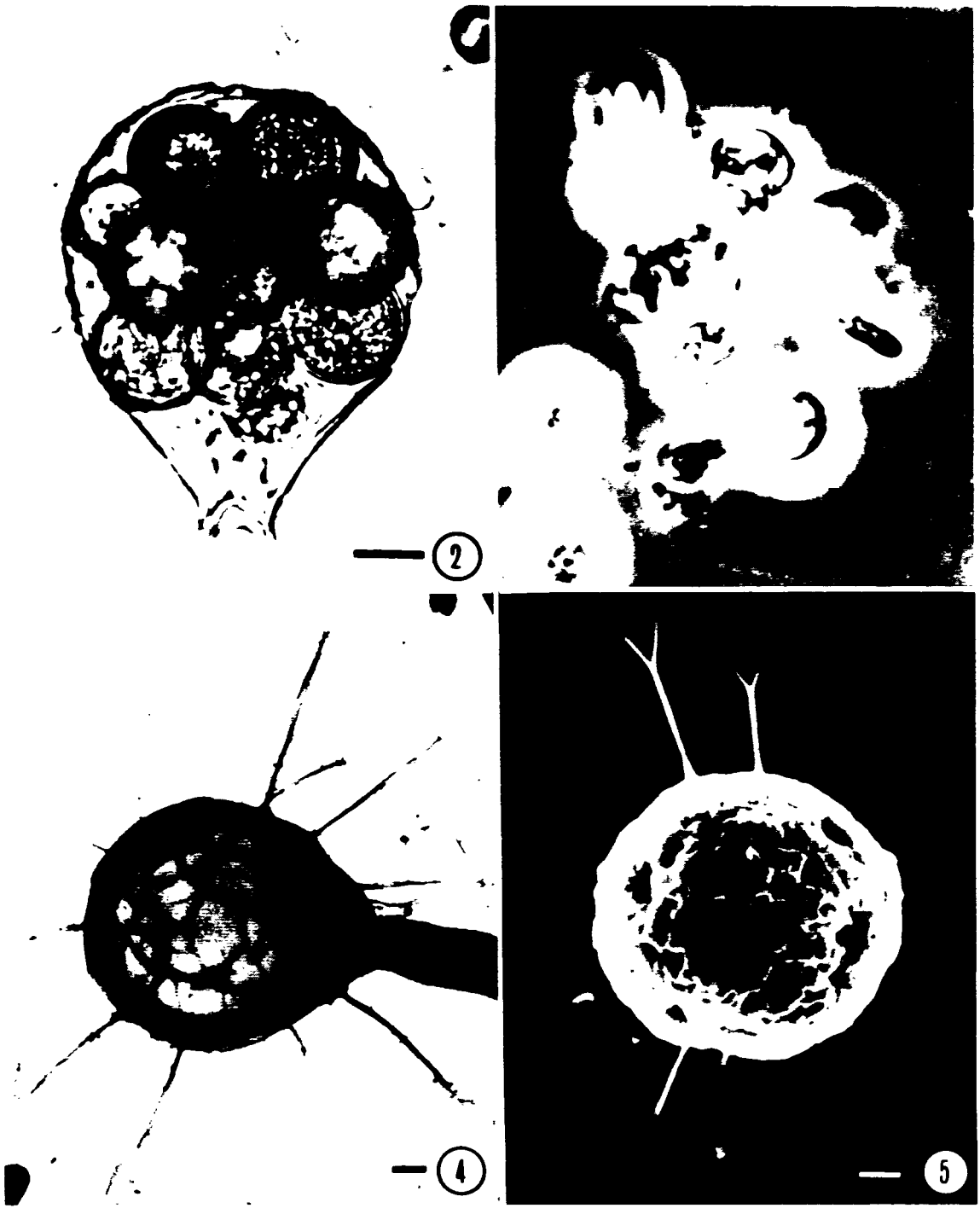
release, (2) the type of zoosporangium proliferation, (3) the degree of self attractiveness of the colony to zoospores. (4) the presence or absence of secondary zoospore cyst ornamentation, (5) the shape and structure of the wall of the oogonium, (6) the origin of the antheridial hyphae, (7) the type of attachment of the antheridial cell, (8) the structure of the oospore, (9) the degree of oospore abortion (self parasitism). and (10) the final growth form of the colony.

Selected samples were examined by both TEM and SEM. For the former study formvar coated grids were placed on the bottom of crystallizing dishes, immediately after removal of actively sporulating colonies. The grids were left for 24 hours, removed, rinsed in distilled water, examined for cysts and placed formvar side up on a damp filter paper in a petri dish. A second small petri dish was placed inside the first dish and several drops of 4% OsO_4 were added to the empty dish. After 4 hours the grids were removed and air dried. The cysts were later shadowed at 30" with platinum-palladium 80:20 (Pickering et *al.,* 1979). Material intended for SEM was collected in the manner described for TEM, after removal from the crystallizing dish and rinsing, the cysts on the grid were fixed with 1% gluteraldhyde in 0.1 M Millonig's buffer (Millonig, 1961) for 1 hour and then transferred to 1% OsO_4 in 0.1 M Millonig's buffer. The cysts were dehydrated with a graded series of ethanol, transferred to freon TF, and critically point dried. The specimens were later sputter coated with gold-palladium.

RESULTS

Six hundred and eighteen isolates of *Saprolegnia* spp. were obtained from 393 surface lesions during this study. Ninety one percent or 557 isolates fit within the currently accepted definition of *Saprolegnia parasitica* Coker (Seymour. 1970; Hatai et *al.*. 1990). that is. isolates with diclinous antheridial hyphae. centric to subcentric oospores, relatively thin walled. unpitted oogonia when formed (Fig. 2) and/or secondary zoospore cysts with ornamentations consisting of bundles of hooks (Figs. 3, 4, 5, 8). Of the remaining 9%. 48 isolates are assignable to *S. parasitica.* but have single hooks mixed with bundles of long hooks (Fig. 9). 2 isolates fit *Saprolegnia ferax* (Gruith.) Thuret. and *6* fit *Saprolegnia diclina* Humphrey (Figs. 7.6).

Figure 2: Oogonium with oospores and diclinous antheridial hyphae. Bar equals 20.0 pm. **Figure 3:** Secondary zoospore gerrnlings of *S. parasitica* with cyst ornaments (darts). Bar equals 10.0 urn. **Figure 4:** Secondary zoospore germling of S. *parasitica* cyst ornament recognizable as bundles of hook-like filaments. Bar equals 1.0 pm. **Figure** *5:* Secondary zoospore cyst of S. *parasitica* with ornaments extending to surface. Bar equals 1 .O pm.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Within the currently accepted definition of S. *parasitica, two* colony forms are recognized. Both forms produced zoosporangia by internal proliferation in a regularly repeated pattern until 20-30 zoosporangia had been produced and the hyphae developed a beaded appearance. In the first colony form 66% produced oogonia after the initial period of zoospore production. Twenty-two percent did not reproduce sexually, but instead produced gemmae, some of which were oogonial in shape and position, while others were zoosporangial in shape and position. Both sexually reproducing and asexually reproducing beaded forms were also moderately "self attractive," that is, many primary and secondary zoospores returned to the colony to encyst and germinate. Zoospores that left the colony also germinated quickly throughout the crystallizing dish. "Self parasitism" and abortion were extensive in the beaded forms with sexual reproduction. As a result few oospores survive in laboratory cultures. A few of these isolates showed low zoosporangia production and high zoospore "self attraction," that is, forming cuffs of primary zoospore cysts around points on the hyphae of the parent colony. In the isolates with high self attraction. only a few of the primary zoospore cysts germinated to eventually form secondary zoospore cysts. which were recognized by cyst wall ornamentation.

Isolates of the second colony form developed narrow hyphae which continued to elongate. creating a colony 3 to 4 times the diameter of the first colony type. These colonies did not form gemmae nor did they form oogonia immediately after the initial zoospore production period. A few isolates of the second or filamentous colony type underwent sexual reproduction with the oogonia scattered throughout the colony. These colonies also produced few secondary zoospore cysts.

From one location. Hoods Canal Hatchery, only six isolates appear to fit S. *parasiticu* in its most common form. In the other 36 isolates the secondary zoospore cysts had appendages consisting of both small bundles of hooks and individual hooks reminiscent of S. *diclina* (Fig. 9). Isolates with both small bundles of hooks and individual hooks were also present at South Santiam Hatchery.

Figure *6:* Secondary zoospore cyst of *S. diclina* with short single hook-like ornaments. Bar equals 1.0 μm. **Figure 7:** Short hook-like ornaments of *S. diclina* secondary zoospore cyst. Bar equals 1 μm. **Figure 8:** Distal end of a single bundle of filaments with hook-like tips of a *S. parasitica* secondary zoospore cyst. Bar equals 1.0 μm. **Figure** 9: Secondary zoospore cyst from Hood Canal Hatchery showing both single short hook-like filament and long bundles of hook-like filaments. Bar equals 1 .O pm.

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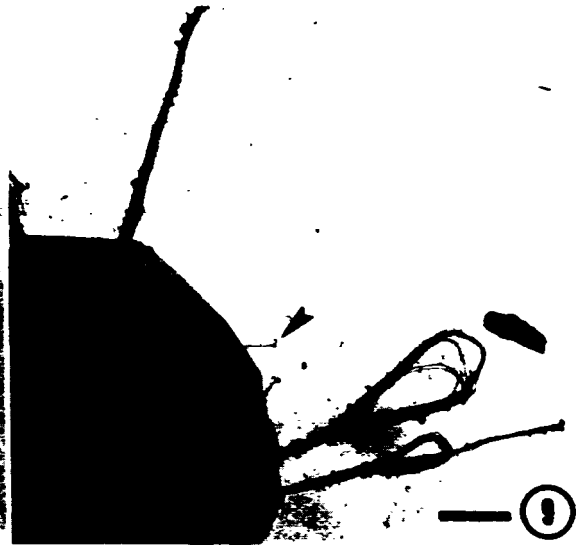

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Growth rate was determined. at least once in duplicate, for all isolates. Two isolates. one from Similkameen Rearing Pond, and one from Green River Hatchery, were measured weekly. as a control. along with each test group of isolates for 35 weeks. These two isolates did not vary. more than 1.6 mm/day in growth rate throughout the 35 week testing period. The Green River sample had a mean growth rate of 13.3 ± 0.8 mm/day (95% C.I.) and the Similkameen sample had a mean of 16.9 ± 0.7 mm/day (95% C.I.). Thus. group to group variability approximates ± 0.8 mm/day. Within group variation was determined for three isolates using 15 race tubes for each isolate. The within group variation approximates ±0.5 mm/day. with the Similkameen isolate having a mean of 17.2 ± 0.5 mm/day (95% C.I.). the Green River isolate having a mean of 13.3 ± 0.4 mm/day (95% C.I.). and the Cowlitz isolate having a mean of 14.0 = 0.5 mm/day (95% C.I.).

Adult Chinook salmon lesions were extensively sampled before holding and treatment at 3 sites: Cowlitz Hatchery. South Santiam Hatchey, and Dexter Holding Pond (Table 1. Fig. 1). Growth rates ranged from 5 mm/day to 17 mm/day with means of 13.9. 13.7 and 14.5 mm/day respectively (Table 2, Fig. 10). Adult Chinook Salmon lesions also were sampled extensively at spawning time after holding and treatment at 4 sites: Cowlitz Hatchery Leavenworth Hatchery. Entiat Hatchery. and Bonneville Hatchery (Table 1. Fig. 1). Growth rates ranged from 13 mm/day to 18 mm/day with means from 14.5 to 15.2 mm/day (Table 2. Fig. 10). One population of early returning spring Chinook salmon from Cowlitz Hatchery. was sampled monthly over a period of 4 months (Fig. 12). The earliest sample had a much broader range of growth rates than any of the succeeding samples from the same population (Table 2. Fig. 12). The samples of 5 August were primarily taken from small lesions and from lesions that had no apparent hyphae. The other samples. taken on other dates. were from large lesions that had resulted in extensive epidermal damage. often with the dermis visible from the exterior. The sample taken from late returning spring Chinook salmon gave results similar to those found for the early return Chinook salmon (Fig. 12).

Gelatin liquefaction rate (Fig. 1 1) was determined for all isolates. Several isolates were chosen to determine both within group and between group variation: (1) the Hupp Springs isolate gave a within group mean of 0.7 ± 0.1 gm/day gelatin liquefaction rate (95% C.I.) and a between group mean of 0.70 ± 0.24 gm/day (95% C.I.), (2) the Cowlitz (27 Aug. 1991) isolate gave a within group mean of 1 .l ± 0.1 gm/day (95% C.I.) and a between group mean of 1 .I = 0.1 gm/day (95% C.I.), (3) the Cowlitz (3 Sept. 1991) isolate gave a within group mean of 1 .O ± 0.2 gm/day (95% C.I.) and a between group mean of 1.1 ± 0.24 gm/day (95% C.I.). and (4) a Danish isolate (*S. parasitica,* Olson. this volume. Chap. 8) gave a within groups mean of 0.9 ± 0.13 gm/day (95% C.I.) and a between group mean of 0.9 ± 0.15 gm/day (95% C.I.).

The 3 sites representing samples from before holding and treatment had a greater range of values than the post holding and treatment sites in a manner similar to that shown by growth rate (Table 2. Fig. 11). Cowlitz showed a decrease in variability between the first and second sampling periods also in the manner of that shown by growth rate (Fig. 12).

Several small collections were made from fish whose lesions that were not responding to formalin treatment (Fig. 13). These involved young fish ranging from 3 months to 12 months of age and in the case of Leeburg Hatchery (Table 1, Fig. 1), adult brood stock. These isolates are similar in growth rate and gelatin liquefaction to those made from returning adult Chinook salmon.

TABLE 2. Sample Descriptive Statistics

Location	Date	Growth Rate (mm/day)			Gelatin Liquefaction (g/day)		
		N	x	95%C.I.	N	$\bar{X}$	95% c. I.
Cowlitz Hatchec (Early Spring Chinook)	28 May 1991	69	13.9	10.6-17.2	57	0.88	0.35-1.41
	2 July 1991	40	15.5	14.4-16.8	39	0.96	0.74-I I8
	5 Aug 1991	22	14.4	12.0-16.8	22	0.96	0.64-1.13
	3 Sept 1991	66	14.9	13.5-16.4	66	0.90	0.70-1.06
Cowlitz (Late Spring Chinook)	27 Aug 1991	49	14.9	13.5-16.3	48	I .03	0.93-1.13
South Santiam t Hatchec	25 June 1991	66	13.7	9-I-18.2	62	0.88	0.58-1.02
Dexter Holding Pond	26 June I991	35	14.5	11.1-17.9	53	0.95	0.61-1.29
Leav enworth t hatcheq	25 June 1991	58	15.1	13.8-16.4	58	I .08	0.74-I .42
Entiat Hatchery	31 Aug 1991	44	14.5	13.1-15.9	44	0.95	0.69- I .2 I
Bonneville Hatchery	I5 Nov 1991	59	15.2	14.0-16.3	59	I.14	0.93-I .34

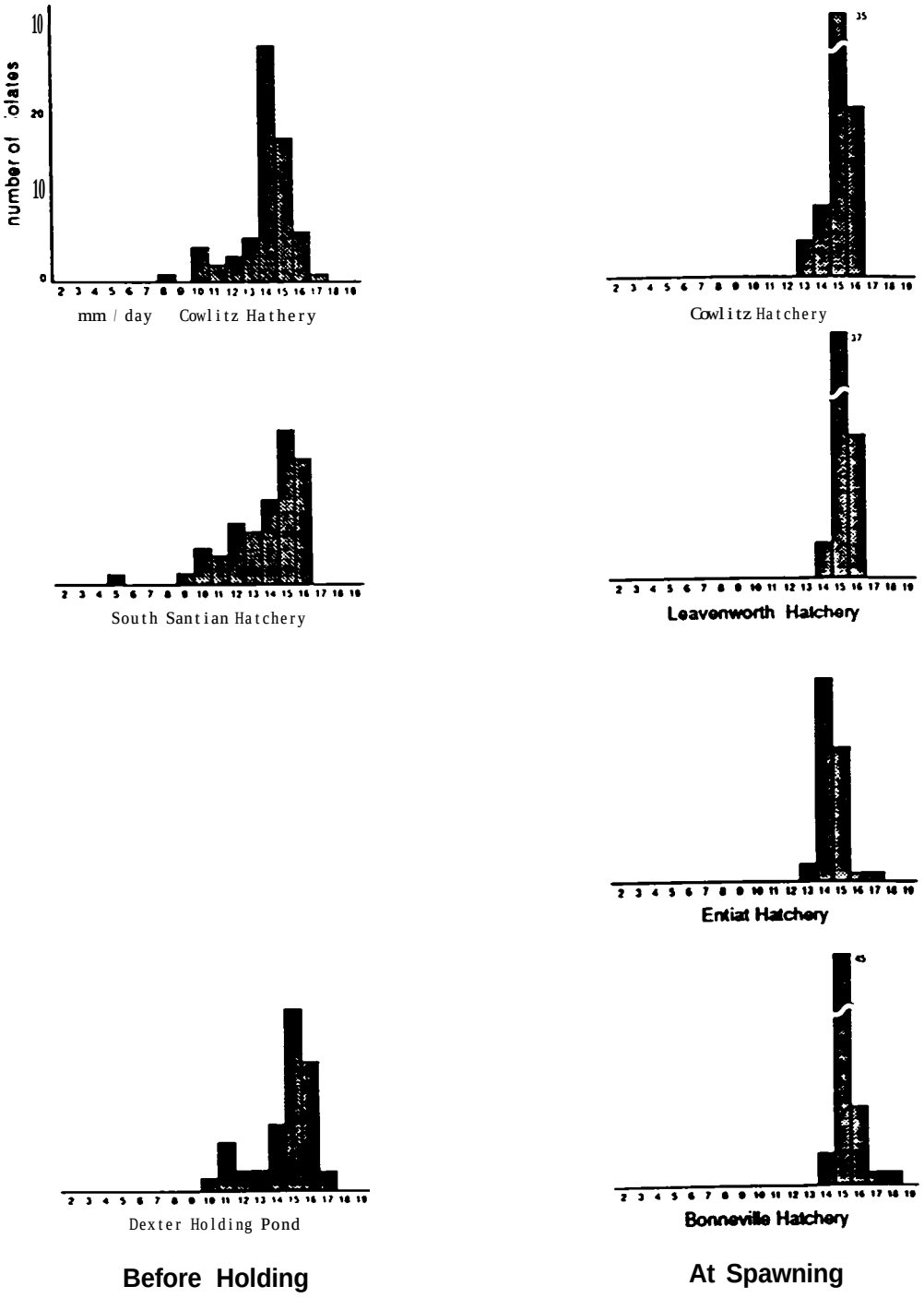

Figure 10. Growth rate histograms of isolates taken before holding and treatment and of isolates taken at spawning after holding and treatment.

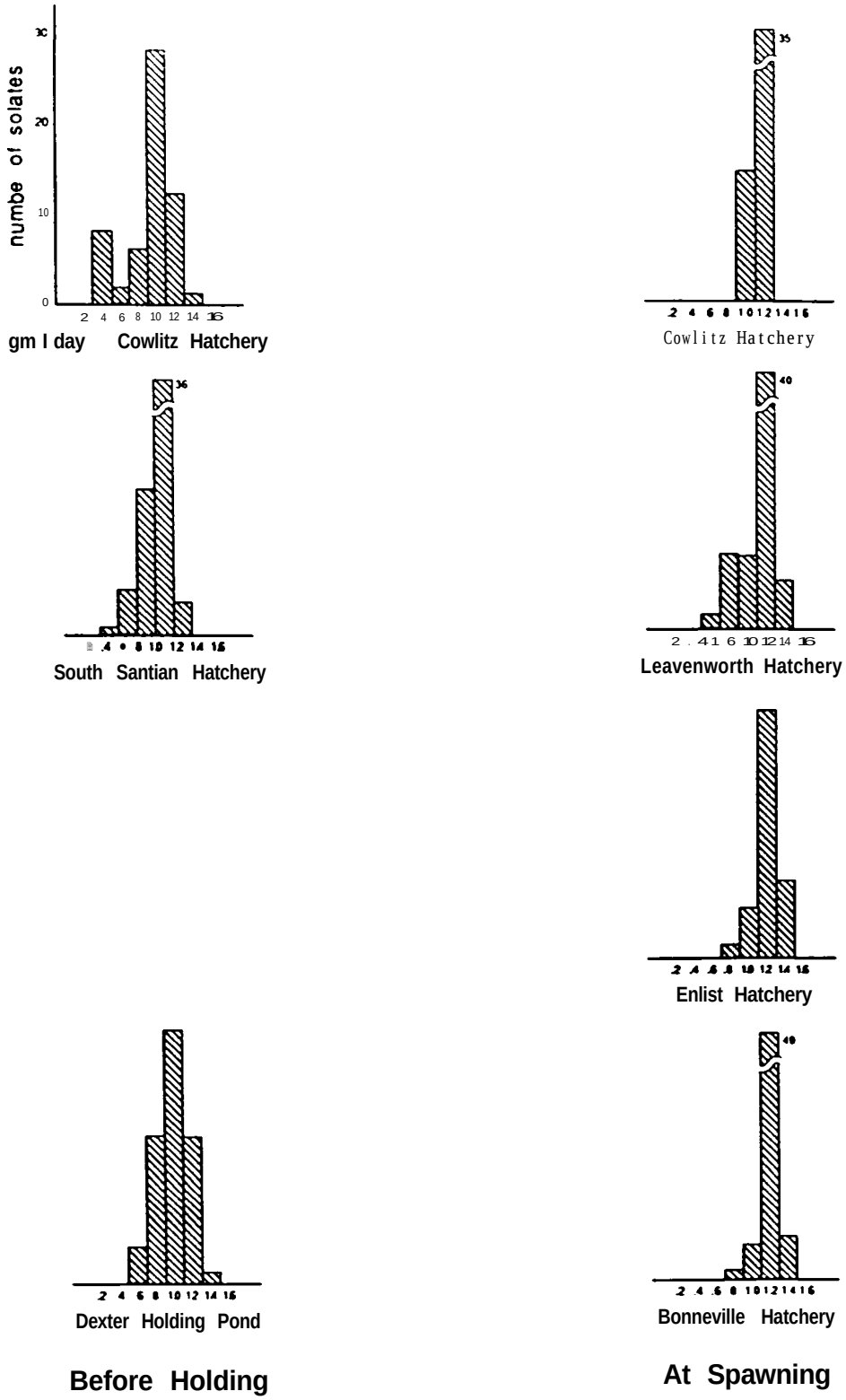

Figure 11. Gelatin liquefaction rate histograms of isolates taken before holding and treatment and isolates taken at spawning after holding and treatment.

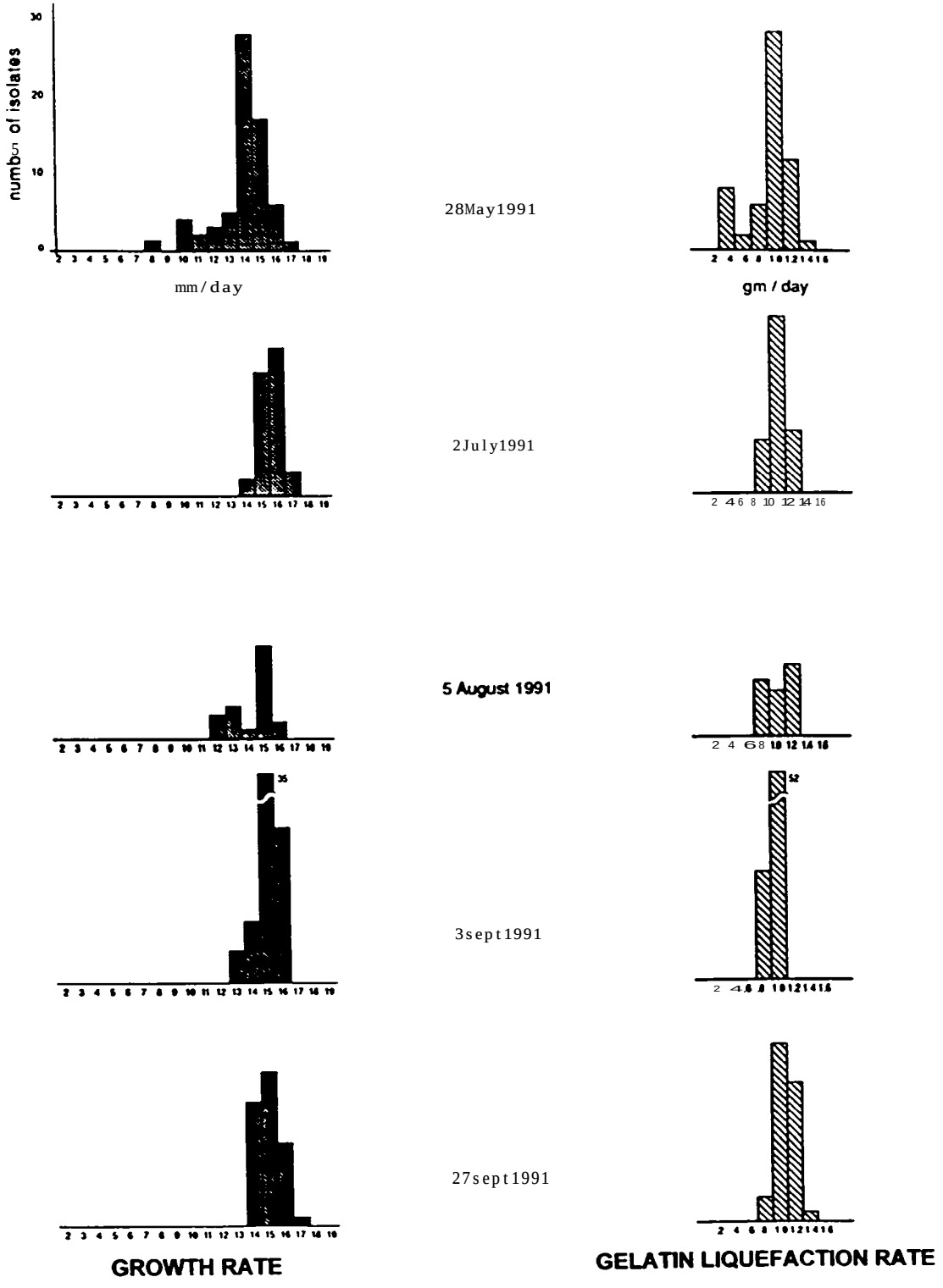

Figure 12. Growth rate and gelatin liquefaction rate histograms of isolates taken from early returning adult Chinook Salmon at Cowlitz Hatchery at monthly intervals. One sample (27 August 199 1) of late returning Chinook salmon is included.

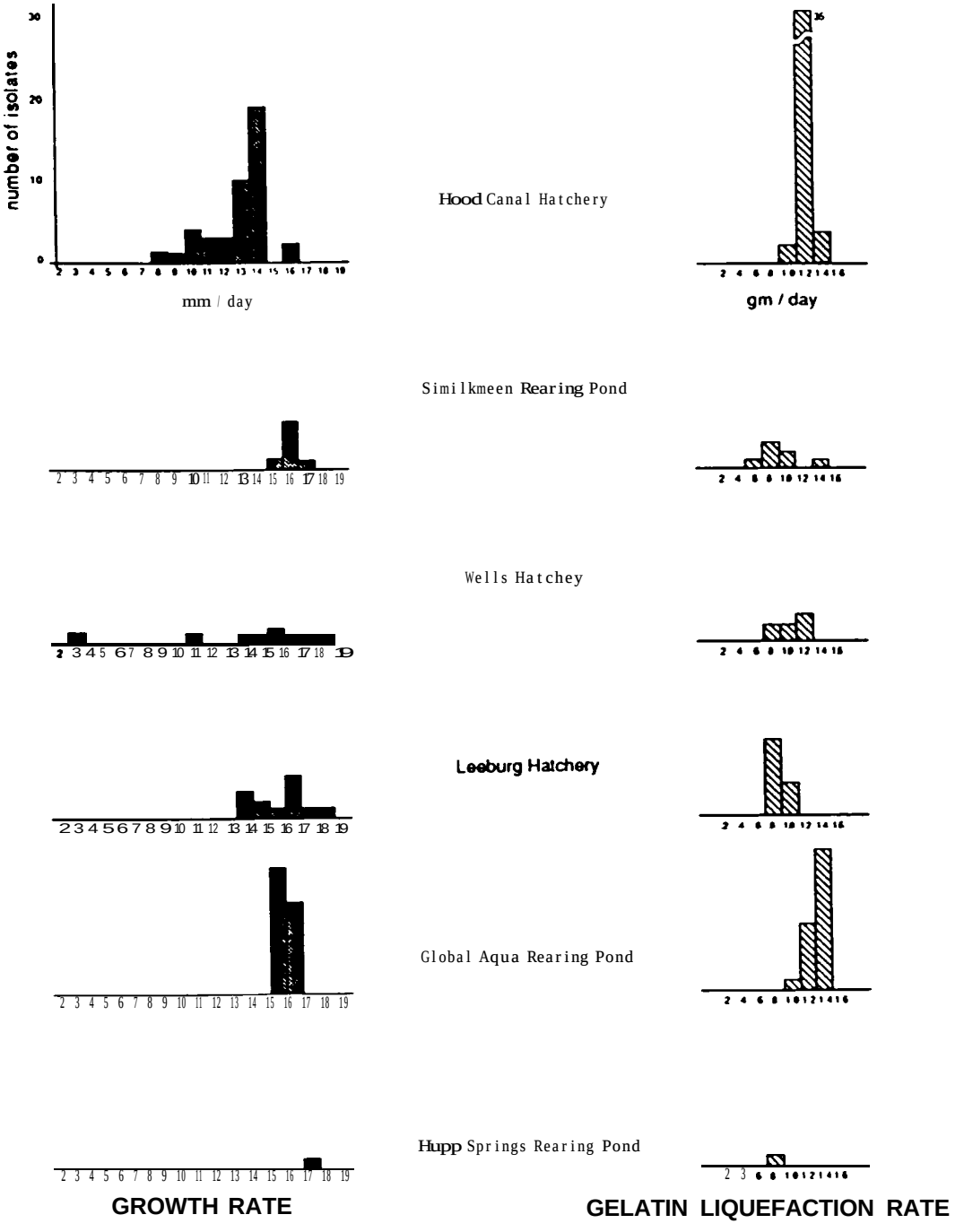

Figure 13. Growth rate and gelatin liquefaction rate histograms of isolates from sites where saprolegniasis was not responding to treatment.

Within lesion variation in growth rate yields the same pattern observed in the growth rate measurements. The subsampled lesions from sites previous to holding and treatment had the largest within lesion variation (Table 3). The sites where sampling was done at spawning time had the least within lesion variation in growth rate. Within lesion variation was not apparent in gelatin liquifaction rates and did not seem to vary with the length of time the fish were held (Table 3).

Within lesion variation using the presence or absence of sexual reproduction as a measure of within lesion variation gave equivocal results. The repetitive samples from Cowlitz showed a disappearance of the asexually. reproducing isolates from the lesions of the early arriving spring Chinook salmon. The late arriving spring Chinook from Cowlitz Hatchery and the spring Chinook from Leavenworth hatchery had mixed lesions with both sexually and asexually reproducing fungi present after holding and treatment. At the other sites. sexual reproducing fungi were the predominate form in the lesions.

Species representing over forty genera of fungi were isolated from surface and or internal lesions. Isolated fungi representing 25 genera found 5 or more times are listed in Table 4. *Phoma* spp. were the most common and were present in 67 lesions. *Phoma hebarum* Westend. was isolated from 27 surface lesions and found internally at 5 separate localities. *Fusarium* spp. were found at every locality and turned up internally in three separate sites. *A Paecilomyces* sp. was found at only one site, but on three separate occasions. *Cryptococcus* sp. and *Trichosporon* sp. were found in the air bladder and the kidney at two sites each. *Mortierella* spp. were found in the kidney and air bladder at two sites and were apparently responsible for a significant increase in mortalities at one site on two occasions.

DISCUSSION

The majority of isolates collected in this study emphasizing the Columbia River system represent *S. parasitica.* Isolates from either external lesions or from muscle or internal organs of various species of salmonids all appear to be members of the same population (Table 2. Figs. 10, 11, 13). The isolates have secondary zoospore cysts with ornaments composed of bundles of structures (Figs. 4, 8), boat hook like in appearance. The isolates have diclinous antheridial hyphae and thin walled unpitted oogonia containing centric to subcentric oospores (Fig. 2). We were unable to relate any specific morphology to a particular species of salmonid nor were we able to relate any particular morphology to the severity of external or internal lesions.

Differences between the colony type of S. *parasitica* and S. *diclina* have been described by Wood *et al.* (1988). *Saprolegnia diclina* formed a large diffuse type of colony after transfer from Polycell to lakewater, while S. *parasitica* had a more restricted type of colony growth. Our findings indicate that S. *diclina* does indeed grow into a

TABLE 3. WITHIN LESION VARIATION

	COWLITZ 28 MAY 991	2 JULY 1991	5 AUG 1991	3 SEPT 1991	COWLITZ 27 AUG 1991	SOUTH SANTIAM HATCHERY	DEXTER HOLDING POND	LEAVENWORTH HATCHERY	ENTIAT HATCHERY	BONNEVILLE HATCHERY
GROWTH RATE										
Lesion(s) with <1 mm/day variation between isolates	7	6	2	18	10	7	7	13	8	14
Lesion(s) with >l mm/day variation between isolates	16	7	4	2	5	15	11	4	3	3
GELATIN LIQUEFACTION RATE										
Lesion(s) with <0.4 gm/day variation between isolates	15	13	6	20	13	20	15	14	10	20
Lesion(s) with >0.4 gm/day variation between isolates	4	0	0	0	2	2	3	3	1	1
MORPHOLOGY										
All isolates with oogonia	10	12	6	17	2	14	9	2	7	6
All isolates without oogonia	3	0	0	2	2	1	8	2	0	
Isolates Mixed	10	0	0	0	12	2	1	10	0	4 1

TABLE 4. Other Organisms Isolated From Lesions

TAXON	TYPE OF LESION	FISH PATHOGEN	ANIMAL PATHOGEN (other than fish)
Mortierella antarctica	SUR-INT	Chinook [1]	
Mortierella alpina	SUR-INT	Chinook [11]	Mammal 20
Mucor sp.	SUR-INT	Chinook [11]	Mammal 20
			Turtle 21
Candida sp.	INT	Chinook [11]	Mammal 19
		Amago 23	
Cryptococcus sq.	SUR-INT	Chinook 11	Mammal 19
		Tench 17	
Trichosporon sp.	INT	Chinook 11	Mammal 19
Rhodotorula sp.	SUR		Mammal 19
Chaetomium sp.	SUR		Mammal 19
Petriella sp.	SUR		Mammal 19
Acremonium sp.	SUR		Mammal 19
Alternaria sp.	SUR		Mammal 19
Arthrographis sp.	SUR		
Aspergillus sqq.	SUR	Tilapia 2	Mammal 19
Cladosporium hebarum	SUR-INT	Chinook 11	Frog 18
			Mammal 19
Curvularis sp.	SUR		
Epicoccum sp.	SUR		Mammal 19
Exophiala sp.	SUR	Cutthroat 3	Mammal 19
		Atlantic 4	
		Catfish 5	
Fusarium solani	SUR		Mammal 19
Fusarium sqq.	SUR-INT	Carp 6	Mammal 19
		Tilapia 6	
Gliocladium sp.	SUR		Mammal 19
Ochroconis sp.	SUR	Coho 12	
		Rainbow 13	
		Chinook 14	
		Masou 16	
Paecilomyces sp.	SUR-INT	Chinook 11	Mammal 19
Penicillium sp.	SUR		Mammal 19
Phialophora sp.	SUR	Atlantic 7	Mammal 19

TABLE 4 continued

TAXON	TYPE OF LESION	FISH PATHOGEN	ANIMAL PATHOGEN (other than fish)
Phoma hebarum	SUR-INT	Coho 12 Chinook 9 Rainbow 10	Mammal 19
Phoma eupyrena	SUR-INT	Chinook 11	Mammal 19
Phoma sqq.	SUR-INT	Chinook 11 Ayu 16	
Trichoderma hartzium	SUR		
Trichoderma sp.	SUR	Chinook 11	
Volelella sp.	SUR		
Woronina polycystis	SUR		
Vannella miroides	SUR		
Hartmannellidae	SUR		Mammal 22

SUR - Isolated from surface lesions
INT = Isolated from internal lesions

1 DeCew et *al.*. 1991
2Olufemi and Roberts. 1983
3Carmichael. 1966
4Otis and Wolke. 1985
5Fijan. 1969
6Hörter. 1960
7Ellis et *al.*. *1983*
8Ross et al., 1975
9Wolke. 1975
10Wood. 1974
11This study
12Ross and Yasutake. 1973
13deHong and von An. 1973
14Doty and Slater. 1946
15Hatai and Kubota. 1989
16Hatai et *al.*, 1986
17Pierotti, 1971
18Elkan and Philpot, 1973
19McGinnis et *al.*, 1982
20Smith, 1968
21 Jacobson, 1980
22Markell *et al.*, 1986
23Hatai and Egusa, 1975

diffuse type of colony. A few isolates of ***S. parasitica*** are able to develop into a large diffuse colonies but the presence of secondary zoospore cysts with long bundles of ornaments place these isolates into *S. parasitica* as presently understood.

The two isolates of S. ***ferux*** from Chinook salmon, and the six isolates of *S. diclina* suggest that while they are present in the environment their relatively low frequency suggests that they are less important than *S. parasitica* in these lesions. The great predominance of *S. parasitica* as parasites of salmon has been reported before (Neish, 1977; Willoughby, 1978; Neish and Hughes. 1980). The isolates from Hood Canal Hatchery and South Santiam Hatchery.. with secondary zoospore cysts that had both bundles of hooks and single hooks. are referred to *S. parasitica* as described by Beakes (1982). These isolates did not undergo sexual reproduction. Treatment at Hoods Canal Hatchery with one third salt water and two thirds fresh water was ineffective in controlling this outbreak of saprolegniasis: although such a treatment had been effective at this site in the past (Chapman. 1991). Slow growing isolates (Fig. 13) of this population were present after treatment with salt water.

Repeated linear growth rate measurements of *S. parasitica* isolated from adult Chinook salmon resulted in a bimodal curve in those samples collected before holding and treatment. The small peak at the slow growing end of the curve is not present in any of the samples taken after holding and formalin treatment. Gelatin liquefaction rate and the within lesion variation measurements representing both before and after treatment support the contention that a component of the population is lost after holding and treatment. It seems likely that formalin treatment of saprolegniasis is responsible for this shift. However, a natural replacement by. some strains over others and/or the effect of fish defenses is equally possible.

Linear growth rates and gelatin liquefaction rates follow a similar population distribution pattern. However, there does not appear to be a relationship between isolates that have slow growth on defined medium and isolates that have a slow gelatin liquefaction rate. It should be emphasized that slow or fast growth on a chemically defined media or liquefaction of 12% gelatin may not reflect growth rate on or invasive ability in salmon.

There are many other fungi, bacteria and other protists that inhabit surface lesions typical of saprolegniasis. On careful examination of the surface lesions septate hyphae, yeasts, and amoebae can be found among the hyphae of *Saprolegnia*. Certainly these organisms contribute to the overall structure of the lesion. Some of the fungi and protists have a clear relationship to *Saprolegnia*. ***Woronina*** *polycystis* and *Mortierella alpina are* recognized as parasites of *Saprolegnia* sp. (Willoughby, 1988, 1992). The relationship of *Epicoccum* sp.. *Fusarium* sp., and *Gliocludium* sp. have yet to be understood, however, recent observations suggest that they diminish the growth of *S. parasitica* in water culture. The internal presence of *Mucor* spp. and *Phoma* spp. originating from surface lesions appear to be following the path of the invading *Suprolegnia.* The presence of *Cladosporirtm hebarum* and *Fusarium* spp. following *Saprolegnia* is of more concern due to the possibility of endotoxins. in particular, fusariotoxins which have been reported to affect fish (Marasas et *al.*. 1967: Bamburg et *al.,* 1968; Diener et *al.,* 1976; Posten et *al.*,

1982). The presence internally of *Fusarium* sp.. *Cyptococcus* sp., *Trichosporon* sp., *Candia* sp., and *Paecilomyces* sp.. without *Saprolegnia*, suggests that these fungi potentially pose a threat to juvenile salmonids as already has been demonstrated for *Phoma* spp. and *Mortierella* spp.

Woronina polycystis Comu an obligate parasite of *Saprolegnia*, was recovered from 3 hatchery sites and isolated into monoxenic culture. The potential use of this pathogen as a bio- control agent of *Saprolegnia pararsitica* is under current study.

In summay, the majority of isolates represented in the samples taken in this study appear to be from one population of *Suprolegnia parasitica.* Members of this population are the primary fungal agents present in saprolegniasis of returning adult Chinook salmon. They are the primary agents present in the external and internal saprolegnoid lesions of other species of adult salmonids and in the external lesions of juvenile salmonids, both Chinook and other species.

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chapter 10

SAPROLEGNIA IN NORWEGIAN FISH FARMING

Finn Langvad[1]

ABSTRACT

Saprolegniasis in the hatchery is controlled by regular use of malachite green once or twice a week. Infections in fish are normally cured with malachite green, but very aggressive infections occur which do not respond well to such treatment. There are two peak periods during the year, early autumn and spring when spore concentrations increases considerably. Oomycetous fungi are also shown to be capable of infecting saltwater fish like sprat when salinity is low. Preliminary experiments using lectins/enzyme-colloidal gold and TEM seem to indicate a stronger cellulosic wall layer in S. *purasitica* than in the saprophytic strain S 1. The presence of a loose outer wall layer of N-acetyl-D-galactosamine is discussed.

Key **words:** *Saprolegnia parasitica,* salinity, hyphal wall, cellulose, N-acetyl-D-galactosamine, saprolegniasis

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INTRODUCTION

Norway is almost ideally suited for fish fuming with its many fjords, providing sheltered waters for the farms. Fish farming. especially of Atlantic salmon. has been a highly successful story over the past 20 years. Almost as much as 90% of the farmed Atlantic Salmon is exported. and the export value amounts to about 3 billion NOK. which is quite a lot for a small country like Norway.

Do we have fish diseases in Norwegian fish farming? Yes. of course we have fish diseases. Do we have problems with *Saprolegnia* on Atlantic salmon? Yes, we have that as well.

SAPROLEGNIA IN THE HATCHERY

Suprolegnia is a constant problem. especially in the hatchery. The most popular type of equipment used for hatching Atlantic salmon eggs is open shallow trays (Fig. 1). The advantage of these is that dead eggs, organic material. etc. can easily be removed. Nevertheless. even if a high hygienic standard is maintained infections with *Saprolegnia* are unavoidable. In Norway, the use of malachite green against fungal infections is not banned. In the hatchey. malachite green is used as a prophylactic once or twice a week. If hatch cylinders are used instead of trays. removal of dead eggs is not possible, and treatment with malachite green is in these cases even more essential than with the open tray system. Thus. successful operation of a hatchery for Atlantic salmon in Norway is not thinkable without the use of malachite green.

SAPROLEGNIA IN FISH

Suprolegnia infections of smolt are not normally a big problem. Infections do occur from time to time. but can be cured with malachite green. However, on some occasions we have experienced very aggressive infections in salmon smolt which have been very difficult to cure. In one special case smolt were treated several times a week, but with little effect (Fig. 2).

This smolt farm. located on the Island of Stord in South-West Norway, utilized a natural lake for their smolt production. Instead of using tanks, which is the most common procedure. they had nets placed directly in the lake. The water quality was good with a pH = 6.5-7.0. We have so far no explanation of the possible cause of these very aggressive attacks and why they did not respond so well to malachite green.

Figure 1. The most popular type of equipment used for hatching Atlantic salmon eggs in Norway consists of open shallow trays. Dead eggs and organic debris may easily be removed. The trays may be stacked to reduce space.

Figure 2: Salmon parr heavily infected with *Saprolegnia*. Infections of this type did not respond well to treatment with malachite green. From an original Ektachrome slide.

SEASONAL FLUCTUATIONS

Following the problems of the smolt farm mentioned above, we decided to measure the Oomycetes spore concentration in this lake monthly for one year to see if there were any peak periods with especially high spore concentrations which again could explain the heavy attacks. This philosophy was based on the assumption that a high spore concentration represents a greater infection pressure on the fish than a lower concentration. The result of this investigation for the period August 1998 to June 1989 is presented in Fig. 3. As can be seen, there was a periodicity with two maxima, one in early autumn and one in spring. We used 6 sampling sites, but only the one with the highest maximum (2569 spores per liter) is presented here. The numbers found are in the same order of magnitude as found by Hallett and Dick (1981) for the Whiteknights Lake and by Willoughby (1962) for Lake Windermere. But for Whiteknights Lake, a periodicity was found, with three maxima rather than two (Hallett and Dick, 1981) as in our investigation.

So the spore concentrations found were not especially high. Although there were no serious attacks during the period of measurement it was nevertheless an interesting observation that spore concentrations just outside the cages were not higher than the other sampling sites.

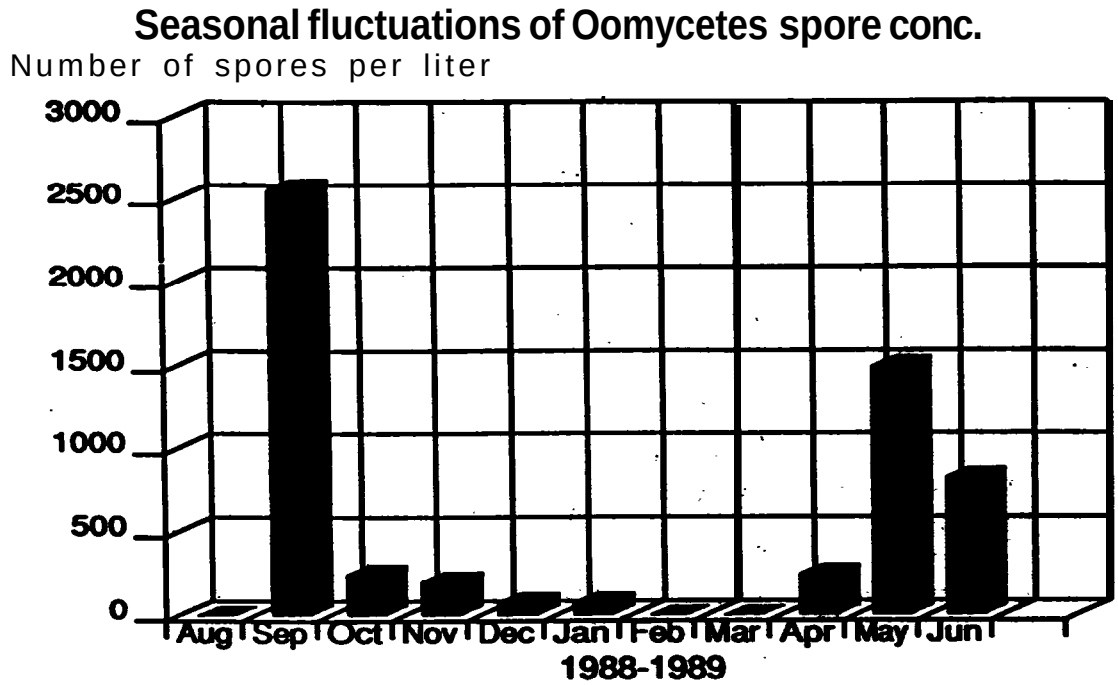


Figure 3: The concentration of oomycete spores seem to vary through the year with two peak periods, one in early autumn and one in spring.

SAPROLEGNIA AND SALT

Salt water has sometimes been used as a treatment against Saprolegnia infections. However, the fungus can tolerate relatively high.salt concentrations without any appreciable reduction in growth. With 1.75% NaCl present in the growth medium the growth is still about 65% of the control without salt. With 3.5% salt the growth is completely checked (Fig. 4).

Two years ago one of the hydro-electrical plants in South-West Norway let out a large amount of surplus water into the narrow Hylsfjord in the area of Ryfylke. The salinity dropped drastically. This led to mass-infection of Sprat (*Clupea sprattus*) with an Oomycetes type of fungi (Fig. 5), and masses of dead sprat were floating along the beaches of the fjord. This shows that given the right conditions even what we regard as typical salt water fishes may be infected with Oomycete.

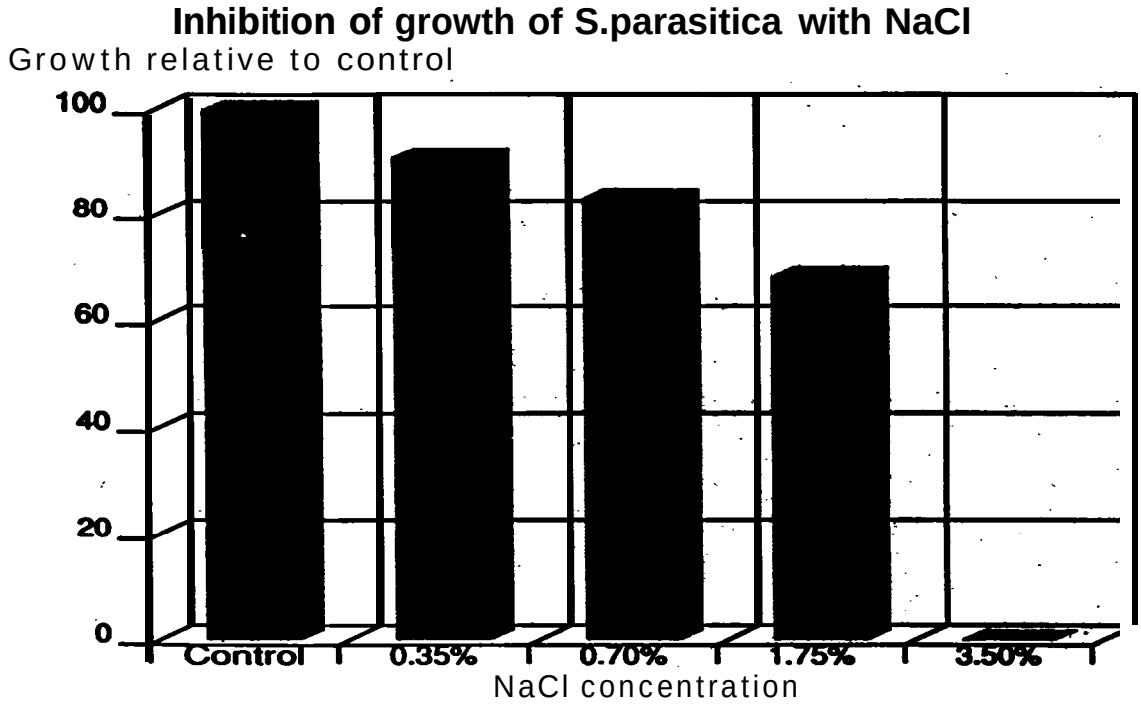


Figure 4: Saprolegnia can tolerate relatively high concentration of NaC1. With 1.75% NaCl present in the growth medium the growth is still about 65% of the control . without salt. With 3.5% NaCl the growth is completely checked.

Figure 5: Infection of sprat (Clupea sprattus) with an Oomycetous fungus. From a fjord in South-West Norway. where large amount of surplus water from a nearby hydro power plant resulted in reduced salinity in the fjord. From an original Ektachrome slide.

AGGLUTININS IN THE MUCUS LAYER

Since our attempt to detect any fungicidal effect of the mucus failed, we tried to find out if the effect of the mucus layer may be an effect of agglutination, using the technique described by Manocha and Chen (1991). With this technique the potential agglutinin is used in a series of two fold dilutions, and the agglutinating activity expressed as the reciprocal of the greatest dilution at which agglutination could still be detected under the microscope as aggregation of the spores. This work is not finished yet, but we have been able to demonstrate what we assume to be agglutination up to a dilution of 8x. Agglutination may be the same as the powerful morphogen described by Wood et al., (1988).

I would like to stress that this work is at its early beginning in my laboratory.

ATTACHMENT OF *SAPROLEGNIA* TO THE FISH

I now would like to draw your attention to one area of research where almost nothing has been done up to *know as* far *as Saprolegnia* and its infection in salmon is concerned. This

is the study of **mechanisms of attachment** and **specificity of attachment** of the fungal parasite to its host. Within plant pathology this area has for some time now been acknowledged as very important for the understanding of fungal infections (Manocha and Chen, 1990).

The process of parasitism in host-fungal parasite systems usually consists first of all of **attachment** and **recognition**. Attachment of fungal parasites to their host cell is a prerequisiteforfurtherparasiticevents.Attachment may involve parasite recognition of specific surface topography of the host or binding between complementary molecules on both host and parasite cell surfaces. As part of recognition, attachment may happen before, after, or together with the phenomenon of recognition between host and parasite (Manocha and Chen,1990).

There is a notable degree of specificity of attachment in host-fungal parasite interactions. Although a diverse array of potential fungal parasites are present in the environment, only a few of them are capable of infecting a specific host. This is certainly the case with saprolegniasis in salmon, where it seems to be only *Saprolegnia parasitica* which is capable of starting an infection.

Studies on the mechanisms underlying the specificity of attachment of fungal parasites to their hosts are still in their infancy. However, in recent years, remarkable progress in morphological and biochemical studies on this subject have been made (Manocha and Chen,l990).

In most fungi, carbohydrates represent 80-90% of the dry matter of cell walls (Bartnicki-Garcis, 1968). Due to their cell-surface location the type of polysaccharide is obviously very important with regards to cell attachment and host wall penetration.

Chemical and biochemical analysis during the last 20 years have disclosed that polysaccharides of fungal cell walls could be subdivided into several broad categories including amino sugars, hexoses, pentoses, hexuronic acids and heteropolysaccharides (Benhamou, 1988). Although such studies have provided precise information about the structure of the carbohydrate containing molecules, the functional specialization of the cell wall components is reflected also by their specific location within the wall structure. Thus, "in sizu" localization of these macromolecules has become an essential complement tobiochemidstudies.

Such a precise localization of cell wall components is possible with the combination of two techniques, namely the use of lectins and enzymes conjugated to colloidal gold and transmission electron microscopy. Lectins are highly specific carbohydrate binding proteins. Both lectins and enzymes can be conjugated to colloidal gold of a certain size. When ultrathin sections of the fungi are treated with lectin-colloidal

gold or enzyme-colloidal gold, very precise localization of different carbohydrates in the cell wall is possible.

For details concerning the use of this technique on fungi, the interested reader is referred to the works of Harris and Szaniszlo (1986), Benhamou et *al.* (1987), Benhamou (1988), Benhamou (1989), Benhamou, Chamberland and Noel (1990), and Clay, Benhamou and Fuller (1991).

STUDIES ON THE CELL WALL OF SAPROLEGNIA

We started to use this technique on different *Saprolegnia* isolates only one month ago. So the results presented today must be regarded as preliminary. Probes used in the study are found in Table 1. Ultrathin sections of *Saprolegnia parasitica* Coker and a saprophytic strain (Sl) were treated with the probes listed in Table 1, and examined under the transmission electron microscope (JEOL 100 S).

Localization of Cellulosic ß-(1,4) Glucans.

Figure 6 shows details of the cell wall of a hypha of *S. parasitica* treated with the Cellulase-gold complex. The fairly intense labeling of the wall can clearly be seen. It can also be seen that it is mostly the outermost part of the wall which is labeled, indicating that most of the cellulose is located there. Ultrastructural detection of cellulosic ß-(1,4)

Table 1. Lectin/enzyme Colloidal gold probes used

Lectin, enzyme	Source	Substrate specificity
Cellulase	*A. niger*	Cellulosic ß-(1,4) glucans
Wheat germ agglutinin (WGA)	*Triticum vulgare*	N-acetyl-D-glucosamin (chitin)
Helix pomatia agglutinin (HpA)	Roman snail	N-acetyl-D-galactosamin
Concanavalin A (Con A)	*Canavalia ensiformis* (Jack bean)	a-D-mannose, a-D-glucose

glucans in the walls of fungi with this technique has earlier been done by Benhamou *et al.* (1987), Benhamou, Chamberland and Noel (1990), and Clay, Benhamou and Fuller (1991). Application of the Cellulase-gold complex to the saprophytic strain S1 resulted in a much weaker labeling (Fig. 7), where the cellulose seems to be located more in the middle of the wall. This difference in location and amount of cellulosic compound may be of importance regarding infection in fish. One might speculate that the stronger outer cellulosic layer of *S. parasitica* may protect it from agglutination or lysis from other components, whilst the saprophytic strain is more liable to attack. Of course, the presence of cellulose in the cell wall of *Saprolegnia* is a well known fact. Sietsma (1969) found that cellulose represented 21% and ß-(1,3)/ß-(1,6) glucans 79% of the cell wall carbohydrates. However, the localization and difference between species/strains has not been known before.

Localization of *N-Acetyl-D-Galactosamin* Residues.

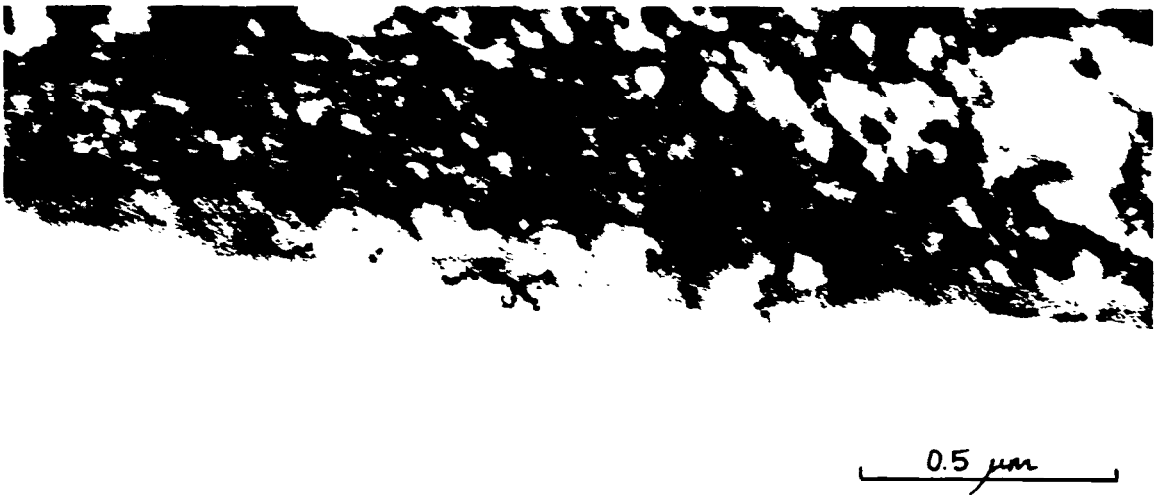
Application of the *Helix pomatia agglutinin* (HpA)-gold complex resulted in a relatively intense labeling of a very loose outer wall layer, lacking in some parts of the hypha (Fig. 8). One may speculate that this loose outer layer may participate in host recognition, but at the moment we have no data to support that view. We have so far not done enough to tell if there is a difference between the two strains with respect to intensity of labeling with the HpA-gold complex, we expect to finish that part later this autumn. N-acetyl-D-galactosamin has been shown to be present in the hypha wall of *Verticillium albo-atrum* (Benhamou, 1988).

Localization of a-Mannose and a-D-Glucose Residues.

The Con A-gold complex resulted in no labeling of the wall of *S. parasitica* or the S1 strain indicating that α- D-mannosyl and α-D-glycosyl is not present in the wall of these fungi.

Localiztion of Chitin.

The application of the WGA-gold complex did not give any labeling of the wall, which is in agreement with the well known fact from chemical and biochemical analysis that chitin is absent from the cell walls of these fungi. Our research within this field will be continued and even extended using more lectins. The detailed results of this will be reported later.



Figures 6 and 7: Transmission electron micrographs (TEM) of cells of *Saprofegnia parasitica* and the saprophytic *Suprolegnia* strain Sl. treated with cellulase-gold complex. **Fig.** 6: Relative intense labeling of the wall of *S. parasitica.* **Fig.** 7: Much weaker labeling of the walls of the Sl strain.

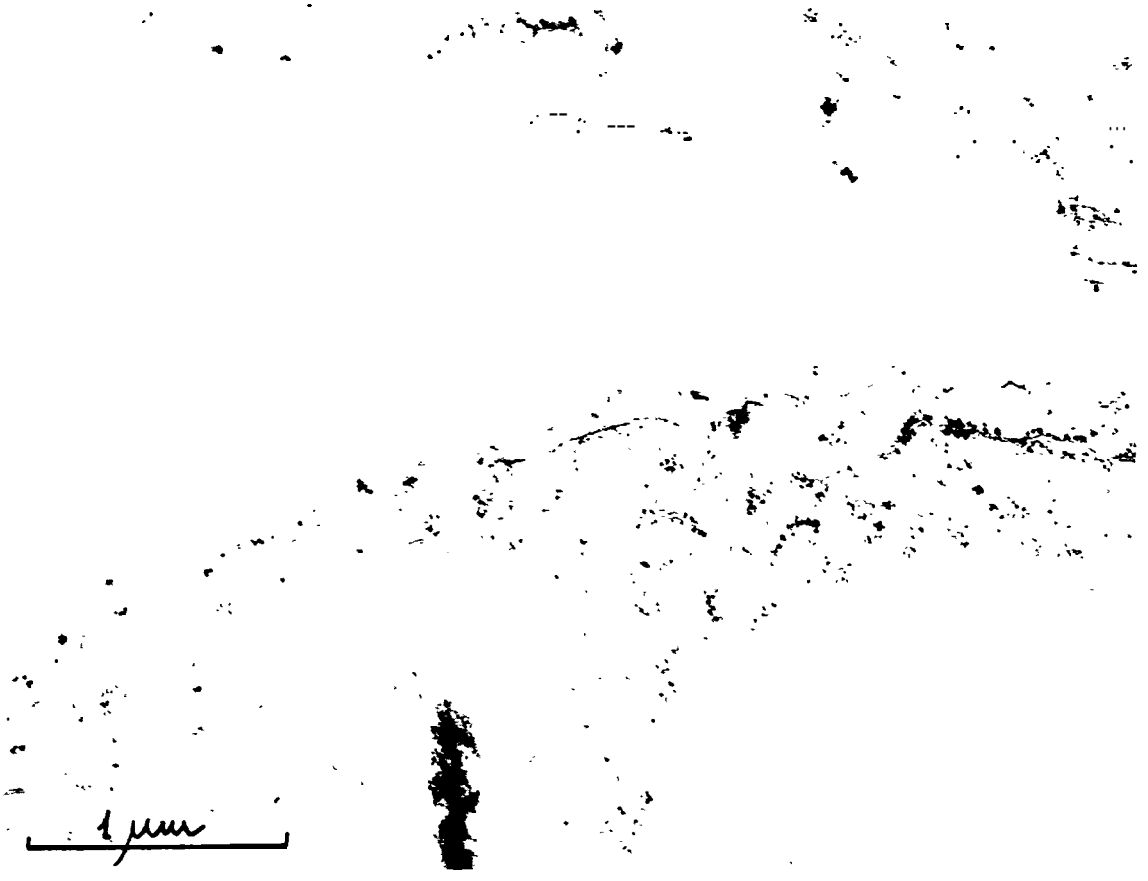


Figure 8: Transmission electron micrographs (TEM) of cells of the Sl strain treated with *Helix pomatia* agglutinin, showing relative intense labeling of a loose outer wall layer.

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chapter 11

SAPROLEGNIA IN IRELAND

P.R Smith[1]

ABSTRACT

The situation *with* respect to *Saprolegnia* infections in salmon hatcheries in Ireland will be discussed with respect to their impact on fish health and the impact of effluent quality regulations on the use of malachite green to control such infections. Peat based biofilters have been designed which are capable of removing a variety of pollutants from effuent water. Analysis of the performance of such filters in a pilot plant at a commercial hatchery has demonstrated that 99.75% of the malachite green in the effluent could be removed and the operation of the filters is cost effective. Preliminary studies on competition between *Saprolegnia diclina* and aquatic bacteria will be reported. These studies will include characterization of the bacteria and the source of their isolation and the correlation of their competitive abilities with their siderophore production.

Key words: *Saprolegnia*, peat biofilters, bacterial competition.

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SECTION IV

REGIONAL STUDIES

chapter 12

CURRENT RESEARCH NEEDS

George J. Mueller

The following research directions were suggested by some of the symposium participants. With the exception of the first group of questions on the systematic and taxonomic status of *Saprolegnia parasitica* the order of presentation does not reflect the importance of the research need. All of the participants submitting suggestions recognized and placed first the need for a clear statement of *the status* of *S. parasitica.* This list includes all of the suggestions submitted, but individual participants may not agree with the entire list.

SYSTEMATICS and TAXONOMY

- *Is Suprolegniu parasitica* Coker recognizable as a distinct species?
- *Is* the North American *S. parasitica* the same species as the European, Japanese or other isolates?
- Is *Suprolegniu parasitica* Coker a valid name or does the name need to be suppressed?

The taxonomic status of this species requires resolution. Extensive morphological, biochemical, and behavioral information is currently available. Molecular level studies that are presently underway will provide additional descriptive clues. It should be possible to re-describe the species and establish a type. If necessary, additional species should be erected for isolates from areas other than eastern North America.

INFECTIVITY and VARIABILITY

- Is the infective stage of *Suprolegniu parasitica* the zoospore, the cyst, or the germling?
- Is there a reliable method for testing infectivity?
- Is the variability between isolates correlated with infectivity?

Before preventative treatment can be rationally designed the infective stage or stages must be known. A reliable infectivity technique should provide the conditions to determine the infective stage. The *ami-momi* technique provides a basis for testing infectivity following physical damage to the epithelium This technique needs to be extended to other species of salmonids. Social stress. environmental stress, or a combination of both may also be useful for infectivity testing. Infectivity resulting from both physical damage and from stress needs to be understood. Once infectivity can be reliably assessed. then. strain variability can also be assessed.

PATHOGENICITY and IMMUNOLOGY

- Is there evidence for deep fungal infections of salmonids in general?
- Is there evidence for toxin production?
- Is there an interaction between the living components *(Suprolegniu,* other fungi, bacteria. and various hosts) resulting in changes in pathogenicity?
- Is vaccination a possibility to protect cultured salmonids?

There is evidence for deep fungal infections in Atlantic salmon caused by *Suprolegniu parasitica* Several species of fungi can be isolated from the deep infections along with S. *parasitica.* Other species of fish need to be examined for deep infections. All species of cultured salmonids need to be examined for signs of toxin production by *S. parasitica* and associated wound inhabitants. The classical immune response of salmonids to saprolegniasis requires reexamination to determine the possibility of vaccination.

TREATMENT and BIOLOGICAL CONTROL

- Is the treatment of saprolegniasis with formalin or other substances optimal?
- Is the use of bacterial antagonists to *Suprolegniu parasitica* a practical approach?
- Is the use of parasites such *as Woronina polycystis* and *Olpidiopsis incrussutu* a possible means of bio-control?

Treatment with formalin needs to be examined in a systematic manner to determine the minimum doses needed for treatment and for prevention of infection. There is a need to examine the pathological effects of treatment on the fish. The apparent association between bacteria with anti-fungal properties and the absence of saprolegniasis needs to be confirmed. The anti-fungal substances produced by these bacteria require identification. Examination of *Olpidiopsis incrassata* and *Woroninu polycystis* as potential bio-control agents is required.

chapter 13

BIBLIOGRAPHY *SAPROLEGNIA* IN SALMON

Gilbert C. Hughes], George J. Mueller[2], and Tae-Joo Choi[1]

Despite long and continuing interest in saprolegniasis and the salmonid fishes, no previously published bibliography or reference list has dealt specifically with the saprolegnian fungi associated with salmonids or any other group of fishes. Many of the pertinent papers were included in the "References" section of **Fungal Diseases of Fishes** by Neish and Hughes (1980) but that list was far from comprehensive. It is our objective here to present as comprehensive a bibliography as possible, including papers dealing with all of the many and varied aspects of associations between saprolegnian fungi and salmonid fishes. It should be noted, however, that we make no claims that this list is a complete one. Some papers have been inadvertently omitted; others have been deemed of such marginal interest that their inclusion would be of limited value. We have primarily emphasized papers dealing with the interactions between the fungi and the fishes.

For related topics. such as saprolegnian taxonomy and classification or malachite green and fungal control, we have included a few of the major papers that will lead the reader to the literature on these subjects. Additionally, papers representative of other fungal diseases of fish have been included. We have made no attempt to treat these related topics comprehensively in the bibliography.

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The citation style used in the bibliography may seem inconsistent to some extent. Probably the major deviation is in the citations of references to work prior to 1900. A number of these papers have not been seen and we have cited them as they are entered in Lindau & Sydow's *Thesaurus litteruturae mycologicae et lichenologicue (* 1908- 19 17). In this generally reliable compendium inclusive pagination numbering is not given but rather only the first page of the paper. We have followed this convention in citing papers from this source.

Like any bibliography this one could be improved. It could certainly be more complete. However, we are not striving for ultimate perfection here but rather for a useful list of the papers dealing with saprolegniasis and the salmonid fishes. If this bibliography serves to direct curious mycologists and fisheries biologists alike to the extensive and fascinating literature dealing with these associations of fishes and fungi. it will have fulfilled all our hopes for it.

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