OVERSEAS DEVELOPMENT ADMINISTRATION
NRED AQUACULTURE RESEARCH PROGRAMME

STUDIES ON THE BIOLOGY OF THE EUS APHANOMYCES

PROJECT R5997

Project Completion Report
1 April 1994 - 31 March 1997

Institute of Aquaculture
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RNRRS PROJECT COMPLETION SUMMARY SHEET

DATE REPORT COMPLETED: 9/5/97
PROJECT NUMBER: R5997
RNRRS PROGRAMME: Aquaculture
PROGRAMME MANAGER: Prof R.J. Roberts
RNRRS PROGRAMME PURPOSE: Reduce the impact of fish disease
RNRRS PRODUCTION SYSTEM: Aquaculture
COMMODITY BASE: Freshwater fish
BENEFICIARIES: 'The rural poor'
TARGET INSTITUTIONS: AAHRI[1], Bangkok
GEOGRAPHIC FOCUS: Southeast Asia

	Planned	Actual
START DATE:	1/4/94	1/4/94
FINISH DATE:	31/3/97	31/3/97
TOTAL COST:	£126,060	£172,051[2]

1. Project purpose:

1.1 As described in project memorandum:
To provide an understanding of the general biology and ecology of the fungal pathogen of epizootic ulcerative syndrome (EUS) in fish, and resolve questions over its relationship with strains from other similar diseases.

1.2 Following the retirement of Dr L.G. Willoughby, the ecological element of the studies was terminated, and Dr K.D.Thompson initiated work to examine the immune mechanisms of fish against infection by the EUS fungal pathogen.

2. Outputs:

2.1 As described in project memorandum:

2.1.1 "A definitive detailed description of the biology of the pathogenic Aphanomycete(s) and its non-pathogenic co-habitants". The EUS fungal pathogen, *Aphanomyces invaderis*, was characterised and compared with its non-pathogenic co-habitants in terms of: pathogenesis in snakeheads and crayfish; the effect of temperature and culture medium on growth; extracellular enzyme production; chemical susceptibility; ultrastructure; immunocytochemistry, zoospore physiology; protein and carbohdrate electrophoresis banding patterns; lectin binding characteristics; polyclonal antibody reactivity by Western blot analysis; biochemical fingerprinting using pyrolysis mass spectra (PyMS); and molecular studies involving random amplification of polymorphic DNA (RAPD).

2.1.2 "Definition of the taxonomic relationship of the pathogen and its peers". The studies described above succeeded in distinguishing *A. invaderis* from its non-pathogenic co-habitants. Dendrograms constructed from this data showed that *A. invaderis* demonstrates more similarities with the destructive crayfish plague fungus, *Aphanomyces astaci*, than it does with Asian saprophytic *Aphanomyces* spp.

2.1.3 "Definition of the taxonomic relationship between EUS fungus and fungi from RSD[3], MG[4] and menhaden disease". All initial studies showed no consistent differences between *A. invaderis*

[1] AAHRI = Aquatic Animal Health Research Institute

[2] At the end of year 2, the consultant on the project (Dr L.G. Willoughby) retired on the grounds of ill-health. Consequently the position was reappointed to a post-doctoral research position (held by Dr K.D.Thompson), which led to increased salary and overhead costs.

and RSD and MG isolates. Later RAPD analysis confirmed this, revealing extreme genetic homogeneity between all the isolates, such that they were not only considered con-specific, but also part of the same clonal lineage. Isolates of fungi obtained from diseased menhaden (UM[5]) in the USA were shown to be incapable of growth within snakeheads, and biologically most closely related to the saprophyte, *Aphanomyces laevis*. It is considered very likely that the true UM pathogen has not been isolated, and that another fungus may bear a closer relationship to *A. invaderis*.

2.1.4 "Understanding of the fungus's survival mechanism over the lethal temperature season". Despite repeated attempts to induce temperature-resistant sexual structures in *A. invaderis*, none have been observed in any isolate. Further ecological studies were discontinued following the retirement of Dr Willoughby. It is considered likely that the fungus does not survive for long outside susceptible host fish, but definitive information on this aspect of the fungus requires a further research phase.

2.2 <u>Additional outputs</u>

Further work, undertaken by Dr Thompson, revealed that non-specific antibodies were produced by EUS infected snakeheads and trout; and a significant cellular response to *A. invaderis* was demonstrated by trout macrophages, both *in vivo* and *in vitro*. These are encouraging results for the possible future development of vaccines or other immunostimulants to protect fish against *A. invaderis* infection.

3. Contribution of outputs to project goal:

Significant progress has been made on various aspects regarding the fungal aetiology of EUS. Most notable is the confirmation that only one species of fungus (*A. invaderis*) is responsible for EUS lesions, and that it is conspecific, and probably clonal, with MG and RSD isolates. The extreme genetic homogeneity between isolates from as diverse locations as Bangladesh (EUS), Japan (MG) and Australia (RSD) gives a strong indication that it is the fungus that has spread across Asia causing ulcerative disease outbreaks in fish, and not some other biological agent, including viruses. Comparisons can be drawn with the crayfish plague fungus, *A. astaci*, and its spread across Europe. This enables future work on the control of EUS to be directed towards the diagnosis and treatment of the fungal aetiology. Other project outputs on the biology of EUS have also made significant progress towards this goal. Most of the techniques adopted were capable of diagnosing *A. invaderis* in culture (in particular: temperature-growth profiles, Western blots, PyMS and RAPD-PCR), and the chemical treatment studies indicated that outside the fish, *A. invaderis* is differentially more susceptible to several fungicides than other saprolegniacean fungi tested. Humoral and cellular defence mechanisms in fish were shown to be active against *A. invaderis,* but further studies are required to show whether protective immunisation can be induced. Zoospore behavioural, ultrastructural and immunocytochemical studies revealed particular characteristics of *A. invaderis* that may be relevant to its pathogenicity. Unusually, some zoospore stages lack a thick outer coat and cross-react with monoclonal antibodies specific to a non-saprolegniacean *Phytophthora* plant pathogen.

4. Publications:

4.1 Four papers have been published, or are in press (listed below), three other manuscripts have been submitted and three more are under preparation.

4.1.1 Lilley, J. H., Hart, D., Richards, R.H., Roberts, R.J., Cerenius, L. and Söderhäll, K. (1997) Pan-Asian spread of single fungal clone results in large scale fish-kills. Veterinary Record (in press)

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[3] RSD = red spot disease

[4] MG = mycotic granulomatosis

[5] UM = ulcerative mycosis

4.1.2 Lilley, J.H. and Inglis, V. (1997) Comparative effects of various antibiotics, fungicides and disinfectants on *Aphanomyces invaderis* and other saprolegniaceous fungi. Aquaculture Research, 28 (in press)

4.1.3 Lilley, J. H. and Roberts, R. J. (1997) Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. Journal of Fish Diseases, 20: 135-144.

4.1.4 Thompson, K. D., Lilley, J. H., Chinabut, S. and Adams, A. (1997) The antibody response of snakehead, *Channa striata* Bloch, to *Aphanomyces invaderis*. Fish and Shellfish Immunology (in press)

4.2 Publications that have been produced by Dr Willoughby during the period of this project are listed below.

4.2.1 Willoughby, L. G. and Roberts, R. J. (1994) Loss and recovery of zoospore motility in an isolate of *Aphanomyces* from a diseased fish. Mycological Research 98(12), 1463-1464.

4.2.2 Willoughby, L. G. and Roberts, R. J. (1994) Zoospore motility, its loss and recovery, in an isolate of *Aphanomyces* from a diseased fish in Thailand. In: Salmon Saprolegniasis. (Ed. by G.J. Mueller) Pp. 99-108. Bonneville Power Administration, US Department of Energy, Portland, USA.

4.2.3 Chinabut, S., Roberts, R. J., Willoughby, L. G. and Pearson, M. D. (1995) Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with the specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. Journal of Fish Diseases 18, 41-47.

4.2.4 Willoughby, L. G., Roberts, R. J. and Chinabut, S. (1995) *Aphanomyces invaderis* sp. nov., the fungal pathogen of freshwater tropical fishes affected by epizootic ulcerative syndrome (EUS). Journal of Fish Diseases 18, 273-275.

4.2.5 Willoughby, L. G. and Chinabut, S. (1996) Self-staling in *Aphanomyces invaderis,* the fungal pathogen of freshwater, tropical fish affected by epizootic ulcerative syndrome (EUS). The AAHRI Newsletter 5(2), 2-3.

4.2.6 Willoughby, L. G. (1997) *Saprolegnia polymorpha* sp. nov., a fungal parasite on Koi carp, in the UK. Nova Hedwigia (In press)

4.2.7 Willoughby, L. G. (1997) *Achlya diffusa* (Fungi, Oomycota) from fish ponds in Thailand. Nova Hedwigia 64(3-4), (in press)

5. Internal reports:

5.1 Twelve quarterly reports and three annual reports were submitted to ODA.

5.2 A report on a joint ACIAR[6]-ODA-NACA[7]-AAHRI mission to advise on EUS outbreaks in Pakistan from 9-19 March 1997 has been prepared and will be submitted to ODA.

6. Other dissemination of results:

6.1 An oral presentation entitled "The aggressive *Aphanomyces* pathogen of Asian freshwater and estuarine fishes" was given at the Sixth International Marine Mycology Symposium, Portsmouth in July 1995.

6.2 A poster presentation entitled "Comparative studies of the EUS *Aphanomyces* and other fungi associated with fish diseases" was given at the World Aquaculture Society Conference, Bangkok in February 1996.

6.3 Another poster presentation entitled "Immunocytological study of zoospore and cyst surface characteristics in fish and invertebrate pathogenic Oomycete 'fungi'" in cooperation with G.W.

[6]ACIAR - Australian Centre for International Agriculture Research

[7]NACA =Network of Aquaculture Centres in Asia-Pacific

Beakes, A.R. Hardham, J. Kerwin and E. Peterson was given at the Symposium on Fungal Physiology and Biochemistry, Nottingham in April 1997.

6.4 In March 1997, JHL joined a team from AAHRI, NACA and NSW[8]-Fisheries that presented two sessions of training seminars to Punjabi and Sindhi fisheries officers and fish farmers at Lahore and Karachi, during an EUS mission to Pakistan.

7. Follow-up indicated/planned:

7.1 A follow-up project has been proposed that will make use of molecular and imunological tools developed during the present project. The proposed project is directed towards developing control strategies for EUS and increasing resistance in susceptible fish.

7.2 A revised EUS manual is currently being prepared in collaboration with workers from AAHRI, ACIAR and NACA.

7.3 A regional workshop on EUS is scheduled for February 1998 to be held at AAHRI.

8. Authors of this report:

J.H. LILLEY DR K.D. THOMPSON

[8]NSW = New South Wales, Australia

EXECUTIVE SUMMARY

Aphanomyces invaderis is the recently-named Oomycete fungus that has been shown to be involved in a highly damaging disease of wild and cultured, freshwater and estuarine fishes, that has spread across Southeast and South Asia over the last two decades (epizootic ulcerative syndrome: EUS). The present study shows that *A. invaderis* is the only species, out of a number of isolates from EUS-affected areas in Thailand, that is capable of invading snakehead fish muscle tissue and reproducing EUS lesions, and is therefore pathognomic to the disease. *A. invaderis* is characterised and distinguished from the saprophytic isolates by means of: growth at various temperatures; growth on different media; extracellular enzyme production; chemical susceptibility; ultrastructure; immunocytochemistry, zoospore physiology; protein and carbohdrate electrophoresis banding patterns; lectin binding characteristics; polyclonal antibody reactivity by means of Western blot analysis; biochemical fingerprinting using pyrolysis mass spectra (PyMS); and molecular studies involving random amplification of polymorphic DNA (RAPD).

A. invaderis is shown to be indistinguishable from pathogenic *Aphanomyces* isolates from two other fish diseases, namely Japanese mycotic granulomatosis (MG) and Australian red spot disease (RSD) using the techniques described above. RAPD analyses, in particular, show that a wide range of EUS, MG and RSD isolates are not only conspecific, but probably constitute a single genetic clone. This strongly suggests that it is *A. invaderis*, and not any other biological aetiology, that has spread across Asia causing ulcerative disease in fish. It is recommended that the name *A. invaderis* is used to describe all EUS, MG and RSD pathogenic isolates. This work also shows that *Aphanomyces* isolates obtained from outbreaks of ulcerative mycosis (UM) of American menhaden, is distinct from *A. invaderis*, and more similar to the saprophytic fungus *A. laevis*. It is conjectured that the real UM pathogen has not been studied and that this may show greater similarity to *A. invaderis*. In comparison to the other species tested, *A. invaderis* is most similar to *Aphanomyces astaci*, the crayfish plague fungus which spread across most of Europe at the beginning of this century.

Snakeheads and trout are shown to produce non-specific antibodies in response to infection by *A. invaderis*. The cellullar immune response is also studied and shown to be important in terms of the susceptibility of fish. *In vitro* assays are used to investigate the factors involved in macrophage activity against the fungus. The potential for the development of protective vaccines or immunostimulants to combat EUS is discussed.

Outside of infected fish, *A. invaderis* is shown to be more susceptible to chemical treatment than other saprolegniacean fungi tested. Zoospore behavioural, ultrastructural and immunocytochemical studies also revealed some unusual characteristics of *A. invaderis* that may have relevance to its pathogenicity. In particular, encysted primary zoospores were shown to lack a thick outer coat and have fewer peripheral vesicles than other related fungi and secondary zoospores cross-reacted with monoclonal antibodies specific to a non-saprolegniacean *Phytophthora* plant pathogen.

ABBREVIATIONS

AAHRI - Aquatic Animal Health Research Institute, Thailand
ACIAR - Australian Centre for International Agriculture Research
ATCC - American Type Culture Collection
APW - autoclaved pond water
CDA - Czapek Dox agar
CMA - cornmeal agar
CVA - canonical variate analysis
DAB - 3'3 diaminobenzidine tetrahyrochloride
ECP - extracellular products
EDTA - ethylenediaminetetraacetic acid
EUS - epizootic ulcerative syndrome
FITC - fluorescein isothiocyanate
FMA - snakehead fish-meat-extract agar
FME - fish meat-extract (Hatai *et al*, 1977)
GP - glucose-peptone medium
GP-PenOx - glucose-peptone-penicillin-oxolinic acid medium
GP-PenStrep - glucose-peptone-penicillin-stretomycin medium
GPY - glucose-peptone-yeast medium
GY - glucose yeast medium (Dykstra *et al*, 1986)
H&E - haemotoxylin and eosin
HCA - hierarchal cluster analysis
HRP - horseradish peroxidase
HSW - high salt wash buffer
IFAT - indirect fluorescent antibody technique
IgG - immunoglobulin G
IHC - immunohistochemistry
im - intra-muscular
IMI - International Mycological Institute
ip - intra-peritoneal
LSW - low salt wash buffer
MAb - monoclonal antibody
MEA - malt extract agar
MG - mycotic granulomatosis
NACA - Network of Aquaculture Centres in Asia, Bangkok
NIFI - National Inland Fisheries Institute, Bangkok
NSW - New South Wales
OD - optical density
PAb - polyclonal antibody
PAS - periodic acid - Schiffs
PBS - phosphate buffer saline
PCR - polymerase chain reaction
PDA - potato dextrose agar
PG-1 - peptone-glucose-1 medium
pi - post-injection
PMSF - phenylmethylsulphoxylfluoride
PyMS - pyrolysis mass spectrometry
RAPD - random amplification of polymorphic DNA
RFLP - restriction fragment length polymorphism
rRNA - ribosmal RNA
RSD - redspot disease
SAPU - Scottish Antibody Production Unit
SC - subcutaneous
SDA - Sabouraud dextrose agar
SDS-PAGE - sodium dodecyl sulphate polyacylamide gel electrophoresis
SEAFDEC - SE Asian Fisheries Development Centre, Iloilo, Philippines
SEM - scanning electron microscope
TBS - tris buffer saline
TEM - transmission electron microscope

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APPENDIX FIVE - Paper two - Lilley J.H. and Inglis V. (1997) Comparative effects of various antibiotics, fungicides and disinfectants on *Aphanomyces invaderis* and other saprolegniaceous fungi. Aquaculture Research 28: (in press)

APPENDIX SIX - Paper three - Thompson K.D., Lilley J.H., Chinabut S. and Adams A. (1997) The antibody response of snakehead, *Channa striata* Bloch, to *Aphanomyces invaderis*. Fish and Shellfish Immunology (in press)

APPENDIX SEVEN - Paper four - Lilley J.H., Hart D., Richards R.H., Roberts R.J., Cerenius L. and Söderhäll K. (1997) Pan-Asian spread of single fungal clone results in large scale fish-kills. Veterinary Record (in press)

1. BACKGROUND

1.1 Introduction

Epizootic ulcerative syndrome (EUS) has been the cause of large-scale fish-kills in culture and capture fisheries in Southeast Asia and the subcontinent region for over 15 years (Chinabut, 1995). Outbreaks have been particularly severe in rice-field fish, an important dietary component of many rural communities. The nature of the spread of EUS across Asia has always indicated that an infectious biological agent was the cause, and since the early studies, a number of viruses, bacteria, fungi and parasites have been recovered from affected fish (~~Frerichs, 1995)(Boonyaratpalin, 1989)(Roberts et al, 1993)~~(reviewed by Frerichs, 1995; Boonyaratpalin, 1989; Roberts *et al*, 1993; and Tonguthai, 1985). In recent years a slow-growing, thermolabile fungus of the genus *Aphanomyces* has been shown to be capable of migrating through the tissues of susceptible fish and reproducing the characteristic EUS lesions (Roberts *et al*, 1993). At the time of the start of this study, EUS was defined at an ODA-sponsored regional seminar in Bangkok as "a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious aetiology characterised by the presence of invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response" (Roberts *et al,* 1994). Soon after, Willoughby *et al* (~~Willoughby~~ 1995) named the fungus *Aphanomyces invaderis*.

It was acknowledged at the 1994 meeting that the pathology of two other fish diseases, Japanese mycotic granulomatosis (MG) (Egusa, 1992) and Australian red spot disease (RSD) (Callinan *et al*, 1995a) appeared indistinguishable from EUS. Ulcerative mycosis (UM) (Dykstra *et al*, 1989) of fish in Western Atlantic estuaries was also recognised as being very similar to the above diseases. Consequently, EUS research priorities, and the focus of the present study, involved establishing whether *A. invaderis* is the only species responsible for the fungus-related pathology of EUS, and if so, to characterise it and compare it with *Aphanomyces* isolates from RSD, MG and UM, and fungi of other aquatic animal mycoses.

1.2 The history of epizootic ulcerative mycoses in Asia

1.2.1 Mycotic granulomatosis (MG)

The first report of an ulcerative condition similar to EUS came in summer 1971, in farmed ayu (*Plecoglossus altivelis*) in Oita Prefecture, Japan (Egusa and Masuda, 1971, cited by Egusa, 1992). The characteristic pathology involving a granulomatous response to invasive hyphae was soon recognised and the disease was named mycotic granulomatosis (Miyazaki and Egusa, 1972). It rapidly spread to several parts of the country and affected many species of fish, predominantly cultured ayu and goldfish *(Carassius carassius auratus)*; and wild crucian carp *(Carassius auratus),* bluegill *(Lepomis macrochirus)* and grey mullet (*Mugil cephalus*) (Miyazaki and Egusa, 1972; 1973a; b; c). Significantly, common carp *(Cyprinus carpio)* was not affected. Hatai *et al* (1977) isolated the invasive fungus from affected fish and subsequently called it *Aphanomyces piscicida* (Hatai, 1980). Although serious MG epizootics have not been reported in Japan since 1973, outbreaks have continued to occur periodically, and recently Hatai *et al* (~~Hatai~~ 1994) reported a similar disease in imports of ornamental dwarf gourami (*Colisa lalia*) from Singapore.

1.2.2 Red spot disease (RSD)

In 1972, outbreaks of a cutaneous ulcerative condition called red spot disease (RSD) affected estuarine fish, particularly grey mullet (*Mugil cephalus*), in Queensland, Australia (McKenzie and Hall, 1976). The disease later progressed to coastal rivers in New South Wales (Callinan *et al*, 1989), Northern Territory (Pearce, 1990) and Western Australia (Callinan, 1994a). Seasonal outbreaks continue to occur and the cost to commercial fishermen has been estimated at $Aus 1 million annually, not including losses associated with discarded catches and possible long-term declines in fish stocks (Callinan *et al,* 1996).

An *Aphanomyces* fungus was recovered from diseased fish by Fraser *et al* (~~Fraser~~ 1992) and was shown to reproduce the disease in fish using bath challenges, but only when the skin of experimental fish was artificially abraded (Callinan, 1994b). Therefore, some other factor was considered to be involved in the disease process. Virgona (~~Virgona 1992)~~ 1992) showed that RSD outbreaks in estuarine fish in the Clarence river, NSW were associated with lower catchment rainfall and Callinan *et al* (~~Callinan 1995~~1995b) related this to runoff from acid sulphate soils. Ultrastructural examination of fish gills and skin showed that the low pH and elevated concentrations of monomeric aluminium, representative of estuarine acidification, induces significant lesions in fish (Sammut *et al*, 1996). In aquarium trials, Callinan *et al* (~~Callinan~~ 1996) subsequently induced RSD in fish exposed sublethally to artificially acidified water (at both pH 3 and pH 5) and pathogenic *Aphanomyces* spores, even at low concentrations of monomeric aluminium.

1.2.3 Epizootic ulcerative syndrome (EUS)

Following the outbreaks of MG and RSD, there has been a tangible spread westwards across Asia of a fish condition associated with dermal ulceration and involving large scale mortalities in a number of fish species. The condition was given its present name, epizootic ulcerative syndrome (EUS), in 1986 at the Consultation of Experts on Ulcerative Fish Diseases in Bangkok (FAO, 1986). Outbreaks of EUS have been reported in 18 countries of the Asia-Pacific region, although not all are confirmed EUS according to the Roberts *et al* (~~Roberts~~ 1994) definition. The most recently affected was Pakistan in early 1996 (Figure 1.1).

In 1975-6, an ulcerative disease outbreak, believed to be EUS, occurred in the rivers of southern Papua New Guinea (Haines, 1983). In 1982-3, there were high mortalities in gudgeon (*Ophieleotris aporos* and *Oxyeleotris heterodon*) from inland areas and mullet from estuaries in northern Papua New Guinea (Coates *et al,* 1989). Introduced tilapia (*Oreochromis mossambicus*) are common in these areas, but they proved resistant. Preserved affected fish were later examined by Roberts *et al* (~~Roberts~~ 1986) and confirmed as pathologically identical to EUS.

In 1980 an epizootic haemorrhagic condition occurred in Java, Indonesia affecting primarily cultured cyprinid and clarid fish although whether this was EUS is uncertain (Roberts *et al*, 1986). Typically ulcerated snakeheads and catfish have subsequently been reported in the Indonesian states of Sumatra, Sulawesi and Kalimantan (Widagdo, 1990). Invasive hyphae have been identified from sand gobies (*Oxyeleotris marmoratus*) from eastern Kalimantan (Rukyani, 1994), and D. Bastiawan isolated *A. invaderis* from an EUS-affected sand goby from Java in 1993.

Roberts *et al* (1986) discussed unconfirmed accounts of ulcerated walking catfish (*Clarias batrachus*) in Singapore in 1977 and of subsequent occurrences thereafter. Despite Singapore's status as a centre of trade in EUS-susceptible ornamental fishes (Lilley *et al*, 1992) there have been no records of high EUS losses to this industry.

An incidence of high fish mortalities occurred in southern peninsular Malaysia in 1979 (Shariff and Law, 1980, cited by Roberts *et al*, 1986), but the first reported typical EUS outbreaks were in December 1980, in rice-field fishes in northern Malaysia (Jothy ~~1981~~, 1981, cited by Roberts *et al*, 1986). These have recurred annually ever since, albeit to a lesser extent (Shariff and Saidin, 1994). Major species affected are snakeskin gourami (*Trichogaster pectoralis*), striped snakehead (*Channa striata*), climbing perch (*Anabas testudineus*) and walking catfish (Shariff and Saidin, 1994).

Significant, well-documented epizootics have occurred annually in Thailand since 1981 (Ulcerative Fish Disease Committee, 1983; Chulalongkorn University, 1983; 1985; 1987). The second (1982-3) and third (1983-4) outbreaks were particularly devastating as they affected the intensive fish culture systems of central Thailand as well as wild fish in natural waterways. Some

of the most severe mortalities were in snakehead (*Channa striata*) farms and rice-field fish and direct economic losses in 1982-3 alone were estimated at US$ 9 million (Tonguthai, 1985). The original outbreaks started towards the end of the rainy season (September) and persisted throughout the cool season to March. Outbreaks now tend to be restricted to the coolest months of December and January. During the last season (December 1996) EUS was experienced in NE, central and southern provinces (S. Kanchanakhan, pers. comm.). Further discussion of mycological studies carried out in Thailand is given in Section 1.4.1.

Myanmar, Lao PDR and Cambodia, all bordering Thailand, first reported major outbreaks of EUS in 1983 or 1984 (Lilley *et al*, 1992). Subsequent epizootics were less extensive (eg EUS affected 35 Burmese townships in 1984-85 and 11 townships in 1989-90, Soe, 1990), but given the importance of susceptible fish to rural communities in these countries, the impact continues to be significant.

Several accounts of EUS-affected fish have also come from Vietnam, China and Hong Kong although these are still not validated. The first report of ulcerated snakeheads in Vietnam, and therefore the most likely first occurrence of EUS in that country, came from the Mekong delta in 1983 (Xuan, 1990). Ulcerated *Labeo rohita* were first observed at the Pearl River Fisheries Institute in Guangzhou, South China in 1982 (Lian, 1990). Clarid catfish were affected in the same area in 1987-8 (Lian, 1990) and *Carassius auratus* were reportedly affected over much of Eastern China in 1989 (Guizhen, 1990). Wilson and Lo (1992) reported seasonal mortalities of over 70% of snakeheads (*Channa maculata*) in late summer in Hong Kong since 1988.

Laguna de Bay in the Philippines, the largest and most productive lake in SE Asia, experienced a serious outbreak of EUS in December 1985. An estimated 5-40% of snakeheads, gobies, gouramies, catfish, crucian carp, *Arius* sp and *Therapon* sp were ulcerated whereas milkfish, bighead carp, and tilapia were unaffected (Llobrera, 1987). The disease continued to spread to at least 11 other provinces affecting wild fish in lakes, rice-fields and swamps and pond cultured fish (Bondad-Reantaso *et al*, 1994). Rejection of ulcerated fish, and reduction in market prices of all fish, significantly reduced the income of fishermen. Mullet, goatfish (*Upeneus bensai*), croaker (*Johnius* sp), *Psettodes* sp and *Scanthophagus argus* in a lagoon in Buguey Province suffered an outbreak in 1990 which was confirmed as EUS by histological examination (Bondad-Reantaso, 1990; S. Chinabut, pers. comm.). These brackishwater and marine species provided an explanation as to how the disease may have spread between the islands. The severity of outbreaks has decreased since 1993. Several *A. invaderis* isolates were obtained as described by Paclibare *et al* (1994) and provided for the present study. Further studies in the Philippines on bacterial, fungal and environmental involvement in EUS are discussed in other sections.

A major outbreak of EUS in western Sri Lanka occurred in December 1987, prior to any outbreaks on the subcontinent mainland (Costa and Wijeyaratne, 1989). It is suspected that the disease was imported from SE Asia in shipments of infected fish, possibly ornamental angel fish (*Pterophyllum scalare*), which have suffered from high disease-related mortalities (Balasuriya, 1994). Snakeheads with large necrotic ulcers were the most visible sign of the disease, but tilapia, the main commercial species was not affected. EUS was reportedly still active in Batticaloa lagoon in 1996 (P. Vinobaba and M. Vinobaba, pers. comm.).

In the last few years, EUS has had a serious effect on fisheries throughout the subcontinent, causing losses in important capture fisheries areas and damaging confidence in an aquaculture industry still in the early stages of development. The disease was first reported in Chandpur district of Bangladesh in February 1988, this first outbreak lasted for 13 months during which time it progressed rapidly throughout the country, aided by the flood of September 1988 (Barua, 1994). Ulceration was observed in many wild species, predominantly snakeheads, *Puntius, Clarias, Mystus and Mastacembelus.* Cultured Indian major carp were also affected, although mortalities due to the disease were probably restricted to fingerlings (Roberts *et al,* 1989). Direct

of the most severe mortalities were in snakehead (*Channa striata*) farms and rice-field fish and direct economic losses in 1982-3 alone were estimated at US$ 9 million (Tonguthai, 1985). The original outbreaks started towards the end of the rainy season (September) and persisted throughout the cool season to March. Outbreaks now tend to be restricted to the coolest months of December and January. During the last season (December 1996) EUS was experienced in NE, central and southern provinces (S. Kanchanakhan, pers. comm.). Further discussion of mycological studies carried out in Thailand is given in Section 1.4.1.

Myanmar, Lao PDR and Cambodia, all bordering Thailand, first reported major outbreaks of EUS in 1983 or 1984 (Lilley *et al*, 1992). Subsequent epizootics were less extensive (eg EUS affected 35 Burmese townships in 1984-85 and 11 townships in 1989-90, Soe, 1990), but given the importance of susceptible fish to rural communities in these countries, the impact continues to be significant.

Several accounts of EUS-affected fish have also come from Vietnam, China and Hong Kong although these are still not validated. The first report of ulcerated snakeheads in Vietnam, and therefore the most likely first occurrence of EUS in that country, came from the Mekong delta in 1983 (Xuan, 1990). Ulcerated *Labeo rohita* were first observed at the Pearl River Fisheries Institute in Guangzhou, South China in 1982 (Lian, 1990). Clarid catfish were affected in the same area in 1987-8 (Lian, 1990) and *Carassius auratus* were reportedly affected over much of Eastern China in 1989 (Guizhen, 1990). Wilson and Lo (1992) reported seasonal mortalities of over 70% of snakeheads (*Channa maculata*) in late summer in Hong Kong since 1988.

Laguna de Bay in the Philippines, the largest and most productive lake in SE Asia, experienced a serious outbreak of EUS in December 1985. An estimated 5-40% of snakeheads, gobies, gouramies, catfish, crucian carp, *Arius* sp and *Therapon* sp were ulcerated whereas milkfish, bighead carp, and tilapia were unaffected (Llobrera, 1987). The disease continued to spread to at least 11 other provinces affecting wild fish in lakes, rice-fields and swamps and pond cultured fish (Bondad-Reantaso *et al*, 1994). Rejection of ulcerated fish, and reduction in market prices of all fish, significantly reduced the income of fishermen. Mullet, goatfish (*Upeneus bensai*), croaker (*Johnius* sp), *Psettodes* sp and *Scanthophagus argus* in a lagoon in Buguey Province suffered an outbreak in 1990 which was confirmed as EUS by histological examination (Bondad-Reantaso, 1990; S. Chinabut, pers. comm.). These brackishwater and marine species provided an explanation as to how the disease may have spread between the islands. The severity of outbreaks has decreased since 1993. Several *A. invaderis* isolates were obtained as described by Paclibare *et al* (1994) and provided for the present study. Further studies in the Philippines on bacterial, fungal and environmental involvement in EUS are discussed in other sections.

A major outbreak of EUS in western Sri Lanka occurred in December 1987, prior to any outbreaks on the subcontinent mainland (Costa and Wijeyaratne, 1989). It is suspected that the disease was imported from SE Asia in shipments of infected fish, possibly ornamental angel fish (*Pterophyllum scalare*), which have suffered from high disease-related mortalities (Balasuriya, 1994). Snakeheads with large necrotic ulcers were the most visible sign of the disease, but tilapia, the main commercial species was not affected. EUS was reportedly still active in Batticaloa lagoon in 1996 (P. Vinobaba and M. Vinobaba, pers. comm.).

In the last few years, EUS has had a serious effect on fisheries throughout the subcontinent, causing losses in important capture fisheries areas and damaging confidence in an aquaculture industry still in the early stages of development. The disease was first reported in Chandpur district of Bangladesh in February 1988, this first outbreak lasted for 13 months during which time it progressed rapidly throughout the country, aided by the flood of September 1988 (Barua, 1994). Ulceration was observed in many wild species, predominantly snakeheads, *Puntius, Clarias, Mystus and Mastacembelus.* Cultured Indian major carp were also affected, although mortalities due to the disease were probably restricted to fingerlings (Roberts *et al,* 1989). Direct

losses to fisheries during the first outbreak in 1988 were estimated at US$ 3.4 million (Barua, 1990). Extensive local media coverage about the disease at that time fuelled a widespread, but unfounded, fear of possible public health risks from fish consumption, resulting in further losses to fish traders. EUS incidences subsequently declined, but there are reports that, as from 1995, the severity of the disease is again on the increase in Bangladesh (G.U. Ahmed, unpublished report). In January 1993, *A. invaderis* was isolated from farmed Indian major carp (*Labeo rohita*) in NW Bangladesh and wild fish in the productive flood plain area of NE Bangladesh (Plate 1.2)

Incidences of EUS in India have been comprehensively reviewed (Zoological Society of Assam, 1988; Kumar *et al*, 1989; Jhingran and Das, 1990; National Workshop on Ulcerative Disease Syndrome in Fish, 1990; ICSF, 1992; Das and Das, 1993; Ninawe, 1993; Mohan and Shankar, 1994; Das, 1994). Discussion of some of the research work presented is given in other sections. The NE Indian states were the first to report losses in May 1988. The disease spread through rivers, reservoirs and paddy fields to most states, affecting some Indian major carp farms as well. EUS had a serious impact on fish in low salinity areas of the rich brackishwater fisheries of Chilka Lake, Orissa in November 1990 (Raman, 1992), and the reservoirs and backwaters of Kerala in June 1991 (Sanjeevaghosh, 1991). To date only Jammu & Kashmir, Punjab and Gujarat have not reported outbreaks.

Bhutan and the eastern Terai of Nepal were first affected in 1989 and by 1993, EUS had spread to Himalayan valley regions including Pokhara and Kathmandu where cold water species, including *Tor* spp, were affected (Phillips, 1989; Shrestha, 1994). It is estimated that 20-30% of Nepalese pond fish production (about 3000 mt) is lost every year through EUS (Pantha, unpublished report).

The latest country to be affected by EUS was Pakistan, where EUS was confirmed in snakeheads in Lahore Division, Punjab in April 1996 (Kanchanakhan, 1996a). The blotched snakehead or mud murrel (*Channa punctata*) was the most commonly affected species; *Puntius* spp, *Labeo rohita* and *Cirrhinus reba* were also reportedly affected (N. Akhthar, pers. comm.). An estimated 20% of farms were affected in Sialkot Division, Punjab with the incidence being higher in ponds that were inundated by flooding in 1996 (NACA, 1997). Although reported losses have not been high, possibly due to the extensive use of tub-well water for fish farms and elevated salinity in parts of Punjab (NACA, 1997), EUS is now established in the upper Indus watershed, and given that Pakistan has the largest canal system in the world based around the Indus, there are fears of a rapid spread to other areas and potentially serious future impacts.

1.3 Species affected

Many species (>100) have been reported to be affected by EUS (Lilley *et al*, 1992), but only relatively few reports have been confirmed by demonstrating the presence of mycotic granulomas in histological section or by isolation of a pathogenic *Aphanomyces* fungus from internal tissues. Table 1.1 lists these, including species from MG or RSD outbreaks.

Similarly, some commercially important species are considered to be particularly resistant to EUS, but few studies have been undertaken to confirm these observations and investigate the mechanism of resistance. Species reported to be unaffected by EUS outbreaks include Chinese major carps, tilapias and milkfish (*Chanos chanos*). Hatai (1994) experimentally injected catfish (*Parasilurus asotus*), loach (*Misgurnus anguillicausatus*) and eel (*Anguilla japonica*) with hyphae of MG-*Aphanomyces* and found them to be refractory to infection. Wada *et al* (1996) and Shariffpour (pers. comm.) experimentally injected common carp (*Cyprinus carpio*) with zoospores of MG- and EUS-*Aphanomyces* respectively, and demonstrated that fungal growth was suppressed by an intensive inflammatory response. M. Khan (pers. comm.) showed that tilapia (*Oreochromis mossambicus*) did not mount such a marked response, but EUS-*Aphanomyces* were nonetheless incapable of sustained growth within the muscle.

Some authors have commented that the most severely affected species in natural outbreaks are generally bottom dwellers (Llobrera, 1987)(; Chondar & Rao, 1996) or posses air-breathing organs (Roberts *et al*, 1994), but examination of Table 1.1 shows that this is by no means always the case.

In the case of snakeheads, no particular size group appears to be more susceptible, with affected fish ranging from 40g to 900g (Cruz-Lacierda and Shariff, 1995). However, there is a possibility that size may be significant in other species. For example Indian major carp, and rohu (*Labeo rohita*) in particular suffer high mortalities as fingerlings (Roberts *et al*, 1989) but larger fish, although appearing ulcerated, have not demonstrated typical mycotic granulomas in muscle tissue, and are not reported as dying in large numbers (NACA, 1997).

Plate 1.1

(a) Publications on EUS from SE Asia and South Asia

(b) Fish kill in natural water body in Thailand

(Figure 1.1 Map of Asia)

Plate 1.2

(a) EUS-affected fish from an important flood-plain fishery near Srimanagal, Bangladesh

(b) EUS-affected cultured rohu (*Labeo rohita*) juvenile from Bangladesh

Table 1.1 Species susceptible to EUS (or MG† or RSD‡) as indicated by the presence of typical mycotic granulomas in histological section or isolation of pathogenic *Aphanomyces* from muscle or internal organs (numbers correspond with references given below; *denotes artificial challenge)

Latin name **(common name)**	Jap†	Aus‡	Ino	Tha	Lao	Mya	Phi	Ban	Ind	Pak	Sco
Acanthopagrus australis (yellowfin bream)		5									
Carassius auratus (crucian carp)	9										
Carassius carassius auratus (gold fish)	9										
Channa maculata (=Ophicephalus maculatus) (Formosan snakehead)	9							12			
Channa marulia (=Ophicephalus marulius) (river murrel - India)								4			
Channa micropeltes (=Ophicephalus micropeltes) (red snakehead)				4							
Channa punctata (=Ophicephalus punctatus) (mud murrel - India)								8		7	
Channa striata (=Ophicephalus striatus) (striped snakehead)				3*,8	4	4	1		10		
Cirrhina mrigala (mrigal) - fingerlings								12			
Clarias batrachus (walking catfish)							1				
Clarias gariepinus (African catfish)							2*				
Colisa lalia (dwarf gourami)	6										
Fluta alba (swamp eel)				4							
Glossogobius giurus (bar-eyed goby)							1				
Labeo rohita (rohu) - fingerlings								12,8			
Lepomis macrochirus (bluegill)	9										
Liza diadema (mullet)		11									
Mastacembalus armatus (armed spiny eel)								4			
Mastacembalus pancalus (guchi - Bangladeshi)								4			
Mugil cephalus (grey mullet)	9	5					1		10		
Notopterus notopterus (grey featherback)				4							
Oncorhynchus mykiss (rainbow trout) - marginally susceptible	6*										13*
Osphronemus goramy (pla raet - Thai)				4							
Oxyeleotris marmoratus (sand goby)			8								
Oxyeleotris sp (gudgeon)											
Plecoglossus altivelis (ayu)	9										
Puntius gonionotus (silver barb)				4							
Puntius sophore (punti - Bangladeshi)										4	
Puntius sp (puntius)								12	10		
Rhodeus ocellatus (tairiku-baratanago - Japanese)	6*										
Rohtee sp (keti - Bangladeshi)						4					
Scardinius erythrophthalmus (rudd) - marginally susceptible	6*										
Sillago ciliata (sand whiting)		5									
Trichogaster pectoralis (snakeskin gourami)				4							
Trichogaster trichopterus (3-spot gourami)				8							
Tridentiger obscurus obscurus (Japanese trident goby)	9										
Xenentodon cancila (round-tailed garfish)								8			

COUNTRY KEY: Jap = Japan; Aus = Australia; Ino = Indonesia; Tha = Thailand; Lao = Lao PDR; Mya = Myanmar; Phi = Philippines; Ban = Bangladesh; Ind = India; Pak = Pakistan; Sco = Scotland

REFERENCE KEY:

1 Callinan *et al* (1995)
2 Callinan (pers. comm.)
3 Chinabut *et al* (1995)
4 Chinabut (pers. comm.)
5 Fraser *et al* (1992)
6 Hatai (1994)
7 Kanchanakhan (1996a)
8 Lilley and Roberts (1997)
9 Miyazaki (1994)
10 Mohan and Shankar (1995)
11 Pearce (1989)
12 Roberts *et al* (1989)
13 Thompson (pers. comm.)

1.4 The pathology of EUS, MG, RSD and UM

A brief account of the gross clinical signs and histopathology of EUS is given in Table 1.2 alongside descriptions of MG, RSD and UM, in order to show the similarity between the progression of each of these diseases in fish.

The initial signs of EUS are similar to many other fish disorders, involving reduced appetite, lethargic swimming behaviour, and in the case of snakeheads, swimming with the head out of the water. Petechia and/or small red or grey erosions can rapidly develop and expand into large ulcers, with associated loss of scales, haemorrhaging and oedema. In the case of RSD, Callinan *et al* (1989) commented that erythematous dermatitis lesions and intermediate-type lesions subsequently resolved, whereas necrotizing dermatitis lesions consistently developed into dermal ulcers. Healing lesions are scarce among UM-affected fish, however, and Noga *et al* (1988) concluded that that disease was routinely fatal. The most common lesion reported for all of the four diseases described in Table 1.2 is the advanced lesion or dermal ulcer.

Snakeheads are often the most visible sign of an EUS outbreak, as aside from being highly susceptible, they can survive with much more severe, chronic lesions. These may completely destroy the peduncle or erode into the abdominal cavity, sometimes exposing the swim bladder. Head erosion is a common feature of diseased snakeheads and specimens have been found with exposed optic nerves or loosened maxillae and mandibles. A graphic photographic account of naturally and experimentally infected snakeheads is available in Kanchanakhan (1996b).

Callinan *et al* (1989) examined 2560 RSD-affected mullet (*Mugil cephalus*), each with an average of 1.5 dermal ulcers, and found lesions generally occurred more often in the posterior and dorsal areas of the fish than the anterior and ventral regions. A total of 361 EUS-affected snakeheads (*Channa striata*) from two separate studies, one in Thailand (Chinabut and Lilley, 1992) (see Plate 1.3), and one in the Philippines (Cruz-Lacierda and Shariff, 1995) showed that multiple lesions were common and occurred on all parts of the body, including the head, opercula and fins. The first study showed that dorsal and ventral surfaces were equally affected (80% of fish in both cases); and the second showed a slight preponderance of lesions on the head and towards the posterior sections of the fish. A similar investigation of UM-affected menhaden (*Brevoortia tyrannus*), however, showed that the posteroventral portion of these fish was most frequently affected (67% of all lesions in 424 fish were located here) (Noga *et al*, 1988). It was speculated that this may be due to differences in the immune capability of this part of the fish, or the release of some chemoattractant from the perianal area that stimulates fungal zoospores to attach.

Table 1.2 Classification of lesions in EUS and equivalent descriptions from similar disease conditions

EUS of snakehead (*Channa striata*): Lilley *et al*, 1992	**MG-like disease of dwarf gourami (*Colisa lalia*): Wada *et al*, 1994**	**RSD of mullet (*Mugil cephalus*): Callinan *et al*, 1989**	**UM of menhaden (*Brevoortia tyrannus*) Noga *et al*, 1988**
Petechia. Acute dermatitis forming rosacea.		**Erythematous dermatitis**: Yellow skin with irregular reddening. Scales fractured. Usually <10 mm Ø. Epidermis present at margins and irregularly over lesion. Epidermis hyperplastic, oedematous and infiltrated by mononuclear cells. *Stratum spongiosum* mild to severe congestion, oedema and mononuclear cell infiltration. Other tissues normal. No hyphae or granulomas.	
1. Small haemorrhagic surface lesions. Epithelial necrosis with surrounding oedema, haemorrhage of the underlying dermis and some inflammatory cell infiltrate. Few hyphae sometimes seen.		**Intermediate-type dermatitis**: Approx. 10mm Ø. Epidermis absent over lesion, though sometimes evidence of regeneration, scales usually retained. Mild to moderate chronic active dermatitis with some fungal hyphae and granulomas down to skeletal muscle. Often muscle necrosis.	**Early Type I**: Flat, red or yellow-red area <5 mm Ø. Primarily macrophage response. Aseptate hyphae (7-12 μm Ø) surrounded with small granulomas, appeared to originate near a scale pocket. Fungus invasion associated with necrosis of skeletal muscle and myophagia. Some multinucleated giant cells.
2. Mild epithelial necrosis. Surrounding oedema, haemorrhage of underlying dermis, and inflammatory cell infiltration with severe necrotizing myopathy spread over a wide area below the active skin lesion. Hyphae enclosed in epithelioid capsules. No disruption of internal organs.	**Type 1:** Small, discoloured pale areas (3-10mm Ø) with irregular reddening. Granulomas in *stratum compactum* and muscle, surrounding sparsely-branching aseptate hyphae (8-20 μm Ø). Severe floccular degeneration of skeletal muscle fibres. **=Moderate MG lesion (Miyazaki and Egusa, 1972)**	**Necrotizing dermatitis**: yellowish-grey to red, ovoid domed areas (10-40mm Ø). Epidermis and scales usually absent, dermis swollen and macerated. Few hyphae trailed from lesion (not cotton-wool-like). Moderate to severe, locally extensive, necrotizing, granulomatous dermatitis. Large numbers of sparsely-branching, aseptate hyphae (12-18 μm Ø) usually within granulomas down to skeletal muscle. Severe floccular degeneration of muscle.	**Early Type II lesion**: Raised 15-20 mm Ø smooth area with small area of scale loss. Small ulcerated area in centre. Intense inflammation, large coalescing granulomas with necrotic core sometimes identifiable as fungal hyphae.
3. Advanced lesion, with large bacterial ulceration, massive necrotizing granulomatous mycosis of underlying muscle involving branching, aseptate Oomycete coated with epithelioid cells. Hyphae may invade abdominal viscera, particularly in the case of major carp (Roberts *et al*, 1989) and spiny eels (Chinabut, 1990) Internal organs other than those infiltrated with hyphae show only mild pathological changes.	**Type 2**: Open ulcers (up to 10mm Ø) leaving crater-shaped cavity. Large amounts of epidermis and *stratum compactum* sloughed off. Granulomas and hyphae in deeper muscle tissue; commonly throughout the viscera; also within vertebrae and costae; and rarely, hyphae (without cellular response) found in the brain. **=Severe MG lesion (Miyazaki and Egusa, 1972)**	**Dermal ulcer**: About 10-40mm Ø. Margins sharply defined. Skeletal muscle exposed up to 10mm below surface. In some cases bone or viscera exposed. Moderate to severe diffuse granulomatous myositis. Hyphae, within granulomas, rarely penetrated internal organs. Some dermal ulcers showed evidence of healing.	**Advanced lesion**: Open ulcers up to 25mm Ø, containing white friable material consisting of hyphae and necrotic muscle. Hyphae surrounded by intense granulomatous inflammation. Occasional foreign-body-type multinucleated cells. Myophagia and muscle fragmentation often without associated fungus. Often lesions affect viscera or cross to contralateral flank. **End-stage lesion**: Necrotic core sloughed leaving crater-shaped cavity with non-oedematous periphery. Many with large numbers of hyphae, others heavy Gram -ve bacterial infections associated with necrotic cell debris. Some osteoblastic activity. Some mild fibroplasia.
			Healing lesion: <5 mm Ø. Usually overlying peritoneal cavity. Smooth, non-ulcerated areas of tissue loss. Epidermis intact and mildly hyperplastic. Regenerating scales. Mild to moderate granulomatous inflammation. Few hyphae.

Plate 1.3

(a) EUS-affected snakeheads with multiple dermal ulcers

(b) Adult rohu from Pakistan with dermal ulcers but the fungus does not extend into the muscle

1.5 Causative agents

Given the nature of the spread of EUS across the region, an infectious biological causative agent has always been suspected. This was confirmed by the successful transmission of EUS to healthy fish by cohabitation with infected fish, feeding of infected material, and exposure to affected water (Balasuriya *et al*, 1990; Subasinghe and Jayasinghe, 1990; Cruz-Lacierda and Shariff, 1995).

1.5.1 Mycology

Almost since its appearance in Southeast Asia, fungi have been known to be involved in the aetiology of EUS. Limsuwan and Chinabut (1983) described a "severe chronic granulomatous mycosis" in histological sections of lesions from diseased fish in Thailand. At this time *Achlya* and *Saprolegnia* spp were identified from affected fish (Pichyangkura and Bodhalamik, 1983; Limsuwan and Chinabut, 1983) but these were later dismissed as secondary agents (Tonguthai, 1985). Roberts *et al* (1993) subsequently isolated the fungus, that was later to be named *Aphanomyces invaderis,* from within the muscle of EUS-affected fish in Thailand. It was shown to be slow-growing and thermolabile when compared to other local saprophytic *Aphanomyces,* and when mycelium of isolates RF6 and RF8 were placed below the dermis of striped snakeheads (*Channa striata*), they were shown to be capable of migrating into the tissues and causing typical EUS lesions (Roberts *et al*, 1993). Chinabut *et al* (1995) further showed that at 26°C and 31°C, challenged snakeheads staged a marked inflammatory response to fungal invasion and by day 14 healing became well established, whereas at 19°C there was only a limited macrophage response and by day 19 all the fish had died. The seasonal nature of EUS has indicated that low temperature is an important factor in the disease process and this study provides an explanation of how it affects the development of lesions.

Nonetheless, as *Saprolegnia* and *Achlya* are commonly observed in squash preparations of EUS lesions (Pichyangkura and Tangtrongpiros, 1985; Ninawe, 1993), and these, along with culturally fast-growing *Aphanomyces* strains, have been isolated from EUS affected fish (Willoughby and Lilley, 1992; Qureshi *et al*, 1995), Noga (1993; 1994) called for clinically relevant challenge studies to rule out the possibility that the fungus seen invading tissues is not simply one of any number of environmental opportunists. Such trials were undertaken for this study and are described in Section 3.2.

An account of the characteristics of *Aphanomyces invaderis* as described by Willoughby *et al* (1995) is given in Section 3.1.3, along with the published details of the Japanese MG-fungus *(Aphanomyces piscicida)* and the Australian RSD-associated *Aphanomyces*.

Callinan *et al* (1995) compared RSD-*Aphanomyces* isolates with EUS isolates from the Philippines and found them to be similar in terms of morphological and cultural characteristics and peptide banding profiles. Hatai *et al* (1994) recently isolated an *Aphanomyces* sp from dwarf gourami *(Colisa lalia),* and found it to resemble previous MG isolates, but neither strain has been compared directly with EUS or RSD fungi. Callinan (1994b) and Wada *et al* (1994) have made recommendations for direct comparative studies of the pathogenesis and biological characteristics of the fungi involved in all three diseases. Work described in subsequent sections here attempts to address these recommendations.

1.5.2 Environmental factors associated with EUS

It may be the case that in EUS-affected areas of SE and South Asia where water is acidic and/or poorly buffered (eg Udon Thani, NE Thailand and East Pegu, Myanmar: Roberts *et al,* 1986; East Kalimantan, Indonesia: Rukyani, 1994; and North Luzon, Philippines: Callinan *et al,* 1995b) the disease process is the same as reported for Australian estuaries, with acid-induced dermatitis leading to fungal infection. Further evidence for the importance of pH come from statistical studies on EUS outbreaks in Bangladesh which have indicated that routine use of agricultural lime ($CaCO_3$), which is known to stabilise the pH of water, reduces the likelihood of EUS

outbreaks (Hossain *et al*, 1992); Ahmed and Rab, 1995). However, regional EUS sampling surveys have clearly shown that some outbreaks occurred in areas that did not experience acid water conditions (eg Chiang Mai, N Thailand and Mandalay, Myanmar: Roberts *et al*, 1986; Mymensingh, Bangladesh and Nakorn Ratchasima, NE Thailand: Phillips and Keddie 1990; and parts of South India: Callinan *et al*, 1996) and in such cases, other pathological agents, as described below, may have a more significant affect.

Relatively low water temperatures, which have been widely associated with EUS outbreaks, are thought to cause immunosupression such that fish are unable to prevent extensive fungal invasion (Chinabut *et al*, 1995).

1.5.3 Other pathological agents associated with EUS

Despite overwhelming evidence that fungi are the cause of the granulomatous response and the progression of lesions in EUS-affected fish, some workers have attached a causative role in these developments to nocardioform bacteria (Chakrabarty and Dastidar, 1991); Dastidar and Chakrabarty, 1992) or myxosporean parasites (Tika Ram *et al*, 1994; Sanaullah *et al*, 1996). Although there is no reason to doubt the diagnosis of these organisms in some EUS-affected fish, they can not be considered to be the cause of typical EUS lesions.

A variety of other bacteria, viruses and parasites have been isolated from EUS-affected fish, some of which may have a role in the progression of EUS, either by facilitating infection by *A. invaderis*, or as secondary opportunists.

In areas where acid water does not occur, and therefore the acid-induced dermatitis described by Callinan *et al* (1996) can not effect EUS, some microbiological, or other environmental agent(s), is probably required to facilitate infection by *A. invaderis*. Rhabdoviruses isolated from EUS-affected fish were originally considered candidate primary pathogens (Frerichs *et al*, 1986), but were subsequently found to be incapable of reproducing ulcers in snakeheads (Frerichs *et al*, 1993), and comprised of at least two viral species (Kasornchandra *et al*, 1992; Lilley and Frerichs, 1994). However, Kanchanakhan (1996b) showed that rhabdovirus infection resulted in skin damage in juvenile snakeheads, and may be an important precursor to fungal invasion. He reported 100% (17/17) infection of fish injected with a rhabdovirus prior to immersion in *A. invaderis* zoospores, whereas only 3/18 fish exposed to spores alone, developed EUS. A birnavirus isolated during the 1983-4 outbreak in Thailand was also shown to be capable of inducing sub-lethal dermatitis in snakeheads (Saitanu *et al*, 1986)

Similarly, Subasinghe (1993) showed that EUS transmission to naive snakehead fry, by feeding with infected material or exposure to affected water, was enhanced by concurrent heavy infestation with *Trichodina*. Skin parasite infestations have been seen to precede EUS outbreaks on several occasions, but the species of parasite varies between localities; *Epistylis* was observed on fish prior to the 1982-3 outbreak in Thailand (Tonguthai, 1985) and significant levels of *Lernea* was reported in the 1996 outbreak in Pakistan (NACA, 1997).

Bacteria, and *Aeromonas hydrophila* in particular, have been consistently isolated from EUS ulcers (Llobrera and Gacutan, 1987; Pal and Pradhan, 1990), some strains with particularly virulent (Torres *et al*, 1990; Suthi, 1991; Karunasagar *et al*, 1995) or cytotoxic (Yadav *et al*, 1992) capabilities. Although some bacteria certainly contribute to the development of necrotic ulcers (Lio-Po *et al*, 1992), they are generally considered to be secondary opportunists. This conclusion is supported by studies in Bangladesh showing that a large variety of bacteria, including aeromonads, are part of the natural flora in fish farms water (Karim and Chowdhury, 1995) and the mucus and kidneys of healthy, cultured *Labeo rohita* (Baqui and Chowdhury, 1995).

Initial research focus on RSD was similarly directed towards bacterial pathogens, and *Vibrio anguillarum* was originally considered significant (Burke and Rodgers, 1981). A further

assessment of the bacteria and parasites involved in RSD dismissed them as primary agents (Callinan and Keep, 1989), and subsequent research focused on mycological and environmental aspects of the disease.

1.6 Other similar diseases or mycoses of aquatic animals

Mycotic granulomatosis (MG) and red spot disease (RSD), and the extreme similarity of these diseases to EUS, have already been described above in some detail. Other mycotic fish diseases caused by agents of varying or unknown relationship to *Aphanomyces invaderis*, or ulcerative diseases of undetermined aetiology are reviewed here.

1.6.1 Ulcerative mycosis (UM)

Noga (1994) postulated that ulcerative mycosis (UM) of coastal fish populations of the western Atlantic may be part of the same syndrome as EUS, given the similarities in clinico-pathological features of both diseases (Table 1.2) and that predominantly *Aphanomyces* fungi are recovered from UM-diseased fish (Dykstra *et al*, 1986). However, fish challenged with these *Aphanomyces* isolates have failed to reproduce the disease whereas fish developed UM when lesion material is used as an inoculum, suggesting that some other, unidentified agent is required for infection (Noga 1993).

UM was first observed in April 1984, in menhaden (*Brevoortia tyrannus*) in the Pamlico River, North Carolina and in November of that year a massive kill was reported (Noga and Dykstra, 1986). Epidemics of similar diseases were later recognised in estuaries along the eastern seaboard of USA from Connecticut (Noga, 1993) to Florida (McGarey *et al*, 1990), although it is uncertain whether these were first occurrences and represented a spread in the disease. Several fish species were shown to contract UM-like diseases in Pamlico river (Noga *et al*, 1991) but the incidences of these was markedly lower than in menhaden (Levine *et al*, 1990). In menhaden, a larger proportion of age-0 fish were shown to be affected than age-1 fish (Levine *et al*, 1991). Levine *et al* (1991) also provided evidence that specific regions of low salinity within the Tar-Pamlico estuary harboured higher levels of diseased fish, and Noga (1993) observed that the most damaging outbreaks in the Pamlico River coincided with years of unusually high rainfall and reduced salinity (1984 and 1989). Outbreaks have continued to occur, some of which have resulted in mortalities of millions of fish (Noga, 1993) and infection rates of menhaden up to 100% (Levine *et al*, 1991).

Noga *et al* (1996) showed that sublethal exposure to toxins produced by a recently identified "phantom" dinoflagellate, also responsible for high mortalities in the Pamlico river (Burkholder *et al*, 1992), can result in dermatitis and subsequent ulcerative mycosis infection.

Several species of dead and moribund ulcerated fish, predominantly catfish (*Bagre marinus*) and mullet (*Mugil* spp), have also been observed in and around the estuaries feeding the Baia de Sepetiba in SE Brazil (P.C. Scott, pers. comm.) but no post mortem was carried out, and it is unknown if a mycosis was involved.

1.6.2 Other ulcerative diseases in the Asia-Pacific region

There are many accounts of ulcerative disease outbreaks in fish in the region, some of which, although not confirmed as EUS, tend to fit well with the known characteristics of the disease and have been generally associated with EUS. These were discussed in Section 1.2.3. Two other accounts which show gross clinical signs similar to EUS are included here.

Munday (1985) reported the presence of severely ulcerated red cod (*Pseudophycis barbatus*) in the River Tamar near Launceston, Tasmania in November 1980 and 1981. Although a variety of bacteria and parasites were identified from the fish, pollution was considered the main cause of the disease. Munday (pers. comm.) now believes ulcer disease was the same syndrome as EUS

although it occurred in higher salinity water, but he adds that now Launceston's sewerage system has been improved, the disease is no longer reported.

Schuurkamp and Hortle (1987) described an ulcerative disease outbreak in freshwater herring (*Nematalosa flyensis*) and 3 other river species in the backwaters of Western Province, Papua New Guinea in 1985 and 1986. Only bacteriological examination was carried out and a number of bacteria were isolated.

1.6.3 Other aphanomycoses of fish

The first reported *Aphanomyces* infection of fish, where the fungus was considered to be the primary disease agent, occurred in November 1942 in several aquarium tanks in the University of Illinois (Shanor and Saslow, 1944). Guppies (*Lebistes reticulatus*) and other ornamental species first showed signs of an abnormal dorsal hump, and within a few days hyphae began to protrude from the dorsal musculature. All infected fish died soon after lesions developed. A sterile *Aphanomyces* fungus was the only agent isolated.

An aphanomycosis was reported in Uttar Pradesh, India in 1974, causing mortalities of about 90% in culture ponds of Indian major carp, *Cirrhinus mrigala* (Srivastava, 1979). A sterile *Aphanomyces* species was isolated from hyphae protruding from the body of affected fish, and found to be capable of colonising experimentally injured areas of fish, eventually killing them. Although there was no indication that the fungus was invasive within the muscle, and the infection appears secondary to the physical injury of the fish, the disease fungus is significant as it was given the name *Aphanomyces pisci*. However, cultures are now apparently unavailable from either the IMI culture collection where it was lodged, or from the author.

The two accounts above give some information about the growth characteristics of the isolates concerned, and are discussed in comparison to *A. invaderis* in Section 3.3.4.

There are several other reports of superficial infections of fish by a large number of saprolegniacean fungi (see Section 1.6.7). Reported incidences of *Aphanomyces laevis* acting as a wound parasite on fish are given by Vishniac and Nigrelli (1957), Scott and O'Bier (1962), Scott (1964), Srivastava (1980a), Ogbonna and Alabi (1991) and Khulbe *et al* (1995). Similar accounts involving unspeciated *Aphanomyces* are given by Scott and O'Bier (1962), Scott (1964), Willoughby (1970), Pickering and Willoughby (1977), and Srivastava (1980a). Finally there is one report of *Aphanomyces stellatus* affecting eels (Hoshina *et al,* 1960, cited by Neish and Hughes, 1980).

1.6.4 Crayfish plague

Aphanomyces astaci has been the cause of catastrophic mortalities in European crayfish populations since it was first reported in 1860 in Italy (Ninni, 1865, cited by Alderman and Polglase, 1988). Early workers claimed parasites were the cause (Harz, 1881; as described by Southgate, 1983), and when *A. astaci* was identified by Schikora (1903; 1906, cited by Alderman and Polglase, 1988), it was dismissed by other workers as being a saprophyte, probably *Saprolegnia* or *Achlya*. A bacterial aetiology was also described, and it was not until Nybelin's work in 1934 (cited by Alderman *et al,* 1987) that *A. astaci* was accepted as the primary cause. *A. astaci* is now known to be a highly infectious agent that results in 100% mortality of susceptible hosts (Alderman *et al,* 1986); it releases a number of enzymes important in pathogenesis (Söderhäll, 1978); is capable of invading the musculature of infected European crayfish (Southgate, 1983); and has been reported to be similarly slow-growing and fastidious in culture to *A. invaderis* (Unestam, 1965); Unestam and Gleason, 1968). Most of this more recent work on crayfish plague has been carried out in Sweden (where it is known as kräftpesten), which has been transformed from one of the largest exporters of crayfish (Marren, 1986) to the world's largest importer of crayfish (Swahn, 1994) since the introduction of the disease in 1908. The first confirmed occurrence of crayfish plague in the UK was as recent as 1981 (Alderman *et al,* 1984).

UK isolates of *A. astaci* were included in most of the comparative studies of *A. invaderis* described here, and for the first time characterised by comparing polymorphic DNA fragments with those of established *A. astaci* groups from Sweden (Section 3.10).

1.6.5 Aphanomyces in other aquatic animals

In November 1964, a sterile *Aphanomyces* isolate was isolated from the skin of a freshwater dolphin (*Inia geoffrensis*) that had developed several dermal lesions having been flown from the upper Amazon in Peru to San Francisco (Fowles, 1976). Reported growth characteristics of this isolate are compared to *A. invaderis* in Section 3.3.4.

Valairatana and Willoughby (1994) also reported *Aphanomyces* growing as a wound parasite on the surface of a soft shell turtle (*Trionyx cartilogineus*) in Thailand. This isolate (T1SA) is included among a number of "saprophytic" *Aphanomyces* studied here.

1.6.6 Invasive saprolegniasis

The differential ability of particular strains within the *Saprolegnia parasitica-diclina* complex to colonise fish has been reported ever since the taxon *S. parasitica* was established (Coker, 1923; Willoughby, 1978; Hatai *et al*, 1990; Hatai and Hoshiai, 1993; Beakes *et al*, 1994). However, "saprolegniasis" is typically an infection of the epidermis or dermis of fish (see next section) and bears little relationship to the fungal involvement in EUS. In some cases, though, hyphae of *Saprolegnia* have been reported extending into the underlying musculature and internal organs.

Agersberg (1933, cited by Southgate, 1983), Bootsma (1973), Nolard-Tintigner (1973), Hatai and Egusa (1977), Bruno and Stamps (1987), Hatai and Hoshiai (1992) and Wada *et al* (1993) all described the penetration of musculature and/or internal organs of small fish due to natural or experimental challenge by *Saprolegnia*. Davis and Lazar (1940, cited by Southgate, 1983) described a similar mycosis of rainbow trout fry and named the causative fungus *Saprolegnia invaderis*. However Seymour (1970) later excluded this taxon as he considered it a representative of *Saprolegnia ferax*.

Neish (1977), demonstrated that *Saprolegnia* hyphae could also penetrate the muscle of adult fish (maturing sockeye salmon, *Oncorhynchus nerka*). Puckeridge *et al* (1989) later described a mycotic dermatitis in bony bream (*Nematalosa erebi*) in South Australia whereby *Saprolegnia* (predominantly *S. parasitica*), considered to be the primary infective agent, penetrated deep in the muscle of advanced lesions. Bly *et al* (1992) reported a similar condition in channel catfish (*Ictalurus punctatus*). In contrast to EUS lesions however, there was no extensive degeneration of muscle tissue or any significant inflammatory response by the fish in all these cases. Whereas the sockeye salmon incidents were considered to be related to increased levels of plasma corticosteroids in the maturing fish, the latter two cases clearly followed rapid reductions in water temperature during cold winter periods, and in this respect, show similarities to EUS. Bly *et al* (1992) described experimental bath challenges with zoospores of *Saprolegnia* sp from local outbreaks in which a rapid decrease in temperature from 22°C to 10°C sufficiently immunosuppressed juvenile catfish to cause 92% infection and 67% mortality within 21 days. They later showed that similar conditions occurred during natural outbreaks (Bly *et al*, 1993). It is interesting to note that unlike the local unspeciated *Saprolegnia*, an isolate of *S. parasitica* (ATCC 42062), also tested here in Section 3.2, failed to infect fish.

One mechanism that may be important in enabling particular strains to penetrate deeper into the tissues of the host may involve specific enzyme activity, as investigated in *Saprolegnia* spp by Peduzzi *et al* (1976) and Rand and Munden (1992). Given the extent of muscle tissue degeneration in EUS-affected fish as a result fungal penetration, specific extracellular enzyme activity may be a particular feature of the fungus or fungi involved (see Section 3.3).

1.6.7 Dermatomycoses

Superficial infections of fish by saprolegniacean fungi are commonly observed and have been reported for almost 250 years. William Arderon's 1748 description of saprolegniasis of roach (*Rutilus rutilus*) may well be the first record of a mycotic disease of any vertebrate (Hughes, 1994).

A great number of Oomycete species have been found to be capable of naturally or experimentally infecting fish. These have been reviewed by Scott (1964), Wolke (1975), Neish and Hughes (1980), Alderman (1982) and Hatai (1989). Srivastava (1980a) listed 40 speciated and unspeciated fungi that have been reported on over 100 different species of fish. Intensive investigations of Oomycetes colonising fish have been carried out in Japan (Hoshina *et al*, 1960, Hatai *et al*, 1977b; 1977c); India (Bhargava *et al,* 1971; Srivastava and Srivastava, 1977a; 1977b; 1978; Srivastava, 1980b; Khulbe, 1980; 1983; 1989; Sati *et al*, 1982; Singhal *et al*, 1987; Khulbe *et al,* 1995); Egypt (El-Sharouny and Badran, 1995); Nigeria (Ogbonna and Alabi, 1991); the UK (Willoughby, 1970; Willoughby, 1997a) and the USA (Tiffney, 1939a; 1939b, cited by Hughes, 1994; Scott and Warren, 1964; Scott and O'Bier, 1962).

In their study of fungi growing on perch (*Perca fluviatilis*) in Lake Windermere, Pickering and Willoughby (1977) identified 4 different genera of fungal colonists on a single lesion and described a apparent succession in species according to the type of lesion. Despite this intricate fungal involvement, they did not attribute the primary cause of the lesion to any of them. Ulcerative dermal necrosis (UDN) is a disease of maturing wild salmonids in which fish can develop heavy *Saprolegnia* infections, but even in these lesions, fungal hyphae generally do not penetrate further than the dermis (Roberts *et al*, 1970).

These latter cases are clearly in contrast to the mycotic involvement in EUS. That so many Oomycete fungi have the ability to colonise fish, but relatively few species have been reported penetrating the internal tissues of the host, indicates that very specific adaptations of the fungus and/or specific conditions within the fish are required for fungi to achieve this. The fungus or fungi involved in MG and RSD are considered the only biological causative agent(s) required for infection, and therefore warrant intensive investigation to understand the processes involved in the pathogenesis of these highly damaging diseases. Whether other aetiologies are involved in EUS and UM or not, the fungi involved again show extraordinary characteristics and are clearly central to the virulence of the disease.

2. PROJECT PURPOSE

2.1 As described in the project memorandum:
To provide an understanding of the general biology and ecology of the fungal pathogen of EUS and resolve questions over its relationship with strains from other similar diseases.

2.2 Following the retirement of Dr L.G. Willoughby, the ecological element of the studies was terminated, and Dr K.D.Thompson instead initiated work to examine the immune mechanisms of fish against infection by *A. invaderis*. An account of this work is given in Section 3.8.

3. RESEARCH ACTIVITIES, OUTPUTS AND CONTRIBUTION OF OUTPUTS

3.1 GENERAL INTRODUCTION AND METHODS

3.1.1 Introduction

3.1.1.1 The taxonomical position of *Aphanomyces*

The genus *Aphanomyces* was established by deBary in 1860, its name meaning "imperceptible fungus", apparently due to the delicate appearance of the hyphae on the body of a decaying insect in the water. In Scott's (1961) "Monograph of the Genus *Aphanomyces*", 26 species were listed as well as 2 isolates of doubtful affinities, comprising Shanor and Saslov's (1944) *Aphanomyces* sp isolated from guppies and Matthew's (1935) *Aphanomyces* sp growing on frog's eggs. *Aphanomyces* species can be categorised into those with smooth or roughened oogonial walls (Cutter, 1941). Scott ~~{Scott 1961 #10270}~~(1961) erected three subgenera on this basis: *Aphanomyces* (with smooth oogonial walls, including *A. laevis*), *Axyromyces* (with roughened walls and no ornamentation) and *Asperomyces* (with roughened and ornamented walls, including *A. astaci*). Dick (1973) later questioned the validity of this use of subgenera. However, as oogonia have not been demonstrated in *A. invaderis* or *A. piscicida,* no attempt can be made to allocate subgenera anyway. Early descriptions of oogonia in *A. astaci* are now also considered questionable ~~{Alderman & Polglase 1988 #9410}~~(Alderman and Polglase, 1988).

Aphanomyces is contained within the family Saprolegniaceae: aseptate, normally eucarpic fungi typically demonstrating two zoospore forms (diplanetism) (Dick, 1973). The Saprolegniaceae are the largest family within the order Saprolegniales (class Oomycetes), which is normally characterised by the production, from within a zoosporangium, of zoospores with two flagellae, differing in length and type ~~{Cavalier-Smith 1987 #11980}~~(heterokont). The Saprolegniales also contain cellulose and ß-1,3-1,6-glucans and lack chitin within their cell walls, although chitin has been demonstrated in some other members of the Oomycetes ~~{Bartnicki-Garcia 1987 #3640}~~(Bartnicki-Garcia, 1987).

3.1.1.2 The life-cycle of *Aphanomyces*

A generalised life-cycle of *Aphanomyces* is shown in Fig 3.1.1.

Saprolegniacean genera are distinguished primarily by asexual characters, particularly zoosporangial formation and zoospore release. The zoosporangia of *Aphanomyces* spp are typically no wider than the hyphae. A single row of primary zoospores are formed within a zoosporangium and released from an apical tip at which they immediately encyst and form achlyoid clusters (Fig 3.1.1B). Diplanetism is therefore at a reduced state in *Aphanomyces* spp given that the primary zoospore is not fully released from the sporangium. The only asexual, free-swimming stage of *Aphanomyces* spp is the secondary zoospore (C) which is discharged from the encysted primary zoospores (sometimes referred to as cystospores). The secondary zoospore remains motile for a period depending on environmental conditions and location of a host or substratum. Typically the zoospore encysts and germinates to produce new hyphae (D-E). However, some *Aphanomyces* spp do show a phenomenon known as polyplanetism (Cerenius *et al,* 1984) whereby several tertiary generations of zoospores may be produced from the secondary cyst. This repeated emergence of zoospores was considered to be an adaptation to parasitism among particular *Aphanomyces* species ~~{Cerenius & Söderhäll 1985 #11320}~~(Cerenius *et al,* 1985).

The morphology of sexual reproductive structures traditionally define the species status of a saprolegniacean fungus. An example of the female oogonia and male antheridia are given in Fig 3.1.1F. However, it has proved notoriously difficult to demonstrate sexual structures in many saprolegniacean strains. This phenomenon appears to be more prevalent among the more pathogenic species or strains ~~{Alderman & Polglase 1988 #9410}~~(Alderman and Polglase, 1988).

The reports of fish aphanomycoses listed in Section 1.6, for example, invariably describe sterile isolates. Early descriptions of oogonia in the crayfish plague fungus *Aphanomyces astaci* (Rennerfelt, 1936, as cited by Scott, 1961) have not been adequately substantiated (Unestam, 1969a; Rodgers, 1988) and are believed by some workers to be mistaken ~~{Alderman & Polglase 1988 #9410}~~(Alderman and Polglase, 1988). The common fish parasite, *Saprolegnia parasitica* was originally described as sexually sterile ~~{Coker 1923 #10090}~~(Coker, 1923) and although oogonia have now been identified from this species ~~{Kanouse 1932 #11940}~~(Kanouse, 1932), they remain more rarely observed than in other *Saprolegnia* spp (Wood, 1988). The EUS, MG, RSD and UM pathogenic isolates now provide further examples of important saprolegniacean fungi that cannot be speciated by traditional means due to their sterile nature. The ATCC has recently expanded its computer coding system for the identification of microbial strains to include saprolegniacean fungi in an attempt to address the problem ~~{Jong, Davis, et al. 1991 #10420}~~(Jong *et al*, 1991).

3.1.1.3 Descriptions of *A. invaderis*, *A. piscicida* and RSD-associated *Aphanomyces*

As mentioned above, oogonia have not been observed in any of the three *Aphanomyces* strains associated with EUS, MG and RSD, which can not therefore be speciated according to traditional mycological characteristics. However, this is not a formal requirement, and Willoughby *et al* (1995) and Hatai (1980) have published descriptions of the EUS and MG fungi as *A. invaderis* and *A. piscicida* respectively. A summary of these descriptions, and that of the RSD-associated *Aphanomyces* by Fraser *et al* (1992), is given in Table 3.1.1. It should be noted that Hatai's (1980) description of *A. piscicida* was, however, not validly published according to the International Code of Botanical Nomenclature, for a number of reasons, but principally because no Latin diagnosis was given (Article 36).

3.1.1.4 A few notes on terminology

It should be noted here that *Aphanomyces,* and Oomycetes in general, are no longer phylogenetically regarded as true fungi, but rather fungal-like protists. They are now classed alongside diatoms, brown algae and xanthophytes within the phylum Heterokonta as part of the third botanical kingdom, the Chromista. They are sometimes called pseudofungi, either as a general term or a formal taxon ~~{Cavalier-Smith 1987 #11980}~~(Cavalier-Smith, 1987). Despite these fundamental changes in the taxonomy of this group, the Oomycetes are still most commonly referred to as fungi, and Dick (1997) argues that physiologically they are fungi, and should be called as such. Therefore, for the purpose of this thesis, the organisms studied here shall remain "fungi".

It is clear from section 1.6 that the saprolegniaceans have varying abilities to parasitise fish. *Saprolegnia* sp, for example, may or may not be a primary pathogen and may or may not penetrate internal fish tissues (compare Puckeridge *et al*, 1989 with Roberts *et al,* 1974). In this study, a clear distinction made is between pathogens and saprophytes with regards to EUS. In Section 3.2, pathogenicity is determined by the ability of zoospores to germinate and grow invasively in snakehead muscle tissue, eventually producing EUS-like lesions. As the zoospores are introduced directly into the muscle in these challenge studies, even "pathogens" are not assumed to be primary pathogens; although this possibility is discussed, with particular reference to *A. astaci*, a highly adapted, primary (possibly obligate) pathogen of crayfish. Saprophytic isolates have been obtained from water samples or from the surface of EUS-affected fish, or other diseased aquatic animals, and cannot grow invasively in snakehead muscle. These isolates probably derive nutrients from dead plant and animal material (necrotrophs) and live facultatively on dead tissue in living fish (perthotrophs). Their ability to derive nutrients parasitically from living fish tissue (biotrophs) or actually kill any tissue at all is undetermined. However, any involvement these isolates have in EUS can be considered opportunistic, and secondary to the development of a lesion.

The name *Aphanomyces invaderis* is at this time still only associated with EUS outbreaks. Therefore in the sections describing experiments it is often referred to as the EUS pathogen, in order to distinguish it from the MG pathogen (*"A. piscicida"*) and the RSD pathogen (*Aphanomyces* sp). A discussion of the species *A. invaderis,* and which isolates should be included in the taxon is given in the final section.

3.1.2 Activities

3.1.2.1 Fungal isolates

Tables 3.1.2 and 3.1.3 list the origins of the fungal isolates obtained for the purposes of this study, and currently being maintained at the Mycology lab, Institute of Aquaculture, University of Stirling. Included here are: EUS, RSD or MG *Aphanomyces* pathogens; *Aphanomyces* species associated with other diseases of aquatic animals (principally UM and crayfish plague); *Aphanomyces, Achlya* and *Saprolegnia* isolates obtained from the surface of EUS-affected fish or from EUS-affected water bodies; and the peronosporalean fungus *Phytophthora cinnamomi* obtained for further comparative purposes. Appendix 1 lists the particular fungal isolates used in each of the experiments described. Figure 3.1.2 maps the origin of the EUS, RSD and MG isolates.

3.1.2.2 Isolation of fungi

Fungi were isolated using a variety of techniques. The isolation procedures used for each of the *Aphanomyces invaderis* isolates TA1, RF6 and RF8 are detailed in Roberts *et al* (1993). BH, BR and BS isolations are described in Willoughby and Roberts (1994a).

Isolations of *A. invaderis* by the present author were made as follows. Where multiple samples were available, a medium-sized dermal ulcer overlying muscle tissue at least 1 cm thick was selected. Small fish were cut it in two using a sterile scalpel, slicing through the fish at the edge of the lesion. A hot scalpel was used to sterilise the exposed surface of the muscle. A small-bladed sterile scalpel was used to cut out a circular block of muscle from beneath the lesion. Muscle samples were about 0.5 cm^3 and not visibly discoloured by the overlying ulcer. With larger fish, the whole lesion was removed, the surface of the remaining tissue was sterilised, and a block of muscle was cut from the exposed area. The muscle sample therefore contained the invasive pathogenic fungus, but not any saprophytic fungi associated with the surface of the fish, and minimal bacterial contamination. The muscle block was then placed in a sterile Petri dish of GP-PenOx broth (see Appendix 2a). The Petri dish was left until fungi could be seen growing out from the muscle block (about 10 hours at 25°C). These hyphal strands were cut out using aseptic technique and transferred to a Petri dish of GP-PenStrep agar (Appendix 2a). In this way, bacterial growth was inhibited and the fungus was able to grow free of bacterial contamination. An agar block containing fungal hyphae from the edge of the colony was then placed upside down on a Petri dish of GP agar and repeatedly subcultured until an axenic stock was obtained.

Aphanomyces isolates from Australia, Indonesia and the Philippines were all isolated from ulcerated fish on Czapek Dox agar (CDA) with penicillin G (100 units/ml) and oxolinic acid (100 μg/ml). Emerging hyphal tips were repeatedly transferred to fresh plates of CDA or glucose-yeast (GY) agar (5g/l glucose, 2.5g/l yeast, 15g/l agar) with the same antibiotics until bacterial-free cultures were obtained (Fraser *et al*, 1992; Callinan, pers. comm.). MG-isolate NJM9030 was probably obtained using FME agar (10% fish-meat extract, 1.5% agar) as described by Hatai *et al* (1977) and NJM9201 was isolated on GY agar (but with 10g/l glucose) with penicillin (500 units/ml) and streptomycin (500 μg/ml)(Hatai *et al,* 1994).

Isolations from UM-affected fish were reportedly made either on cornmeal agar containing penicillin (100 units/ml) and streptomycin (100 μg/ml) (Noga and Dykstra, 1986) or on the same media supplemented with 1% yeast extract and 2% D-glucose (Dykstra *et al,* 1986; Dykstra *et al,* 1989). UK *Aphanomyces astaci* isolates were obtained on RGY agar (1g yeast extract, 5g D-glucose 12g agar in 1l river water) with oxolinic acid (10 μg/ml) and penicillin G (6 μg/ml) as

described by Alderman and Polglase (1986). Swedish *A. astaci* isolate J1 was obtained by the method of Unestam (1965) using glucose-blood agar (0.5% glucose, 5% crayfish blood or 20% horse serum, 1.5% agar). All other *A. astaci* were isolated as detailed by Cerenius *et al* (1988) using PG-1 agar (Appendix 2a).

The saprophytic isolates in Table 3.1.2 were easily obtained by techniques described in Roberts *et al* 1993. Previous published information has been given on TF5, TF33, TF41 (Roberts *et al*, 1993), TF54 (Willoughby and Roberts, 1994), T1SA (=SH1: Valairatana and Willoughby, 1994), W2BAC (Willoughby, 1997b), S. aust (=795: Wood, 1988), E3 (Wood, 1988; Burr, 1991), and TP41 (Willoughby *et al*, 1983; Cross and Willoughby, 1989; Bly *et al*, 1992; Beakes *et al*, 1994; Bullis *et al*, 1996; Smith *et al*, 1994).

3.1.2.3 Maintenance of fungi

Significant difficulties have been encountered in the maintenance of the slow-growing EUS, RSD and MG isolates due to the rapid staling of the growth media by these cultures ~~{Willoughby & Chinabut 1996 #12110}~~(Willoughby and Chinabut, 1996). Stocks were initially maintained in flasks containing 200 ml GP broth at 10°C for 6 weeks before subculturing. Given the large amount of incubator space required to maintain all the isolates, cultures were later kept at room temperature on GPY slopes filled with sterile light paraffin oil (BDH) according to Smith and Onions (1994). Given that cultures of the reputedly fastidious *A. astaci* ~~{Unestam 1965 #7540}~~(Unestam, 1965) can now be maintained for longer periods of time on the buffered medium PG-1 (Söderhäll and Cerenius, 1987), a short comparison of these media was undertaken (see Section 3.3.2.4). As a result, all stocks are now maintained on PG-1 slopes under oil at room temperature. Using this technique, cultures of *A. invaderis* have been kept for up to a year before subculturing.

Experimental cultures were maintained at 10°C on GPY agar Petri dishes for a maximum of 7 days. Inocula were taken using a 4mm cork borer.

3.1.2.4 Water quality analysis of water used for preparing APW

Autoclaved, filtered pond water ~~{Cerenius & Söderhäll 1980 #7240}~~(APW) is used to induce sporulation of saprolegniacean fungi as described in section 3.1.2.5 below. Preparation of APW is described in Appendix 2a.

Water used for APW should first be checked for its ability to support saprolegniacean fungi. Studies undertaken in Stirling or Newcastle used water from Airthrey Loch, a small eutrophic loch on Stirling University campus. This was tested in July 1993 for fungal spores using a GP-PenStrep assay technique ~~{Valairatana & Willoughby 1993 #12100}~~(Valairatana and Willoughby, 1993) and a hemp seed baiting technique (Willoughby, 1994). Very high levels of *Saprolegnia* spp and *Achlya* spp were recorded at that time and the water was considered suitable. Subsequent water quality analysis of Airthrey Loch throughout 1994 by Kelly and Smith (1996) recorded high pH levels (up to pH 9.54) over the spring and summer (see Appendix 2b). This was probably in response to macrophyte and algal respiration, and was shown to result in high autochthonous production of phosphorus. These fluctuations in water quality conditions (and pH in particular) may have affected the suitability of the water for *Aphanomyces* sporulation and could have resulted in low yields of zoospores for experiments conducted at that time. Marked seasonal variations in fungal yields associated with pH changes have been widely reported from natural water bodies in both temperate ~~{Waterhouse 1942 #11740}{Roberts 1963 #10530}~~(Waterhouse, 1942; Roberts, 1963) and tropical (Alabi, 1971) localities.

Water used for APW in studies in Bangkok was taken from the fish pond at the National Inland Fisheries Institute ~~{Boonsom & Jokngoen 1992 #330}~~(NIFI). Spore counts conducted over an 8 month period in 1992 using the GP-PenStrep assay and snakehead fish scale baits ~~{Willoughby & Lilley 1992 #5530}~~(Willoughby and Lilley, 1992) revealed high levels of *Achlya* spp and

Aphanomyces spp, particularly over the winter period, when all experiments were carried out (Lilley, 1992). Some water quality data taken in March 1997 are presented in Appendix 2b; this is slightly after the period when experiments were carried out in previous years.

Zoospore reemergence studies undertaken in Uppsala used stocks of Hålsjön Lake water kept since the 1970's, when water quality measurements were taken (Appendix 2b). This water was shown to be a good sporulating medium in experiments at that time (Unestam, 1969a, 1969b; Svensson and Unestam, 1975), and kept in order to standardise procedures.

3.1.2.5 Zoospore production

The production of suspensions of secondary zoospores was required for several of the experiments described in this thesis. However, it proved very difficult to produce consistent yields of zoospores between isolates, and even different cultures of the same isolate. As mentioned above, this may be partly explained by varying water quality conditions of the APW used. However, some batches of APW that had initially induced sporulation, subsequently failed to do so in some cultures. It is commonly observed that repeated subculture of laboratory stocks of fungi can result in reduced sporulation ~~{Unestam & Svensson 1971 #9710}~~(Unestam and Svensson, 1971) and this may have been the case with some of the cultures here. Therefore techniques for producing zoospores were adapted over the course of the study in an attempt to maximise yields.

(a) Initial experiments used a technique based on the description of Willoughby and Roberts (1994b) to prepare suspensions of secondary zoospores. Briefly, 4 mm agar plugs of mycelium were placed in Petri dishes containing GPY broth (Appendix 2a) and cultured for 4 days at 20°C. The resulting mycelium mats were brought through 5 Petri dishes containing sterile distilled water to remove the nutrient media, and finally left overnight at 20°C in APW. Willoughby and Roberts (1994b) used APW during the whole washing and incubation process, but it was found in this study that sterile distilled water could substitute the APW for the washing process without any apparent effect on sporulation. Cerenius and Söderhäll (1980) did likewise for *A. astaci*, but warned that use of distilled water for the incubation process as well resulted in unattached primary cysts and also a reluctance of these cysts to produce motile zoospores.

(b) More effective washing of mycelium mats was later achieved by sequential tranfer through two 500 ml bottles of sterile distilled water, before leaving the fungus overnight in APW ~~{Beakes & Gay 1980 #3910}~~(Beakes and Gay, 1980).

(c) Towards the end of the project, an isolate of *Phytophthora cinnamomi* was obtained for molecular comparison with *A. invaderis*. Sporulation of this fungus is achieved by initial growth in V8 broth ~~{Zheng & Ko 1996 #7600}~~(Zheng and Ko, 1996). Although *A. invaderis* did not grow well in this medium, it was found to produce generally higher yields of zoospores. This media was therefore adopted using 5 day old mycelium mats in combination with the technique described in (b).

(d) *In vitro* studies undertaken at Uppsala University employed the drop culture technique used in that laboratory (Söderhäll *et al*, 1978). Mycelium squares, approximately 1 mm^2, were used to inoculate drops of 0.25 ml PG-1 broth in sterile Petri dishes. After 3 days growth at 22°C, the medium was replaced with a similar drop of APW, which was then changed for new APW every 30 min for 3 hours. The cultures were then incubated overnight at 22°C.

(e) Large volumes of zoospore were produced according to Cerenius *et al* (1988) for use in the crayfish immersion challenges described in Section 3.2.2.7. This technique involved homogenising a large number of mycelium mats in APW.

3.1.2.6 Light microscopy and photography

Routine light microscopy at the Institute of Aquaculture, Stirling was done using an Olympus BX40F microscope. Petri dishes containing zoospore suspensions were generally observed directly under the microscope, with the mechanical stage removed, at x10 with phase contrast optics.

3.1.3 Outputs and contribution of outputs

The methods described in this section were used in the activities described in subsequent sections. The contribution of the resulting outputs will be discussed in the relevant section.

Figure 3.1.1

Generalised life-cycle of *Aphanomyces* spp

A - Vegetative hyphae
B - Primary zoosores forming within a hyphal-sized zoosporangium and encysting at the apical tip to form an achlyoid cluster
C - Motile, laterally biflagellated, hererokont secondary zoospores
D - Encysted secondary zoospore
E - Germling
F - Sexual structures: female oogonium and male antheridium

Table 3.1.1 **Summary of published descriptions of EUS-, MG- and RSD-associated *Aphanomyces***

Fungal strain	*Aphanomyces invaderis*	*Aphanomyces piscicida*	RSD-*Aphanomyces*
Reference	**Willoughby *et al* (1995)**	**Hatai (1980)**	**Fraser *et al* (1992)**
Hyphal Ø	In fish tissue: 11.7 - 16.7 μm On GP agar: 8.3 μm	On isolation from fish: 12-36 μm In FME broth: 5-20 μm	Growing from muscle: broad, thick-walled
Mycelium appearance	Wavy as dies in culture or diseased fish		Colonies on CDA: flat, opaque, no aerial hyphae
Radial growth	On GP agar (per 24h): 4 mm @ 24°C 0 mm @ 37°C	From Hatai & Egusa (1978): 0 mm on FME agar @ 5°C 0 mm on FME agar @ 37°C 0 mm on FME agar & 10ppt NaCl	(per 24h): 6.4 mm on GY agar @ 22°C 3.9 mm on CDA @ 22°C 3.2 mm on FME agar @ 22°C 0 mm on GY agar@ 37°C 0 mm on GY agar & 12ppt NaCl
Oogonia	Not observed	Not observed	Not observed
Chlamydospores	"Problematic"	-	-
Zoosporangial width	Equal to mycelium, about 10 μm Ø	equal to mycelium	-
Zoosporangial type	Terminal on mycelia, often complex. Complex sporangia have 4 lateral evacuation tubes (630-930 μm long), 3 tubes (430-540 μm long) or 2 or 1 tube (330-470 μm long)	No basal septum. Simple, but on rare occasions complex with lateral evacuation tubes. Isodiametric or tapering. 15-250 μm long, usually 20-40 μm	Separated by thin septa. Terminal or intercalary. Terminal sporangia may or may not have lateral evacuation tubes. Intercalary sporangia only have lateral evacuation tubes
Zoosporangial renewal	Sympodial branching below empty sporangium	-	-
1° zoospore comments	Spores that fail to escape from sporangium fuse and germinate readily as a large unit, possible organs of dissemination	Spherical or elliptical. Form in a line	Single row connected by thin strand of cytoplasm
1° cystospore clusters	Achlyoid	Achlyoid. Usually >10 cystospores, rarely >20. Occasionally germinates directly	Achlyoid. Usually 30-50 cystospores
1° cystospore Ø	6.7 - 10 μm	5-23 μm, usually 8-9 μm	-
2° zoospore comments	Motile, biflagellate	Motile, biflagellate. Flagella of equal length	Motile, subsperical, biflagellate. Released within 12 hours of sporangial development @ 22°C. Sporulation poor atat 2ppt NaCl
2° cystospore Ø	-	-	-

Table 3.1.2

Details of EUS, RSD and MG *Aphanomyces* isolates used in this study

Species	Isolate	Isolated from	Disease	Date	Location	Worker
Aphanomyces invaderis	TA1	striped snakehead	EUS	Feb 91	Thailand	L.G. Willoughby
A. invaderis	RF6, RF8	striped snakehead	EUS	Jan 92	Suphanburi, Thailand	L.G. Willoughby
A. invaderis	S1PA, S3PA	striped snakehead	EUS	Jan 94	Suphanburi, Thailand	J.H. Lilley
A. invaderis	G2PA	three spot gourami	EUS	Jan 94	Bang Na, Bangkok, Thailand	J.H. Lilley
A. invaderis	PA1, PA3, PA4, PA5, PA7, PA8, PA10	striped snakehead	EUS	Jan 95	Nonthaburi, Thailand	J.H. Lilley
A. invaderis	96PA	snakehead	EUS	Jan 96	Pichit, Thailand	J.H. Lilley
A. invaderis	BH	round-tailed garfish	EUS	Jan 93	Srimanagal, NE Bangladesh	L.G. Willoughby & J.H. Lilley
A. invaderis	BR	rohu	EUS	Jan 93	Parbatipur, NW Bangladesh	L.G. Willoughby & J.H. Lilley
A. invaderis	BS	mud murrel	EUS	Jan 93	Srimanagal, NE Bangladesh	L.G. Willoughby & J.H. Lilley
A. invaderis	36/1P	sand goby	EUS	Jan 93	Bogor, West Java, Indonesia	D. Bastiawan
A. invaderis	30P	striped snakehead	EUS	Nov 91	Laguna Lake, Sth Luzon, Philippines	J.O. Paclibare
A. invaderis	33P	striped snakehead	EUS	Dec 91	Bautista, Central Luzon, Philippines	J.O. Paclibare
A. invaderis	34P	grey mullet	EUS	Dec 91	Buguey, Nth Luzon, Philippines	J.O. Paclibare
A. invaderis	10D	"snakehead"	EUS	~1992	Philippines	J.O. Paclibare
Aphanomyces sp	3P	grey mullet	RSD	Jun 89	Richmond R., NSW, Australia	G.C. Fraser
Aphanomyces sp	4P	yellowfin bream	RSD	Jun 89	Clarence R., NSW, Australia	G.C. Fraser
Aphanomyces sp	10P	sand whiting	RSD	Jun 89	Richmond R., NSW, Australia	G.C. Fraser
Aphanomyces sp	24P	grey mullet	RSD	May 90	Saltwater Ck, Nth Queensland, Australia	A. Thomas
Aphanomyces piscicida	NJM9030	ayu	MG	1990	Schizuoka, Japan	K. Hatai
A. piscicida	NJM9201	dwarf gourami	MG	1992	Tokyo, Japan (imported from Singapore)	K. Hatai

Species Key: three spot gourami, *Trichogaster trichopterus* (Pallas); round-tailed garfish, *Xenentodon cancila* (Hamilton); rohu, *Labeo rohita* (Hamilton); mud murrel, *Channa punctata* (Bloch); sand goby, *Oxyeleotris marmoratus* (Bleeker); grey mullet, *Mugil cephalus* L.; yellowfin bream, *Acanthopagrus australis* ~~{Bishop, Baker, et al. 1982 #290}~~(Owen); sand whiting, *Sillago ciliata* Cuvier; dwarf gourami, *Colisa lalia* (Hamilton-Buchanan)

Table 3.1.3 **Saprophytes and non-Asian pathogens used for comparative purposes in this study**

Species	Isolate	Isolated from	Disease	Date	Location	Worker
Aphanomyces sp	84-1240 (=ATCC 62427), 84-1249, 84-1282	menhaden	UM	May 84	Nth Carolina, USA	M.J. Dykstra
Aphanomyces astaci	FDL457, FDL458	white-clawed crayfish	crayfish plague	NA	R. Arrow, Herefordshire, UK	D.J. Alderman
A. astaci	J1	noble crayfish	crayfish plague	1962	Ämmern, Östergötlands, Sweden	T. Unestam
A. astaci	PL	signal crayfish	crayfish plague	1970	L. Tahoe, USA	K. Söderhäll
A. astaci	KV	signal crayfish	crayfish plague	1978	Sweden (imported from Pitt Lake, Canada)	K. Söderhäll
A. astaci	PC	red swamp crayfish	crayfish plague	~1994	Spain	J. Diéguez-Uribeondo
Aphanomyces sp	TF5	striped snakehead	EUS	Jan 91	Thailand	S. Chinabut
Aphanomyces sp	TF33	swamp eel	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
Aphanomyces sp	TF41	striped snakehead	EUS	Jan 92	Thailand	L.G. Willoughby
Aphanomyces sp	TF54	striped snakehead	EUS	Jan 93	Thailand	L.G. Willoughby
Aphanomyces sp	T1SA	soft shell turtle	fungal infection	Dec 93	Kasetsart, Bangkok, Thailand	W. Valairatana
Aphanomyces sp	A2SA	aquarium tank	-	Jan 94	Kasetsart, Bangkok, Thailand	J.H. Lilley
Aphanomyces sp	F3SA	striped snakehead	fungal infection	Jan 94	Kasetsart, Bangkok, Thailand	J.H. Lilley
Aphanomyces laevis	ASEAN1, ASEAN3	fish pond	-	Jan 94	Kasetsart, Bangkok, Thailand	L.G. Willoughby
Aphanomyces sp	SSA	striped snakehead	EUS	Feb 94	Suphanburi, Thailand	W. Valairatana
Aphanomyces sp	WSA	fish pond	-	Sep 94	Suphanburi, Thailand	W. Valairatana
Aphanomyces sp	SA11	striped snakehead	EUS	Jan 95	A. Bangkuai, Nonthaburi, Thailand	J.H. Lilley
Achlya sp	S2AC	fish pond	-	Jan 94	Kasetsart, Bangkok, Thailand	J.H. Lilley
Achlya diffusa	W2BAC	fish pond	-	Jan 94	Kasetsart, Bangkok, Thailand	L.G. Willoughby
Achlya sp	AC2, AC5, AC10	striped snakehead	EUS	Jan 95	Bangkuai, Nonthaburi, Thailand	J.H. Lilley
Saprolegnia sp	TF20S, TF23, TF25, TF26,	striped snakehead	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
Saprolegnia sp	TF24	three spot gourami	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
Saprolegnia sp	TF29	swamp eel	EUS	Dec 91	Sakhon Nakhon, Thailand	L.G. Willoughby
S. diclina	TF27	striped snakehead	EUS	Dec 91	Udon Thani, Thailand	L.G. Willoughby
S. diclina	TF31	swamp eel	EUS	Dec 91	Sakhon Nakhon, Thailand	L.G. Willoughby
S. australis	*S. aust* (=795) (=ATCC 42060)	skelly	fungal infection	1977	Ullswater	L.G. Willoughby
S. ferax	P32	lake water	-	1957	Lake Windermere, UK	L.G. Willoughby
S. diclina	E3 (=ATCC 36144)	lake water	-	1960	Lake Windermere, UK	L.G. Willoughby
S. parasitica	TP41 (=ATCC 42062)	brown trout	fungal infection	1970	Hatchery, Windermere, UK	L.G. Willoughby
Phytophthora cinnamomi	IMI 335418	Macadamia	plant pathogen	1989	Australia	P.M. Wood

Species Key: *Aphanomyces laevis* deBary; *Achlya diffusa* Harvey; *Saprolegnia ferax* (Gruithuisen); menhaden, *Brevoortia tyrannus* (Latrobe); white-clawed crayfish, *Austropotamobius pallipes* Lereboullet; noble crayfish, *Astacus astacus* L.; signal crayfish, *Pacifastacus leniusculus* Dana; red swamp (Louisiana) crayfish, *Procambarus clarkii* Girard; swamp eel, *Fluta alba* (Zview); soft shell turtle, *Trionyx cartilagineus* (Boddaert); skelly, *Coregonus lavaretus* L.; brown trout, *Salmo trutta* L.

Figure 3.2.2

Origin of EUS, MG and RSD isolates used in this study

3.2 PATHOGENICITY STUDIES

Some of the work described in Sections 3.2 and 3.3 are published in Journal of Fish Diseases (20, 135-144). The paper, entitled "Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi" is attached as Appendix 4.

3.2.1 Introduction

A. invaderis has already been shown to be capable of reproducing EUS lesions when mycelium of isolates RF6 and RF8 (Roberts *et al*, 1993) or zoospores of RF6 (Chinabut *et al*, 1995) are injected into the muscle of snakehead fish (*Channa striata*). However, as described in the background Section 1.5.1, a number of *Saprolegnia*, *Achlya* and *Aphanomyces* species have been associated with EUS affected fish, and given that normally perthotrophic fungi can invade muscle tissue in some circumstances (Section 1.6.6), there was a need to conduct challenge studies to compare the pathogenic capabilities of several *A. invaderis* isolates with a number of other fungi isolated from EUS lesions, or EUS affected waters ~~{Noga 1994 #10000}~~(Noga, 1994).

The manifest similarities of MG, RSD and UM to EUS have led to recommendations by workers for the implementation of direct comparative studies to establish the relationship between the fungal aetiologies of each case (Wada *et al*, 1994; Callinan, 1994b; and Noga, 1993). Isolates of other fungi involved in invasive mycoses of aquatic animals (ie crayfish plague, Section 1.6.4; and saprolegniasis of fish, Section 1.6.6) were also obtained for comparative studies described here.

A tested challenge model based on that described by Chinabut *et al* (1995) was adopted to address the need for comparative pathogenicity studies as described above. In addition to these studies, preliminary immersion challenges were conducted using artificially injured fish and cold-shocked fish to evaluate the ability of *A. invaderis* zoospores to initiate an infection. Finally, bath challenges using plague-susceptible noble crayfish (*Asatcus astacus*) are described here.

3.2.2 Activities

3.2.2.1 Fish and tanks

Fish challenge experiments were carried out at the Aquatic Animal Health Research Institute (AAHRI), Bangkok. Snakehead (*Channa striata*) fingerlings were obtained locally and held at AAHRI for at least one month before experiments began. The average length and size range of the fish over the trial period was 15.5 ± 2.5 cm.

Experimental tanks were kept in a 22°C constant temperature room. The testing of each fungal isolate was treated as a separate experiment. For each experiment, three fish were introduced to a tank containing 12 litres of water two days before challenge to allow for temperature equilibration. Following challenge, fish were fed once daily with commercial snakehead feed pellets. Snakeheads are air-breathers and no aeration or water exchange was required during the seven-day long experiments.

3.2.2.2 Preparation and assay of inocula for injection challenge studies

Fifty eight fungal isolates as listed in Appendix 1 were selected for study. All isolates were grown at 10°C on Petri dishes of GPY agar (see Appendix 2a). Suspensions of motile secondary zoospores were prepared as described in Section 3.1.2.5(a) using 4 mm agar plugs incubated in GPY broth at room temperature (~26°C) for 48 hours. The resulting mycelial mats were washed in APW (Appendix 2a) and left overnight at room temperature, with the exception of *Aphanomyces astaci* and *Saprolegnia australis* strains which required a lower temperature for sporulation. The resulting zoospore suspensions were filtered through two sterile sheets of Whatman 541 filter paper to ensure that only motile secondary zoospores were collected. The suspensions were assayed to estimate zoospore numbers and used for fish challenges. Control

samples were passed through a syringe and checked under a microscope to ensure that zoospores remained motile.

Zoospore concentrations were determined by performing five 1 ml in 20 ml serial dilutions of each inocula in Petri dishes containing APW, and then adding 0.66 ml of concentrated assay broth (Appendix 2a). These were left for four days at room temperature, after which the dish containing fungus, but with the lowest number of colonies, was used to calculate the zoospore concentration in the original inoculum. As it took four days to obtain an accurate zoospore count, no attempt was made to adjust zoospore concentration at the time of the challenge experiments.

3.2.2.3 Injection challenge experiments

Fish were injected intramuscularly with 0.3 ml of the freshly filtered zoospore inoculum into the flank half-way between the anterior parts of the dorsal and pectoral fins. Negative control fish were injected with APW only. All fish were sacrificed seven days post-injection (pi) and the site of inoculation excised and fixed in chilled 10% buffered formalin.

3.2.2.4 Histopathology

The fixed blocks of tissue from each fish were trimmed, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H&E) and Grocott's stain (Appendix 3). Selected slides were also stained using the periodic acid-Schiff (PAS) reaction (Appendix 3).

3.2.2.5 Preliminary immersion challenge experiment (a) scraped fish

All procedures for this experiment, including acclimatisation of fish, were carried out at 22°C. Twenty 4-mm agar plugs of *A. invaderis* isolate S1PA were used to produce 1 l of a filtered zoospore suspension as described above. Six snakeheads, 2 of which had a 0.5 cm^2 section of the skin scraped with a scalpel, were placed in a plastic bag containing the suspension for 5 hours. The fish were then transferred to a tank containing 15 l of tap water and observed daily. An attempt was made to assay the zoospore suspension at the beginning and end of the 5 hour period, but valid counts were not obtained due to excessive contamination of the assay plates. A few motile zoospores were, however, observed directly under a microscope at the end of the 5 hour challenge.

3.2.2.6 Preliminary immersion challenge experiment (b) cold shock treatment

Forty 4-mm agar plugs of *A. invaderis* isolate PA7 were used to produce sporulating mycelium mats as described above. The mats were placed in a tank containing 14 l of APW at 20°C. Ten snakeheads, acclimatised to normal outdoor temperatures of 26-27°C, were transferred directly to the tank and monitored daily. Water samples from the top, middle and bottom strata of the tank were taken for zoospore assays, however, reliable counts were not obtained due to contamination of the assay plates.

3.2.2.7 Crayfish immersion challenge experiment

A. invaderis isolate PA7 was also tested for its ability to infect noble crayfish, *Astacus astacus*, a species highly susceptible to the plague fungus *Aphanomyces astaci*. This experiment was carried out at the Department of Physiological Botany, Uppsala University, Sweden. Large volumes of motile zoospores were produced according to Cerenius *et al* (1988). Duplicate tanks, each containing two crayfish immersed in 3 litres of PA7 zoospore suspension (at 40,000 spores/ml) were kept at 21°C for 24 hours before transfer to 13°C. A control tank contained two crayfish in a suspension of zoospores of the *A. astaci* strain, Hö (at 10,000 spores/ml).

3.2.3 Outputs

3.2.3.1 Assay of inocula and fish challenge experiments

Zoospore concentrations were very variable, but in general lower for the group of EUS, RSD and MG strains listed in Table 1 (averaging 4,000 spores/ml) than for the other fungi (all the other isolates in combination averaged 12,500 spores/ml). It is significant to note that the zoospore

counts of the 1995 *A. invaderis* isolates, obtained only a week before the trials, were on average over four times higher than zoospore counts of the older *A. invaderis* isolates.

A small number of fish died during the course of the experiments, and in these cases attempts were made to reisolate fungus from within the muscle (see Section 3.1.2.2). This proved possible with nine fish injected with slow-growing *Aphanomyces* at times ranging from 2-14 days pi. Reisolates were kept and given the epithet (K) (denoting Koch's postulate isolate) for later comparative studies. Two *Saprolegnia* isolates, TF25 and TP41, were also reisolated at day 6 and day 5 pi respectively, but these fish showed no gross lesions and no signs of fungal growth in histological section.

3.2.3.2 Histopathology

The host tissue response after 7 days pi fell into two main types. In all cases there was evidence of some mild traumatic damage such as sarcoplasmic degeneration, haemorrhage and cellular inflammatory infiltration. However there was a very distinct difference between the response to the EUS, RSD and MG *Aphanomyces* isolates and that to the other *Aphanomyces*, *Saprolegnia* and *Achlya* spp.

With the EUS, RSD and MG *Aphanomyces* isolates, in all cases there was a typical invasive lesion similar to that described by Roberts *et al* (1993). Numerous delicate fungal hyphae were observed at levels extending from the site of injection, right down to the spinous processes and paravertebral ganglia. In some areas these were associated with very extensive floccular degeneration of muscle sarcoplasm with very little or no myophagia. In other areas there was extensive myophagia and also extensive epithelial and granulomatous accumulation around the developing mycelium. This was accompanied by only limited fibroblastic proliferation and recapillarisation (Plates 3.2.1 & 3.2.2).

None of the other strains showed any evidence of frank pathogenicity. The usual reaction was one of varying degrees of myonecrosis in response to physical damage associated with the inoculum at the site of injection. This was often accompanied by organising haemorrhage. In contradistinction to the usually acellular sarcoplasmic degeneration of the pathogenic strains, the myonecrosis was usually associated with active myophagia, and the entire area showed signs of macrophage activity and fibroblastic proliferation and organisation. There was normally no evidence of fungal hyphae present and it was not possible to demonstrate effete spores. In three cases, one *Achlya* (AC2), one *Aphanomyces* (T1SA) and one *Saprolegnia* (TF29), the reaction was similar to the others except that there was evidence of some limited germination of zoospores with vestigial hyphal growth. In all three cases however, it was found that the inoculum had extremely high zoospore counts with 16,000-64,000 spores/ml, some 2-8 times that of the average for all the isolates.

3.2.3.3 Preliminary immersion challenge experiment (a) scraped fish

During the period up to day 10, both unscraped fish and one scraped fish became moribund, showing signs of "tail rot" with sloughing of epidermal tissue and scale loss. Histology revealed no EUS-associated internal pathology. On day 12, a fish with scale loss, but also mild necrotizing dermatitis at the artificially damaged site was sampled for histology. Sections revealed very few hyphal segments in dermis and muscle tissue underlying the lesion. There was also some associated muscle degeneration. On day 14 a fish with similar clinical signs was sampled for fungus isolation. An *Aphanomyces* fungus was obtained from the muscle tissue and was later diagnosed as *A. invaderis* on the basis of growth characteristics. The last fish, which had also shown mild necrotizing dermatitis around day 12-16, was sampled on day 26. By this time the lesion had healed substantially and this was confirmed by histological section. No hyphal segments could be identified.

3.2.3.4 Preliminary immersion challenge experiment (b) cold shock treatment

During the period up to day 15, five fish were sampled for histology showing signs of "tail rot". On day 18 and day 22, an external fungal infection was observed on two fish. Isolation and examination of the fungi revealed only *Achlya* sp in both cases. On day 23, two of the remaining three fish were sampled showing small petechial lesions on the tail. All fish were examined histologically but no signs of invasive hyphae or granulomatous response were observed.

3.2.3.5 Crayfish bath-challenge experiments

Both control crayfish died within 6 days heavily infected with *A. astaci*. All the PA7-challenged crayfish survived showing no significant pathological signs, and the experiment was terminated after two months.

3.2.4 Contribution of Outputs

In these studies, all *A. invaderis,* MG- and RSD- isolates, and no other fungi, were capable of extensively penetrating muscle tissue and reproducing early EUS-type lesions. Therefore, the possibility that multiple fungal opportunists are involved in producing the distinctive fungus-related pathology of EUS lesions is convincingly ruled out. The distinction made by Hughes (1994) between perthotrophs, parasites and pathogens is clearly relevant here. He suspected that most water moulds are potentially good perthotrophs, and as EUS can result in the exposure of large amounts of necrotic tissue on fish, these lesions provide a substrate for several different competing saprolegniaceans. These vigorous saprophytes are the fungi that are usually isolated from such lesions, and have meant in the case of EUS, that the real pathogen has gone unnoticed for some time. In other mycotic diseases, where the pathogen does not result in such distinctive diagnostic signs as EUS, it may be even more difficult to know whether the true pathogen has been isolated or not.

The pathology induced in snakeheads by the EUS, MG and RSD isolates was indistinguishable, thereby providing evidence that these isolates all constitute the same, fish-pathogenic species. Further studies comparing these fungi *in vitro* are given in the subsequent sections.

Although this study confirms reports of similarities in the histopathology of EUS, MG and RSD, there was no evidence of the invasive nature of the UM fungus. Noga (1993) reported that attempts to reproduce UM in menhaden using this isolate had also failed. Dykstra *et al* (1989) stated that their UM *Aphanomyces* isolates could be divided into three categories according to morphological and growth features. The isolate used here (84-1240) belongs to their first category that grew vigorously and produced sexual stages and could therefore be tentatively diagnosed as *Aphanomyces laevis*. This is consistent with *A. laevis* isolates from Thailand used in the present study which also proved non-pathogenic to snakeheads. The third group described by Dykstra *et al* (1989) however, produced "scant mycelium and few zoospores", and if UM has any relationship to EUS, this category may well represent the invasive UM pathogens. A specific request was made to obtain isolates from this group for the present study, but they are no longer being maintained (Dykstra, pers. comm.).

The slow-growing, pathogenic and sexually-sterile nature of the crayfish plague fungus, *A. astaci*, has led to authors drawing comparisons between this fungus and the EUS and RSD pathogens (Roberts *et al,* 1993; Callinan *et al*, 1995). Further, Diéguez-Uribeondo *et al* (1995) have recently isolated a strain of *A. astaci* that is physiologically adapted to warm temperatures. However, this study clearly shows that *A. astaci* does not have the ability to grow in snakehead fish and *A. invaderis* is unable to infect noble crayfish, one of *A. astaci*'s natural hosts.

Saprolegnia parasitica (or *S. diclina* type 1) isolate TP41 (ATCC 42062) also could not be detected growing in snakeheads. Other challenge studies by Bly *et al* (1992) using the same isolate failed to reproduce even a superficial infection in channel catfish. In that case, cold-shocked catfish were shown to succumb to "winter saprolegniasis" when exposed to zoospores of local parasitic *Saprolegnia* isolates, but not to TP41. Wood (1988) and Cross and Willoughby

(1989), however, used TP41 to induce typical saprolegniasis in salmonids, although this does not involve penetration of the musculature.

Southgate (1983) compared staining techniques of fish and crayfish tissues infected by *S. parasitica* and *A. astaci* respectively, and showed that whereas a variety of procedures were applicable for *Saprolegnia* only Grocott's silver impregnation technique was of use for *Aphanomyces*. Of the three histological staining procedures used in this study, Grocott was the only one that clearly demonstrated *Aphanomyces* hyphae in fish tissue. With careful observation, hyphae could be identified with PAS and H&E although the extent of the infection was far more difficult to ascertain. The use of polyclonal antibody staining techniques is discussed in Section 3.7.

Chinabut *et al* (1995) showed that snakeheads succumbed much more readily to infection by *A. invaderis* at 19°C than at 26°C or 31°C. Two snakeheads injected here with PA7 were left 2 weeks pi at the experimental temperature of 22°C, before sampling. Although these fish had not died, they developed further severe myonecrosis and external haemorrhagic lesions consistent with fish kept at 19°C (Chinabut *et al,* 1995), indicating that the disease was still progressing and that the fungus is also potentially lethal at this temperature.

Preliminary injection challenges were found to be not consistently infective unless an assay count of over 50 spore/ml (15 spores/0.3ml inoculum) was achieved; and earlier immersion trials had failed to induce EUS in healthy snakeheads at a number of undetermined zoospore concentrations (unpublished). Unestam and Weiss (1970) reported on reverse situation involving *A. astaci* challenges against *As. astacus.* They calculated an LD_{50} value of 20,000 spores per 0.05ml inoculum in injection trials and an LD_{50} value of only only 30 spores/ml of water in immersion trials. Alderman *et al* (1987) considered that mortalities would have been 100% in these immersion trials, at even lower zoospore concentrations, if the disease was given enough time to develop.

Despite its clear pathogenic qualities, *A. invaderis* can not be confirmed as the primary cause of EUS unless fungal propagules are shown to breach the fishes skin unaided, as in the crayfish challenges with *A. astaci* described above. Preliminary bath challenges described here succeeded in reproducing early stage infections in a few artificially injured snakeheads but not in cold-shocked snakeheads. Other workers have succeeded in inducing ulcers using RSD isolates in bath challenges with abraded fish (Callinan, 1994), and more significantly, with fish exposed sublethally to acid water (Callinan *et al,* 1996). This latter study confirms that RSD isolates can act as primary pathogens, when combined with particular environmental conditions. As described in Section 1.5.2, acidified water conditions do not occur in many EUS outbreaks, and therefore further investigation of the disease process is required.

A tempting explanation of the initial EUS outbreaks in an area, which tend to have an explosive impact on fish populations, is that EUS-naive fish can be susceptible to infection by *A. invaderis* zoospores whereas fish in EUS endemic areas have developed an innate resistance. Cruz-Lacierda and Shariff (1995) were consistently successful in transmitting the disease to EUS-naive snakeheads by simply exposing them to affected fish or water, whereas similar experiments in Thailand, using farmed fish from an area that has suffered repeated annual outbreaks since 1982, were consistently unsuccessful (unpublished). The snakeheads used in those latter studies were from the same source as the fish studied here. Whether any possible innate resistance in these fish is due to selection processes, or the development of a protective immune response as a result of sublethal exposure, is a matter for further speculation. Studies on the immune response of fish to *A. invaderis* are presented in Section 3.8.

Another, widely attested theory, is that some other biological agent may be important in the disease process. Recent trials have induced 100% (20/20) EUS infection in snakeheads injected

with a rhabdovirus and bathed in *A. invaderis* zoospores, whereas vehicle-only injections with zoospore immersion resulted in 35% (7/20) snakeheads infected (Kanchanakhan, 1996b). These experiments were carried out at 20°C, at AAHRI, using fish from the same source as in present trials, and with a rhabdovirus isolated from the same EUS outbreak that G2PA was obtained. Now that isolation techniques have been optimised, Kanchanakhan (1996b) has reported a high recovery rate of rhabdoviruses from EUS outbreaks in Thailand. However, immersion challenges with suspensions of both rhabdovirus and zoospores have yet to be carried out. Parasite-induced infections described by Subasinghe (1993) also require further analysis, as in that case, the burdens of *Trichodina* required were lethal to fish anyway, and EUS affected fish and water, rather than *A. invaderis* alone, were used to transmit the disease.

One further consideration for fish challenge experiments is that the virulence of *A. invaderis* isolates may become reduced after repeated subculturing. Unestam and Svensson (1971) showed that *A. astaci* isolates lost virulence, zoospore motility and zoospore production capacities after a number of years in culture, but that the first two factors were reversible by selection for these characters. In the present trials, cultures of *A. invaderis* isolated the previous week showed considerably more hyphae in histological section than older isolates, but this is thought to be due to the higher zoospores yields obtained and used for inoculation.

Plate 3.2.1

(a) Floccular degeneration of muscle sarcoplasm showing hyphal segments of 36/1P (H&E, x700)

(b) Extensive penetration of PA7 hyphae (Grocott's stain, x170)

Plate 3.2.2

Extent of pathology caused by MG fungus (NJM9030) after 1 week

(a) Above - muscle degeneration (H&E x170)

(b) Below - associated fungal hyphae (Grocott's x170)

3.3 GROWTH AND CULTURE EXPERIMENTS

3.3.1 Introduction

Growth characteristics at various temperatures and on different media were assessed in studies described here, to examine whether these parameters provided a consistent means of distinguishing pathogens from saprophytes, and whether the EUS, MG and RSD strains could be distinguished in culture. Attempts were made to induce oogonia production to aid in the speciation of isolates. Extracellular enzyme production by means of clearance zones on certain media was also evaluated.

Temperature-growth profiles ~~{Willoughby & Copland 1984 #7590}{Beghdadi, Richard, et al. 1992 #7200}{Barr, Warwick, et al. 1996 #12050}~~(Willoughby and Copland, 1984; Beghdadi *et al*, 1992; Barr *et al*, 1996); growth on different media (Hatai *et al*, 1994); and colonial morphology ~~{Brasier, Hamm, et al. 1993 #7220}~~(Brasier *et al*, 1993) have all provided rapid methods for distinguishing Oomycete species or strains in culture.

Study of the production of extracellular enzymes by saprolegniacean fungi has been shown to be important to the understanding of their trophic capabilities. For example, Unestam (1966) showed that out of a number of Oomycete fungi, only the three plant pathogenic *Aphanomyces* spp had appreciable cellulase and pectinase activity and that the crayfish plague fungus, *A. astaci* showed much greater, and constitutive, chitinase activity. Protease and DNase activity, as analysed here, has been previously studied in other saprolegniacean fungi (Söderhäll and Unestam, 1975; Alberts *et al*, 1989; Smith *et al*, 1994).

3.3.2 Activities

3.3.2.1 Temperature-growth experiments

Standard 4 mm GPY plugs taken from the edge of actively growing colonies were used as inocula for all culture studies. The effect of temperature on growth was tested on GPY at 6, 10, 14, 18, 22, 26, 30, 34, 38 and 42°C. After the plugs were placed on the test media, the linear extension of mycelia was measured on three points at right angles to each other, from the first day that a growing mycelial border was apparent, and daily thereafter until growth began to slow down or the colony had overgrown the Petri dish. Therefore the growth increment was defined as the daily increase of the mean radius of each colony over the period of the exponential growth phase. All experiments were replicated twice and the average is given.

3.3.2.2 Growth on different media

Growth was also compared at 22°C on the following unsupplemented media: Czapek Dox agar (CDA), cornmeal agar (CMA) (Difco Laboratories), Sabouraud dextrose agar (SDA), potato dextrose agar (PDA) and malt extract agar (MEA) (Oxoid), and on fish-meat-extract agar (FMA: 10% v/v snakehead meat extract, 1.2% w/v Oxoid No. 3 agar).

3.3.2.3 Cluster analysis

Numerical taxonomic analysis was performed using the *Aphanomyces* growth data only. The data was not normalised as the same parameter was measured for each variable. A phenogram was constructed by the agglomeration of a squared Euclidean dissimilarity matrix using the unweighted group-average method, UPGMA (Sneath and Sokal, 1973).

3.3.2.4 Viability of fungi on different growth media

GPY agar plugs (4mm) of six *A. invaderis* pathogens (RF8, S1PA, PA3, 30P, 3P, and NJM9030) and six saprophytic isolates of *Aphanomyces* (TF41, A2SA), *Achlya* (S2AC, W2BAC) and *Saprolegnia* (TF26, E3), were inoculated onto 5 Petri dishes containing GPY agar, GPY-Na_2EDTA (0.044g/l) agar, GPY-sodium phosphate (13mM) buffered agar, GPY-Na_2EDTA-sodium phosphate buffered agar or PG-1 agar. Dishes were kept at 10°C and plugs of fungi were taken after 21, 36 and 56 days and inoculated onto GPY agar to test for viability.

3.3.2.5 Clearance zones

Hankin and Anagnostakis (1975) described the use of several solid media substrates to detect enzyme production in fungi. In this study extracellular protease and DNase production were assessed by production of clearance zones on 10% skimmed milk (Oxoid) agar and DNase agar (Oxoid) respectively. Four mm GPY agar plugs of fungi were inoculated onto sterile 0.2 μm polycarbonate membranes (Nuclepore) placed over the top of agar plates as described by (Chang *et al*, 1992). The membrane was peeled off after 7 days at room temperature (19-22°C) and the clearance zone measured in relation to the fungal colony radius. Clearance halos were visualised using DNase agar by flooding plates with 1 N NaCl. Some experiments were run without membranes to see if this made a significant difference or using culture medium in wells in the agar to examine the extent of enzyme release.

3.3.2.6 Pigment production

On receipt of EUS isolate 10D from the Philippines, it was found to produce a grey-brown pigment in broth culture, which after a period of time coloured the medium too. Thereafter all broth cultures kept at 10°C were examined after 2 months and pigment production, assessed visually, was recorded.

3.3.2.7 Attempts to induce production of sexual reproductive structures

Heterothallism (self-sterility) has been demonstrated in some species of *Achlya*, in which mating only occurs when compatible strains are brought together due to the release of particular hormones ~~{Carlile 1996 #12540}~~(Carlile, 1996). This the basis of a short experiment in which several combinations of isolates were grown together on GPY agar (Table 3.3.1). They were examined for oogonia over a period of 3 weeks during which time plugs were taken several times for sporulation as in Section 3.1.2.5(a), and again examined for oogonia. Petri dishes were kept in the dark as Fowles (1976) reported that light inhibited the formation of oogonia in *Aphanomyces* cochloides.

Table 3.3.1 Combinations of isolates grown together on GPY agar plates and then sporulated to test for oogonia production

	TA1	RF6	S1PA	G2PA	BR	BH	10D	84-1240	TF5
TA1									
RF6	X								
RF6(K)		X							
RF8		X							
S1PA	X	X							
S3PA			X						
G2PA	X	X	X						
BR	X	X	X	X					
BH	X	X	X	X	X				
BS					X	X			
10D	X	X	X	X	X	X			
84-1240	X	X	X	X	X	X	X		
TF5	X	X	X	X	X	X	X	X	
TF41									X
TF54									X
T1SA	X	X	X	X	X	X	X	X	X
A2SA									X
F3SA									X

In addition to this experiment, attempts were made to induce oogonia production using hemp seed, different concentrations of neopeptone and GPY (Willoughby, pers. comm.), V8 broth (Appendix 2a) and CMA with 1 g/l peptone (as in Paternoster and Burns, 1996).

3.3.3 Outputs

3.3.3.1 Temperature-growth experiments and growth on different media

Average growth rates of all the isolates under various temperature and nutrient conditions are given in Table 3.3.2. There was no significant difference between the growth rates of the EUS, RSD and MG *Aphanomyces*. All showed optimal growth on GPY at 26-30°C; and failed to survive at 38°C. Further tests showed that they also did not grow at 37°C.

They had a lower growth rate under all culture conditions than any of the Thai saprophytic *Aphanomyces* and were unable to grow on SDA, CMA and MEA. *A. astaci* also failed to grow on SDA, CMA and MEA and was thermo-labile and slow-growing, but was clearly more adapted to colder conditions than the Asian pathogens. The UM *Aphanomyces* also had a preference for colder temperatures and was able to grow on CMA, MEA and relatively well on SDA.

The Thai saprophytic *Aphanomyces* strains were able to grow under all culture conditions tested with optimal growth on GPY at 34-38°C. However growth rates were quite variable within the group, particularly at higher temperatures. In all tests the two *A. laevis* isolates showed a slower growth rate than any of the other Thai saprophytic *Aphanomyces* strains. Growth rates and colonial morphologies on SDA were particularly variable (Plate 3.3.1).

Some *Aphanomyces* isolates produced distinctive colonial morphologies when grown on SDA. The UM fungus (84-1240) and certain Thai saprophytic *Aphanomyces* strains (TF33, ASEAN1, ASEAN3, SA11 and WSA) formed various patterns of radiating lines which appear to be consistent distinguishing features.

3.3.3.2 Cluster analysis

A dendrogram constructed from the growth data is given in Figure 3.3.1.

3.3.3.3 Viability of fungi on different growth media

Percentage viability on GPY agar of the six *A. invaderis* isolates and six saprophytic isolates after growth on 5 different media are given in Table 3.3.3.

Table 3.3.3 Percentage viability of 6 pathogenic *Aphanomyces* and 6 saprophytic fungi after growth on Petri dishes with 5 different media at 10°C

media	21 days		36 days		56 days	
	pathogens	saprophytes	pathogens	saprophytes	pathogens	saprophytes
GPY	100%	100%	17%	100%	0%	100%
GPY-EDTA	100%	100%	33%	100%	0%	100%
GPY-buffer	100%	100%	67%	100%	0%	100%
GPY-EDTA, buffer	100%	100%	83%	100%	0%	100%
PG-1	100%	100%	100%	100%	0%	100%

3.3.3.4 Clearance zones

The extent and transparency of clearance zones given by a number of isolates are showed in Table 3.3.4 and Plate 3.3.2. *A. astaci* and *Achlya* sp showed the greatest protease production compared to growth and the saprophytic *Aphanomyces, Achlya* and *Saprolegnia* spp showed greatest DNase production. Clearance zones shown by EUS, MG and RSD isolates were proportionally slightly larger when polycarbonate membranes were not used. Culture media put in wells in the agar leaked out within a few days without showing any discernible clearance halo with any isolate.

Table 3.3.4 Extent of clearance zones in relation to colony diameter

isolate	skimmed milk agar		DNase agar	
	extent	transparency	extent	transparency
S1PA	x	+	xx	+
34P	x	+	xx	+
10P	x	+	xx	+
FDL457	xxx	+	x	-
84-1240	xx	+	xx	+
TF33	xxx	-	xxx	+
ASEAN1	xx	+	xxx	+
S2AC	xxx	+	xxx	+
TF27	xx	-	xxx	+

x halo less than colony size
xx halo equal to colony size
xxx halo greater than colony size

\+ very transparent
\- not very transparent

3.3.3.5 Pigment production

To summarise the results of pigment production of isolates in culture, 10D retained its ability to colour the medium, and over time a number of other isolates began to show similar characteristics. These included EUS (PA3, PA4, PA5, 30P) and RSD (24P) isolates. No analysis of the ECP was undertaken. No other growth characteristics appeared to be affected.

3.3.3.6 Attempts to induce production of sexual reproductive structures

No oogonia were observed during any of the experiments described. Growth was poor on hemp seeds, but *A. invaderis* could grow on CMA supplemented with peptone.

3.3.4 Contribution of outputs

Growth rate is shown to be relatively consistent among all the EUS, RSD and MG isolates and a good preliminary diagnostic feature of these pathogens. Fowles (1978), in the first direct comparative study of different *Aphanomyces* species, also found temperature-growth profiles to be the most distinctive character studied.

The growth characteristics recorded here agree broadly with those given by other workers. Direct comparison is not possible given that the exact culture conditions vary. The failure of all the EUS, RSD and MG isolates to grow at 37°C on GPY in the present study was a characteristic given by Willoughby *et al* (1995), using glucose-peptone (GP) agar, in their description of *A. invaderis*. The growth data presented by Fraser *et al* (1992) for the isolates 3P, 4P and 10P are similar to that given here, except that in the present study, growth on CDA was very poor in comparison. Growth of RSD isolates on mullet-extract agar (Fraser *et al,* 1992), and of the MG strain on fish-extract agar ~~{Hatai & Egusa 1978 #9260}~~(Hatai and Egusa, 1978), are comparable to the growth of all the pathogens on snakehead-extract agar described here. Hatai *et al* (1994) gave very similar results to those presented here for two pathogenic *Aphanomyces* isolates which grew well on glucose-yeast (GY) agar and PDA but not at all on CDA or malt agar (MA). Hatai and Egusa (1979) also demonstrated the failure of MG isolates to grow on SDA.

Sterile *Aphanomyces* species obtained from guppies, dolphin and Indian major carp (see Section 1.6) are all considered to be distinct from *A. invaderis*, given differences in their reported cultural characteristics. In contrast to *A. invaderis*, the earlier isolates were shown to grow well on hemp seed ~~{Shanor & Saslow 1944 #2780}~~(Shanor and Saslow, 1944); up to 45°C and on CMA ~~{Fowles 1976 #7250}, and Sabouraud agar {Srivastava 1979 #9210}~~(Fowles, 1976), and Sabouraud agar (Srivastava, 1979). These characteristics have more in common with the saprophytic *Aphanomyces* isolates studies here, some of which were also isolated growing parasitically on fish and other aquatic animals.

Given the variability in growth rates and colonial morphology the *Aphanomyces* saprophytes are considered to be comprised of several species. This has been confirmed by molecular studies

undertaken at Glasgow University. Given that most were isolated from fish, several unidentified *Aphanomyces* species are therefore shown to act as perthtrophs and/or wound parasites. The *Achlya* isolates constituted two very different groups, but the Thai *Saprolegnia* isolates appeared fairly homogenous and are possibly all *S. diclina*, although oogonia have been observed in only two isolates. The UK *S. diclina* differed from its Thai equivalents only in terms of its lower temperature preference.

Given the reluctance of *Aphanomyces invaderis* to grow on cornmeal agar, it is possible if this fungus was involved in UM infections, it may not have been isolated, as Noga and Dykstra (1986) describe the use of CMA in their isolation procedure. Dykstra *et al* (1989) subsequently refer to the use of CMA supplemented with 1% yeast extract and 2% D-glucose, which would more likely support *A. invaderis*. Fraser *et al* (1992) showed that the RSD isolates grow on supplemented CMA as described by Dykstra *et al* (1989) and in this study *A. invaderis* were capable of growing on CMA with 1 g/l peptone.

Other studies have relied on SDA ~~{Bruno & Stamps 1987 #8810}~~(Bruno and Stamps, 1987) or CMA (Puckeridge *et al,* 1989; Bly *et al,* 1992) to isolate invasive hyphae in fish. In these cases *Saprolegnia* was obtained, and does appear to be the causative pathogen. Bly *et al* (1992) succeeded in reproducing the disease with their isolates. However, in such cases any involvement of an *A. invaderis*-like agent, with an inability to grow on such media, may have not been recognised. In addition, the isolations of Puckeridge *et al* (1989) were made directly from mycelium on the lesion, which would increase the likelihood of obtaining only wound pathogens. Interestingly, Bly *et al* (1992) did not succeed in obtaining fungal cultures from "systemic infections" using CMA.

Klebs (1899) expounded the theory that in aquatic fungi, sporangial production can be induced by the dilution of the standard growth medium, and oogonial production can be induced by transferring the fungus from a very rich medium to a very dilute one. The first idea is routinely practised for *A. invaderis* in the technique of transferring mycelium to APW. The second was tested by Willoughby (pers. comm.) by growing EUS and MG isolates (RF6 and NJM9030) and saprophytic *Aphanomyces* isolates on x1, x2, x4, x8 or x16 strength GPY agar and then transferring them to APW. However, as in all previous tests, no oogonia resulted. It was noted, however that unlike the saprophytes, the pathogens were unable to grow on x16 GPY agar.

In the current studies, ~~"giant cysts" (Scott, 1961)~~"giant cysts" (Scott, 1961) and chlamydospore-like structures as described by Roberts *et al* (1993) are commonly seen in some of the EUS, MG and RSD isolates, but at no time during the course of this work were sexual structures observed in any of these isolates. Experience with the *A. laevis* isolates (ASEAN1 and ASEAN3) indicates that oogonia are produced more readily soon after isolation, and may lose this ability after repeated subculture. However, some of the EUS isolates tested here were isolated only a matter of weeks before these experiments. Fowles (1976) describes a variety of other methods tested to induce oogonia in an *Aphanomyces* wound parasite, including growth on snake skin, cow's horn, porcupine quill, blonde hair, grass and *Drosophila,* but was similarly unsuccessful.

Alderman and Polglase (1988) observed that asexuality appeared to be more common among the more pathogenic saprolegniaceans. The slow-growing nature of *A. invaderis* may also be an indication of its pathogenicity. A distinction between obligate and facultative microbial parasites is often made, based on the theory that the former can never be cultured in artificial media (Willoughby, 1993). Brook (1966) rejected this idea and pointed out that the reduced ability of an organism to compete with natural saprophytes could restrict it to a parasitic mode of life. Unestam (1969a) suggested that given *A. astaci*'s fastidious nature in culture, it would have little chance of competing with saprophytes outside its natural crayfish host. This was supported by the finding that once all the crayfish in an area are killed by *A. astaci*, and uninfected but susceptible crayfish are introduced one year later, the disease does not recur for a number of years (Unestam,

1969a). This finding also formed the basis of control strategies against crayfish plague (Söderhäll *et al*, 1977). Despite extensive effort in Thailand, no EUS isolates have been obtained other than from diseased fish; and in Australia, Fraser and Callinan (1996) have identified slow-growing *Aphanomyces* from RSD-affected water, but only during active outbreaks. More sensitive, (molecular-based) assays are required to verify this work, but this evidence, when combined with the present studies showing the delicate nature of *A. invaderis* in culture, suggests that *A. invaderis* is adapted to an ecological niche as a fish pathogen and would not survive long as a saprophyte.

According to Lewis (1973), the secretion of hydrolytic enzymes into host tissue is considered an important criterion to differentiate between necrotrophic and biotrophic pathogens. Peduzzi and Bizzozero (1977) suggested that the capability to produce proteolytic enzymes in saprolegnian species provides them with the mechanism of tissue digestion necessary for hyphal penetration and enhanced pathogenicity. In the case of *Saprolegnia,* the conversion from a saprophyte to a fish parasite may be controlled by environmental factors, implying that enzyme production may be induced. *A. astaci,* however, is known to produce chitinase continually, without requiring induction which was considered evidence that that species is an obligate pathogen (Unestam, 1966). The case of A. *invaderis*, which was shown here to produce relatively little proteolytic enzyme on skimmed milk agar, may suggest that induction is required. Callinan (pers. comm.), however, found significant production of clearance zones on skimmed milk agar using RSD-*Aphanomyces*, although it is not known how enzyme activity compared with saprophytic strains.

Table 3.3.2 **Influence of temperature and culture medium on mean radial growth (mm/day) of all isolates**

°C	6	10	14	18	22	26	30	34	38	42	22	22	22	22	22	22
MEDIA	GPY	GPY	GPY	GPY	GPY	GPY	GPY	GPY	GPY	GPY	FMA	PDA	CDA	SDA	CMA	MEA
TA1	0.1	1.1	2.1	2.9	4.3	4.8	4.9	3.6	0.0	0.0	5.0	3.8	0.8	0.0	0.0	0.0
RF6	0.2	0.9	1.6	2.6	3.5	4.3	4.1	2.9	0.0	0.0	2.7	3.4	0.8	0.0	0.0	0.0
RF8	0.1	0.7	1.7	2.8	3.9	4.4	4.4	3.5	0.0	0.0	4.2	3.6	0.9	0.0	0.0	0.0
S1PA	0.1	0.8	1.8	2.4	3.7	5.0	4.8	4.0	0.0	0.0	4.2	3.9	0.9	0.0	0.0	0.0
S3PA	0.2	0.8	2.0	2.6	3.7	4.5	4.9	3.9	0.0	0.0	3.5	3.2	0.8	0.0	0.0	0.0
G2PA	0.1	0.8	2.1	2.7	3.9	4.6	4.8	3.7	0.0	0.0	3.6	3.6	1.0	0.0	0.0	0.0
PA1	0.1	0.8	1.5	2.8	3.9	4.4	5.0	3.6	0.0	0.0	3.6	3.6	0.7	0.0	0.0	0.0
PA3	0.1	0.7	1.7	3.2	3.9	4.4	5.2	4.3	0.0	0.0	3.5	3.5	0.8	0.0	0.0	0.0
PA4	0.1	0.8	2.0	2.8	4.0	4.5	5.1	4.1	0.0	0.0	3.7	3.8	0.7	0.0	0.0	0.0
PA5	0.1	0.7	2.1	2.8	4.0	4.5	4.7	3.6	0.0	0.0	3.5	3.4	0.6	0.0	0.0	0.0
PA7	0.1	0.8	1.8	2.9	4.2	4.6	5.5	4.0	0.0	0.0	3.7	3.9	0.8	0.0	0.0	0.0
PA8	0.1	0.8	1.8	2.9	4.0	4.7	5.0	3.5	0.0	0.0	3.5	2.9	0.7	0.0	0.0	0.0
PA10	0.1	0.7	2.0	2.9	3.9	4.6	4.9	3.7	0.0	0.0	3.6	3.6	0.6	0.0	0.0	0.0
BH	0.2	0.8	1.5	3.4	3.8	4.5	3.8	3.4	0.0	0.0	3.5	3.4	0.8	0.0	0.0	0.0
BR	0.1	1.0	1.7	2.6	3.6	4.3	4.4	3.4	0.0	0.0	4.0	3.2	0.9	0.0	0.0	0.0
BS	0.2	1.1	1.8	2.8	4.1	4.6	4.4	4.2	0.0	0.0	4.2	3.9	0.8	0.0	0.0	0.0
36/1P	0.2	1.0	1.9	2.8	4.1	4.6	4.6	3.7	0.0	0.0	4.1	4.5	1.1	0.0	0.0	0.0
30P	0.1	1.0	2.2	3.0	3.8	4.5	4.0	2.4	0.0	0.0	4.5	3.7	0.6	0.0	0.0	0.0
33P	0.2	1.2	2.3	2.8	4.1	4.5	4.2	2.8	0.0	0.0	4.8	3.9	0.7	0.0	0.0	0.0
34P	0.1	0.5	1.8	2.6	3.2	3.9	3.4	2.0	0.0	0.0	3.4	2.9	1.0	0.0	0.0	0.0
10D	0.1	1.0	2.1	2.4	3.9	4.6	4.6	2.0	0.0	0.0	4.0	2.9	0.6	0.0	0.0	0.0
3P	0.1	0.8	1.6	2.8	3.7	5.0	4.5	3.5	0.0	0.0	3.3	3.5	1.0	0.0	0.0	0.0
4P	0.1	0.5	1.5	2.7	3.7	4.6	4.3	3.5	0.0	0.0	3.2	3.3	0.9	0.0	0.0	0.0
10P	0.1	0.9	2.0	2.8	3.5	4.1	3.7	2.8	0.0	0.0	2.8	3.0	0.9	0.0	0.0	0.0
24P	0.2	0.8	2.1	2.6	3.8	4.7	4.9	3.5	0.0	0.0	3.5	3.4	0.9	0.0	0.0	0.0
NJM9030	0.1	0.5	1.7	3.1	4.0	5.2	5.3	3.8	0.0	0.0	3.6	3.3	1.0	0.0	0.0	0.0
84-1240	1.7	2.6	4.9	6.4	7.8	9.1	7.0	0.0	0.0	0.0	8.6	10.1	8.1	7.2	5.9	6.9
FDL457	1.2	2.3	3.1	3.7	4.3	2.3	0.2	0.0	0.0	0.0	5.6	5.0	0.9	0.0	0.0	0.0
FDL458	1.2	2.4	3.2	3.8	4.7	2.6	0.5	0.0	0.0	0.0	5.4	4.6	0.9	0.0	0.0	0.0
TF5	0.7	2.4	5.3	7.6	10.3	13.8	15.6	17.5	18.7	10.9	9.7	10.2	8.4	4.0	6.5	6.5
TF33	1.2	2.4	5.1	7.3	10.4	13.7	15.5	17.7	15.7	0.9	10.2	11.6	9.7	8.1	8.8	9.4
TF41	0.6	1.9	5.1	6.9	9.4	12.7	15.4	17.2	18.4	9.1	9.0	9.7	7.6	5.3	6.4	6.6
TF54	0.4	1.9	4.5	6.3	9.7	11.9	14.0	15.9	14.9	7.1	7.0	7.2	5.1	1.0	5.6	5.0
T1SA	0.6	2.3	4.4	7.0	9.3	12.7	14.9	17.3	18.8	11.2	10.0	10.9	10.0	5.4	7.9	8.1
A2SA	0.7	2.1	4.9	7.1	9.8	12.8	14.6	16.7	18.6	13.2	10.1	10.6	9.6	4.1	7.0	7.7
F3SA	0.7	2.2	4.6	7.2	10.0	12.8	15.1	16.6	18.3	12.8	10.0	10.8	9.8	4.2	6.9	7.7
ASEAN1	0.2	1.5	3.4	4.0	6.5	9.0	10.3	11.5	11.4	1.2	7.3	6.0	4.9	3.8	4.8	5.0
ASEAN3	0.2	1.4	3.1	5.0	6.5	8.8	10.1	11.4	11.0	1.2	7.4	6.4	5.1	3.1	5.0	5.0
SSA	0.6	2.1	4.3	7.2	10.0	12.5	15.8	18.2	19.5	11.7	10.3	9.7	8.5	4.5	7.8	8.0
WSA	1.4	2.9	5.4	8.3	11.0	14.5	16.8	19.0	18.1	2.8	10.9	13.7	8.6	11.0	9.8	11.1
SA11	2.1	3.6	5.8	8.1	10.3	12.5	14.0	14.8	6.6	0.0	10.1	11.0	9.4	5.6	7.9	8.8
S2AC	0.1	1.6	4.3	5.2	7.8	9.7	12.0	12.8	9.3	0.0	7.6	7.3	2.7	4.1	6.5	6.3
W2BAC	2.0	4.5	8.3	10.6	14.0	17.2	20.2	19.8	11.0	0.0	16.3	19.6	10.2	15.0	13.5	14.6
AC2	0.1	0.9	2.5	5.9	8.0	10.4	11.9	14.5	13.4	0.0	9.0	9.1	4.4	6.5	7.1	7.4
AC5	0.1	0.6	2.4	5.7	7.5	10.3	10.0	13.3	12.0	0.0	9.1	8.4	3.4	5.9	6.7	6.3
AC10	0.1	0.7	2.3	5.6	7.5	10.2	9.8	13.8	11.7	0.0	9.1	8.4	3.5	5.8	6.9	6.5
TF20S	4.5	6.9	11.7	13.4	18.0	22.2	22.5	21.0	5.8	0.0	21.7	21.4	13.8	17.5	19.0	18.8
TF23	4.5	6.9	11.4	14.5	18.3	22.1	24.5	21.1	5.5	0.0	21.5	21.3	14.7	17.1	19.6	18.7
TF24	4.7	6.6	12.4	13.6	18.3	22.2	23.5	21.0	6.0	0.0	22.3	21.3	13.2	17.1	18.5	17.5
TF25	4.7	6.9	11.6	14.4	18.0	21.8	23.0	21.1	5.5	0.0	21.1	21.6	14.7	17.2	19.2	18.5
TF26	4.7	6.9	11.6	14.5	18.0	21.2	23.0	21.2	5.0	0.0	21.1	21.3	13.8	17.7	18.9	18.6
TF29	4.5	6.5	10.3	13.3	17.0	21.1	23.5	21.0	6.8	0.0	20.1	21.9	14.7	17.6	18.5	18.5
TF27	4.3	6.6	10.8	14.0	17.0	21.0	23.0	20.7	6.1	0.0	20.6	22.4	13.4	17.5	18.0	18.1
TF31	4.2	6.5	10.1	13.2	17.1	21.2	23.7	21.2	8.6	0.0	21.0	22.2	15.6	17.2	18.3	19.0
S.AUST	2.5	4.2	6.1	8.3	8.8	10.2	0.8	0.3	0.0	0.0	14.3	16.3	9.9	11.8	10.8	11.2
P32	6.2	8.3	11.7	14.4	17.8	15.9	0.8	0.0	0.0	0.0	20.3	19.8	10.8	13.6	16.0	16.5
E3	6.8	9.1	12.6	15.7	18.5	20.6	5.6	0.8	0.0	0.0	20.6	24.2	13.5	17.9	18.5	18.5
TP41	5.1	7.2	9.6	11.5	13.8	15.8	8.0	0.8	0.0	0.0	18.5	19.2	9.2	13.4	11.9	12.9

Figure 3.3.1 Dendrogram

Plate 3.3.1

Differing colonial morphologies of 3 saprophytic *Aphanomyces* isolates on SDA. These patterns were consistent on several replicate cultures of the isolates

Plate 3.3.2

Clearance zones produced by *A. invaderis* (S1PA), *A. astaci* (FDL457), UM-*Aphanomyces* ("NOGA" = 84-1240), *Aphanomyces* sp saprophyte (TF33), *A. laevis* (ASEAN1) and *Achlya* sp saprophyte (S2AC)

(a) - top - on DNase agar

(b) - middle - on skimmed milk agar

(c) - bottom - polycarbonate membrane showing extent of *A. invaderis* growth (right) and corresponding clearance zone on skimmed milk agar (left)

3.4 CHEMICAL SUSCEPTIBILITY TESTS

The information presented in this section is in press in Aquaculture Research. The paper, entitled "Comparative effects of various antibiotics, fungicides and disinfectants on *Aphanomyces invaderis* and other saprolegniaceous fungi" is attached as Appendix 5.

3.4.1 Introduction

Previous studies on the susceptibility of aquatic fungi to chemical compounds have tended to concentrate on the *Saprolegnia* pathogens of salmonid fish (Willoughby and Roberts, 1992; Marking *et al,* 1994), and the crayfish plague fungus, *A. astaci* (Alderman and Polglase, 1985; Rantamäki *et al,* 1992). Yuasa and Hatai (1994) have also investigated the drug-susceptibility of the MG pathogenic fungus, *A. piscicida*.

Some articles have been published giving recommended treatments for EUS in ponds (Das and Das, 1993; Areerat, 1990) but no studies have investigated the *in vitro* susceptibility of *A. invaderis* to chemical treatment. The present work attempts to characterise isolates of *A. invaderis* in comparison with other fungi, in terms of their susceptibility to various chemical agents. This data can then be used to determine optimum concentrations of antibiotics, fungicides and disinfectants for the respective isolation, treatment and disinfection of these fungi.

3.4.2 Activities

3.4.2.1 Fungal isolates

The 54 fungal isolates used in this study are listed in Appendix 1. The two fungi S1PA(K) and TF25(K) are reisolates of the strains S1PA and TF25 after artificial injection into fish. All fungi were grown at 10°C on GPY agar (Appendix 2a). Four millimetre agar plugs taken from the edge of colonies less than seven days old were used in all experiments.

3.4.2.1 Antibiotic tests

Three antibiotics were tested: penicillin-K (Sigma: 1575 units penicillin-G base per mg), streptomycin sulphate (Sigma: 763 units streptomycin base per mg) and oxolinic acid (Sigma). Each antibiotic powder was added at 10, 50, 100 and 500ppm (equivalent to 16, 79, 158 and 788 units/ml penicillin-G; 8, 38, 76 and 382 ug/ml streptomycin and 10, 50, 100 and 500 ug/ml oxolinic acid) to molten GP agar (Appendix 2a) after autoclaving and cooling to about 50°C. Care was taken to continually mix the media when pouring into the Petri dishes. Control plates contained no antibiotics.

Duplicate agar plugs of each fungus were placed upside-down on five agar plates, each incorporating a different concentration of antibiotic. The plates were kept at room temperature (17-20°C). The growth rate of the fungus was measured as the mean increase in radius of the colony after 72 hours. An average of the duplicate colonies was taken and expressed as a percentage of the growth of the controls.

3.4.2.2 Fungicide tests

Three antifungal agents were selected for minimum inhibitory concentration (MIC) testing. Malachite green (Sigma) was tested at 0.5, 1, 5 and 10ppm; hydrogen peroxide (Solvay Interox Ltd) at 50, 100, 500 and 1000ppm and sodium chloride (BDH) at 5, 10, 20 and 30ppt. Control treatments contained no antifungal agent.

For each fungal strain, 5ml of each concentration of fungicide was pipetted into a different compartment of a multi-compartment "Replidish" (Bibby Sterilin Ltd). Triplicate agar plugs of each fungus were placed in each of the five compartments and left at room temperature (19-22°C) for one hour. For the malachite green and sodium chloride experiments, the plugs were then washed three times in separate Replidish compartments containing distilled water over a period of one hour before blotting on sterile filter paper and placing upside-down on GPY agar. In

the case of hydrogen peroxide, the plugs were transferred to Replidish compartments containing neutralising solution (0.25g/l catalase) for one hour before blotting and placing on GPY agar as with the other fungicide test treatments.

Growth was monitored daily and recorded as "no effect" if growth was apparent at the same time as controls; "inhibition" if growth started after controls; or "control" if no growth took place.

3.4.2.3 Disinfectant tests

Three disinfectants, the iodophore FAM 30 (Evans Vanodine Ltd), sodium hypochlorite (BDH) and Proxitane 0510 containing 5% peracetic acid in hydrogen peroxide (Solvay Interox Ltd) were assessed in terms of their minimum lethal time (MLT) to each fungal isolate.

Three replicate plugs of each fungus were immersed in a single concentration of the test disinfectant for 10 sec, 1 min, 5 min, 20 min or 1 hour and then removed and immersed in a neutralising solution for 20 min before being blotted on sterile filter paper and inoculated onto GPY agar. Triplicate control plugs were placed directly into neutralising solution without treatment and then inoculated onto GPY agar.

FAM 30 was used at a test concentration of 100ppm available iodine, sodium hypochlorite at 100ppm available chlorine and Proxitane 0510 at 100ppm peracetic acid. The neutralising solution for FAM 30 and sodium hypochlorite contained 0.5g/l sodium thiosulphate pentahydrate, 15g/l Tween 80 and 3g/l lecithin; and for peracetic acid it comprised 50g/l sodium thiosulphate pentahydrate and 0.25g/l catalase.

As in the fungicide experiments, growth was checked daily and the treatment was recorded as "no effect", "inhibition" or "control" compared to negative controls. Plates were maintained at room temperature (19-22°C) throughout the experiments.

3.4.3 Outputs

3.4.3.1 Antibiotic tests

The effects of antibiotics on fungal growth are shown in Figure 3.4.1. Oxolinic acid caused slight inhibition of most isolates at concentrations as low as 10ppm, however virtually all fungi continued to grow at 60% of controls or faster, even at the highest concentration of 500ppm. Streptomycin sulphate had the most severe effect, particularly on the EUS, RSD and MG *Aphanomyces* with most of these strains growing at 0-30% of controls when 500ppm was incorporated in the media. Penicillin-K had no significant effect on most fungi even at concentrations of 500ppm.

The effects of the antibiotics on different fungi were quite variable, even between different strains of the same species. All the most recent isolates of *A. invaderis* (PA1, PA3, PA4, PA5, PA7 and PA10) showed lower than average resistance to penicillin. The two *A. astaci* strains (FDL457 and FDL458) were significantly more susceptible to penicillin and streptomycin than most other fungi. The *Saprolegnia australis* isolate was among the most resistant of the fungi to all three antibiotics.

3.4.3.2 Fungicide tests

The only treatment that succeeded in killing all isolates was exposure to 10ppm malachite green for one hour. However, over three quarters of the strains failed to survive treatment with 5ppm malachite green, 500ppm hydrogen peroxide or 30ppt sodium chloride (Figure 3.4.2).

A clear difference in susceptibility to malachite green and sodium chloride between the EUS, RSD and MG *Aphanomyces* isolates and the saprophytic *Aphanomyces* spp was apparent. All the former were killed by exposure to 0.5ppm malachite green or 20ppt sodium chloride for one hour, whereas all the latter survived these treatments.

3.4.3.3 Disinfectant tests

The MLT for all the fungi was 20 min with sodium hypochlorite or Proxitane 0510 and 1 hour with FAM 30 at the specified concentrations (Figure 3.4.3).

All the EUS, RSD and MG *Aphanomyces* isolates were sublethally affected by sodium hypochlorite after 5 min whereas none of the saprophytic fungi were affected by this treatment. The effect of FAM 30 was even more variable between strains: all the fish-pathogenic *Aphanomyces* isolates failed to survive after 1 min, the saprophytic *Aphanomyces* and the *Saprolegnia* spp were killed by 5 min treatments but the *Achlya* spp were all killed only after 1 hour treatments.

3.4.4 Contribution of outputs

A feature of the results of this work is the variability in susceptibility of different fungal species to the compounds tested. Previous workers have used growth in inhibitory compounds as an aid to separating and comparing individual strains and species (Alderman and Polglase, 1984; Coffey and Bower, 1984; Paterson and Bridge, 1994; Yuasa and Hatai, 1994). This study shows that *Aphanomyces invaderis* can be clearly distinguished from the saprophytic *Aphanomyces* spp tested by its greater susceptibility to malachite green, sodium chloride and sodium hypochlorite. In addition, the RSD and MG strains were indistinguishable from *A. invaderis* in all of the tests. The UM *Aphanomyces* (84-1240), differed from *A. invaderis* particularly in terms of resistance to sodium chloride. The unusually high salt tolerance of this strain has been previously reported by Hearth and Padgett (1990).

The three antibiotics tested here have previously been shown to have relatively little effect on some saprolegniacean fungi (Oláh and Farkas, 1978; Alderman and Polglase, 1986) and are therefore suitable for use in fungal isolation media to exclude bacteria. However, at the 100ppm level of streptomycin sulphate and oxolinic acid advised by Willoughby and Roberts (1994), significant growth inhibition of some strains, of EUS, RSD, MG and crayfish plague fungi in particular, does occur. As it is common to use two or more antibiotics in combination when isolating fungi, it is likely that growth may be significantly slowed. Further, Griffin and Coley-Smith (1971) showed that streptomycin toxicity increases with temperature, and in the case of the *A. invaderis* it may be necessary to attempt isolation in temperatures of 30°C or above, resulting in further inhibition in growth of the fungus. The author has therefore adopted a reduced level of 10ppm of these antibiotic preparations for the isolation of *A. invaderis*.

It is possible that some of the variation in susceptibility to the antibiotics between similar strains may be the result of different antibiotic levels used in their isolation. For example, the *A. invaderis* strains PA1-PA10 were isolated using a low penicillin-K concentration of 10ppm, and these are shown to be generally more susceptible to penicillin than other EUS strains. Likewise, both *A. astaci* strains are consistently among the most susceptible of the fungi, and these were isolated as described by Alderman and Polglase (1986) using penicillin-G and oxolinic acid at 10ppm.

Bailey (1983) showed that the agar plug method of testing fungicide activity *in vitro* correlated well with *in vivo* treatments of surface infections of fish. The results of the present fungicide tests should therefore adequately project the activity of these agents against *Saprolegnia parasitica* and the fungi involved in saprophytic infections of EUS-affected fish. The *Achlya* strains examined here have been found to be the most common fungal saprophytes growing on the surface of fish lesions in Thailand (unpublished results), and these, along with the *Saprolegnia* spp, were usually the most resistant species to the compounds tested. As *Saprolegnia* spp are probably most commonly used in studies evaluating new compounds, it is possible that some of the agents dismissed by previous studies may be useful for treating *A. invaderis*.

Given the possible carcinogenic and teratogenic properties of malachite green (Alderman, 1992), an extensive search is underway to find an alternative agent for the treatment and control of

fungal diseases of fish (Bailey, 1984; Marking *et al*, 1994; Fitzpatrick *et al,* 1995). Some substances, including magnesium chloride (Rantamäki *et al*, 1992) and Chitosan (Min *et al,* 1994) have been identified as candidate fungicides. Two compounds currently being considered for use in aquaculture, hydrogen peroxide and Proxitane 0510, are shown here to have some potential for fungicidal treatments and disinfection respectively.

The treatment of several mycoses of fish is difficult because the fungi involved penetrate their hosts and are, to a large extent, protected from exposure to the chemicals. In these cases, a more strategic use of fungicides in culture systems is required, in which the infective stages of the fungus are treated in the incoming water. Willoughby and Roberts (1992) showed that only very low concentrations (0.25ppm) of malachite green are required to kill *S. parasitica* zoospores and cysts and preliminary work by the present authors showed that cysts of *A. invaderis* isolates failed to germinate after exposure for 1 hour at 20°C to malachite green concentrations as low as 0.08 ppm.

EUS has been reported only in tropical and sub-tropical areas and it should be remembered that the antifungal activity of chemicals and their effect on fish varies with temperature. Malachite green, for example, is very toxic to fish at higher temperatures (Alderman, 1985). Chinabut (1993) noted that malachite green is generally more toxic to scaleless fish than scaled fish, but advised that 0.1-0.15 ppm indefinite bath treatments of malachite green should be safe for most tropical fish, preferably in the early morning when temperatures are lowest. Willoughby and Roberts (1992) warned however that given the rapid infection rate by fungi in tropical conditions, one water treatment daily is probably not sufficient to prevent infection.

Further special considerations have to be made with regard to the control and treatment of EUS given that affected cultured fish are often in ponds of very high organic content when the efficacy of many fungicides is reduced. In these cases farm management techniques such as reducing stress to cultured fish, excluding wild fishes and sterilisation of equipment are advised (Lilley *et al,* 1992). The high relative susceptibility of *A. invaderis* to the disinfectants studied here shows that sterilisation can be easily achieved and is a useful management technique which can help prevent outbreaks of EUS.

Pond trials carried out at the Bureau of Fisheries and Aquatic Resources (BFAR) in the Philippines have indicated that 5ppm Coptrol (a copper chelated compound) succeeded in preventing the development of lesions in *A. invaderis*-challenged, abraded catfish (*Clarias gariepinus*), but concluded that this compound is too expensive for use in aquaculture (Callinan, pers. comm.). In the same trials, 0.1 ppm malachite green was partially effective, and agricultural lime (2 $kg/100m^2$) failed to prevent the development of EUS lesions. Agricultural lime ($CaCO_3$) however, just stabilises the pH of water, whereas slaked lime ($Ca(OH)_2$) is known to have an additional biocidal effect and Lilley *et al* (1992) suggested that at prescribed concentrations, this may be a more efficacious treatment for diseased fish.

The spread of EUS to islands and other isolated areas in Asia is probably the result of the uncontrolled movement of fish within the Region (Costa & Wijeyaratne 1989). To minimise the risk of further spread of EUS, one of the effective fungicidal treatments identified in this study should be applied to all fish, including ornamental species, imported to areas free from EUS. This should be combined with a period of quarantine for species susceptible to EUS.

Figure 3.4.1

Growth of test fungi at different antibiotic concentrations as a percentage of controls

(a) left - penicillin-K

(b) middle - streptomycin sulphate

(c) right - oxolinic acid

Figure 3.4.2

Minimum inhibitory concentration (MIC) of three fungicides against test fungi

(a) left - malachite green

(b) middle - hydrogen peroxide

(c) right - sodium chloride

Figure 3.4.3

Minimum lethal time (MLT) of three disinfectants to test fungi

(a) left - FAM 30

(b) middle - sodium hypochlorite

(c) right - Proxitane 0510

3.5 ZOOSPORE BEHAVIOUR

3.5.1 Introduction

Chemotaxis (Cerenius and Söderhäll, 1984a; Rand and Munden, 1993), indirect germination (Willoughby *et al*, 1983) and polyplanetism (Cerenius and Söderhäll, 1985) have all been studied in saprolegniacean fungi as possible adaptations to parasitism.

The cCoulter counter is also evaluated as a method of obtaining rapid size-distribution counts of secondary zoospores (Shoulties and Yang, 1971).

3.5.2 Activities

3.5.2.1 Size-distribution of zoospores

—Coulter counterA selection of EUS (TA1-K, PA7, 36/1P), MG (NJM9201), RSD (24P), UM (84-1240), saprophytic *Aphanomyces* (TF33, WSA, ASEAN3), *Achlya* (W2BAC, AC2), and *Saprolegnia* (TF27, P32, E3, TP41) isolates were analysed. At least two spore suspensions of each isolate were used. These were prepared using (b) or (c) technique described in Section 3.1.2.5. Size distribution data were obtained using a Coulter Multisizer (Coulter electronics). An initial aliquot was taken to obtain counts of motile secondary zoospores. The original sample was then shaken for 1 min in a universal tube, and left for 5 min, before another aliquot was taken for counts of encysted zoospores. At least three counts were taken to allow the sample to stabilise, before readings were downloaded into a computer.

3.5.2.2 Chemotaxis

Chemotaxis in *A. invaderis* was investigated using a technique based of that by Bimpong and Clerk (1970). Test substances were incorporated into 1.2% technical agar (Oxoid no.3). Spore suspensions were prepared as in Section 3.1.2.5. APW (Appendix 2a) was drawn up into a sterilised haematocrit tube, which was then inserted into the test agar. The test agar was pushed further into the tube a further few millimetres using a needle, and the tube was left in a Petri dish containing a test suspension of motile zoospores. After 1 hour zoospore attraction was examined under a microscope and compared to agar-only control plugs.

3.5.2.3 Germination

indirect germination

microcyclesGermination characteristics, and the relative importance of germination to repeated zoospore emergence (polyplanetism) were assessed in three *A. invaderis* isolates (96PA, 36/1P and 33P) in comparison with two unspeciated *Aphanomyces* saprophytes (F3SA, WSA) and *A. laevis* (ASEAN3) from EUS affected areas. This work was carried out at the University of Uppsala, Sweden, and two fungi previously tested in that laboratory were used as controls: *A. laevis* (107-52) and *A. astaci* (Hö).

The technique of Diéguez-Uribeondo *et al* (1994) was adopted, whereby motile zoospore suspensions were obtained as in Section 3.1.2.5(d) and encysted by vortexing for 45 sec. An equal volume of GPY broth was added immediately. After a further 2 hours, the percentage of cysts that had germinated, released zoospores (indicated by empty "ghost" cyst), or were undifferentiated (neither germinated nor empty), were counted under a microscope.

3.5.2.4 Polyplanetism

—This work was carried out at the University of Uppsala with the isolates listed above, and an additional control isolate, *Saprolegnia parasitica* (SPT). Zoospore suspensions were produced and vortexed as described above. After a period of time, which varied between isolates, the next generation of laterally biflagellated zoospores was vortexed and so on until no subsequent zoospore generations were released. At each stage the proportion of germinated, empty and undifferentiated cysts were counted.

3.5.3 Outputs

3.5.3.1 Size-distribution of zoospores

Coulter counts of all suspensions showed a peak at about 2 μm, which probably constituted pond water or paper debris from the paper-filtered APW. This peak was excluded from analyses. The zoospore size distributions clearly distinguished the smaller *Aphanomyces* propagules from those of *Achlya* and *Saprolegnia* (Figure 3.5.1). EUS, MG and RSD isolates were indistinguishable, and often quite variable. However, spore counts of these isolates were generally lower than the other fungi tested, which reduces the accuracy of the machine. The size of saprophytic *Aphanomyces* propagules varied between isolates; WSA as shown in Figure 3.5.1, is clearly intermediate between *A. invaderis* and the *Achlya-Saprolegnia* group. The encysted zoospores of several isolates were measured at up to 0.5 μm larger than motile zoospores.

3.5.3.2 Chemotaxis

Attraction of zoospores to a few test substances are shown in Table 3.5.1.

Table 3.5.?1 Chemotaxis of motile secondary zoospores

	GPY	CDA	1% casein	MEM + 1% FBS	trout blood	salmon blood	tilapia blood
EUS isolate (RF6)	++	-	-	+	+	+	++
MG isolate (NJM9030)	++	-	-	++	+	+	++
A.astaci (FDL457)	-	-	++	++	+	+	+
UM isolate (84-1240)	++	-	+	-	+	+	+
Aphanomyces sp (TF5)	++	-	+	+	+	+	+
A.laevis (ASEAN1)	+	-	+	+	+	+	+

++ strong chemotaxis
\+ some chemotaxis
\- no discernible chemotaxis

MEM minimum essential medium Eagle (ICN)
FBS foetal bovine serum

3.5.3.3 ~~Germination~~

3.5.3.3 Germination

The relative importance of germination and polyplanetism to the various isolates is indicated by the fate of encysted zoospores after the addition of media (Table 3.5.2).

Table 3.5.2 Effect of adding equal volume GPY to spore suspension immediately after vortexing

	% zoospore release (ie empty cysts)	% germination	% undifferentiated cysts
A. invaderis (96PA)	24	71	5
A. invaderis (36/1P)	16	60	24
A. invaderis (33P)	44	56	0
A. astaci (Hö)	56	0	44
Aphanomyces sp (F3SA)	0	100	0
Aphanomyces sp (WSA)	0	92	8
A. laevis (ASEAN3)	0	99	1
A. laevis (107-52)	0	98	2

In other experiments at Stirling University, involving more isolates, no media was added after encystment was induced. In this case, several isolates showed evidence of the production of "starved germlings". Examination of these showed indirect germination commonly occured in several EUS, MG and RSD isolates; a saprophytic *Aphanomyces* sp (WSA) and *Saprolegnia*

isolates (TF20S, TF26, TP41). This phenomenon is where a fine, septate germ tube connects an empty cyst to the viable cytoplasmic hypha (Willoughby, 1977). Less obvious, and more rarely observed evidence of indirect germination was shown by a UK *A. astaci* isolate (FDL458), a UM isolate (84-1240) and saprophytic *Aphanomyces* sp (TF33, TF54). Indirect germination was not observed in a Swedish *A. astaci* isolate (Hö), *A. laevis* (ASEAN1), a saprophytic *Aphanomyces* sp (T1SA) and *Achlya* sp (S2AC). RSD isolate, 24P, was used to show percentage indirect germination out of all germlings in distilled water (59%), 1/10,000 GPY (53%), 1/1000 GPY (43%) and 1/100 GPY (43%), although these percentages were variable among the pathogenic isolates. EUS, MG and RSD isolates, and also the saprophyte, WSA, all demonstrated an abbreviated life cycle (Willoughby, 1977) or "microcycle" in which a cyst germinates but does not grow far before producing another cyst-like structure which presumably is capable of releasing a zoospore, although this was not observed.

3.5.3.4 Polyplanetism

A. invaderis isolates (96PA, 36/1P and 33P) isolates were shown to be capable of producing one generation of motile zoospores from artificially encysted secondary zoospores, but no further generations could be induced. There was however, a fairly high proportion of undifferentiated cysts (neither releasing zoospores, nor germinating). The same was the case for an *A. astaci* isolate (Hö). This contrasts with work by Cerenius and Söderhäll (1984b), who succeeded in inducing three generations from a different *A. astaci* isolate. As reported by Diéguez-Uribeondo *et al* (1994), up to six successive generations of *S. parasitica* (SPT) zoospores could be induced. In concordance with Cerenius and Söderhäll (1985), a higher proportion of *A. laevis* (ASEAN3, 107-52) zoospores germinated than released zoospores. However, other saprophytic isolates (F3SA, WSA) showed a high level of polyplanetism, each producing up to four successive generations of zoospores.

3.5.4 Contribution of outputs

The diameter of hyphae and propagules of EUS, MG and RSD isolates are highly variable (Table 3.1.1) compared with other saprolegniacean fungi. However, size of these structures are one of the few morphological features available to distinguish these isolates from other *Aphanomyces* spp under the light microscope. Therefore, Coulter counter analysis was used as a rapid method to see if a particular size-distribution profile could be obtained for *A. invaderis* zoospores, and whether this was consistent with MG and RSD isolates as well. This method did indicate that propagules of these isolates were generally smaller and had a higher level of variation compared to other species tested, but this made it more difficult to obtain a consistent, normal distribution.

It would be of interest to evaluate the presence of "giant cysts" that appeared to be particularly frequent in sporulating samples of some of the present isolates. These are abnormally large encysted primary zoospores resulting from incomplete cleavage of the protoplasm prior to discharge from the sporangia, which go on to produce two secondary zoospores (Scott, 1961). As with other primary cysts, they are closely associated with the sporangium and difficult to count using the Coulter method.

Specific chemotactic responses are a clear indication of adaptation to a parasitic mode of life. Initial trials using eel mucus in an agar diffusion system as described by Cerenius and Söderhäll (1984a) gave strong indications of a chemotactic response in *A. invaderis*. However, problems were encountered with this technique, and the mucus was unavailable for the present capillary tube trials. These shown significant differences between the response of EUS-MG pathogens and *A. astaci* to GPY and casein. It is hoped to extend these trials using further substances.

The finding of Cerenius and Söderhäll (1985), that the more parasitic *Aphanomyces* isolates showed a higher level of polyplanetism was not sustained in the present studies. The two unspeciated saprophytic *Aphanomyces* (F3SA and WSA) showed a much greater facility for polyplanetism than did either *A. invaderis* or *A. astaci*. Although the latter two species showed a

high proportion of undifferentiated cysts, which may, under more suitable conditions produce zoospores, the three generations demonstrated by Cerenius and Söderhäll (1984b) for *A. astaci* was still less than that induced in the saprophytes here. It may be the case, however, that F3SA and WSA have a more parasitic lifestyle than *A. laevis*, which showed a low level of polyplanetism.

The differentiation between pathogens and saprophytes was equally unclear in the experiments on indirect germination, which Willoughby *et al* (1983) considered to be a possible adaptation to parasitism in saprolegnians. In the present study, indirect germination was shown to be common in *A. invaderis* but unconfirmed, and possibly absent, in *A. astaci*. Saprophytic *Aphanomyces* species showed varying abilities to produce septate germ tubes depending on the isolate. The fact that *Saprolegnia* fungi tested here clearly showed indirect germination, and *Achlya* clearly did not, also indicates that this phenomenon varies between taxa, but its relevance to the lifestyle of the fungus is uncertain.

A clear difference between pathogens and saprophytes was, however, demonstrated by the addition of nutrient media to the encysted zoospores. In this case, none of the four saprophytic isolates produced any zoospores (Table 3.5.2), whereas some *A. invaderis* cysts germinated and some released zoospores. Under these conditions, none of the *A. astaci* cysts germinated. This appears to indicate that *A. invaderis* is intermediate between the saprophytes and *A. astaci* in its requirement for a specific nutrient background before germination takes place. Further studies should investigate what specific requirements are needed to induce 100% germination in *A. invaderis*.

Figure 3.5.1 **Size distribution of (a) secondary zoospores and (b) encysted secondary zoospores**

(a) zoospores

(b) zoospores encysted by agitation

3.6 ULTRASTRUCTURE AND IMMUNOCYTOCHEMISTRY

A manuscript covering work described here is currently in preparation.

3.6.1 Introduction

This study attempts to elucidate some of the ultrastructure and surface binding characteristics of *A. invaderis* zoospores in order to define taxonomic markers particular to the species, and obtain information that may be relevant to pathogenesis.

Electron microscopy studies of the propagules of some Oomycete species have provided information on phylogenetic relationships between groups and valuable insights on the adaptations of those organisms to a particular life-style. Detailed ultrastructural information is available on *Saprolegnia ferax* (Heath and Greenwood, 1971; 1974; Lehnen and Powell, 1991); *Aphanomyces euteiches* (Hoch and Mitchell, 1972a; 1972b); and *Aphanomyces astaci* (Olson *et al*, 1984; Cerenius *et al*, 1984). Kinetosome-associated organelles or K bodies are single-membrane bounded structures occurring in all orders of Oomycetes except for the Peronosporales, and have been shown to act as distinctive taxonomic markers in some species of *Aphanomyces* (Powell *et al*, 1985). These organelles were therefore of particular interest to the present study.

Beakes (1987) observed that even closely related species of Oomycete fungi have evolved subtle variations in their response to environmental cues and encystment behaviour in response to different host preferences. The peripheral vesicles of zoospores of these species have been shown to contain preformed adhesive and attachment components which are released within seconds of contact with a suitable substrate and play a crucial role in the initial stages of infection. In this regard, the surface chemistry of *Phytophthora cinnamomi* (Peronosporales) has been intensively studied by Hardham *et al*, (1994). Further immunocytochemical analysis has been carried out by Berbee and Kerwin (1993) on *Lagenidium giganteum* (Lageniales); Burr and Beakes (1994) on *Saprolegnia parasitica-diclina;* and Lehnen and Powell (1993) on *S. ferax*.

3.6.3 Activities

Most of the work described here was undertaken at Newcastle University, in collaboration with Dr G.W. Beakes. The isolates studied are listed in Appendix 1. In all cases fungi were grown in drop cultures of GPY and induced to sporulate as in Section 3.1.2.5(d). Given the relatively delicate nature of *A. invaderis* zoospores, problems were encountered with their fixation and centrifugation, and a variety of techniques were tested.

3.6.3.1 SEM

Sporulating mycelium was placed in fixative (2.5 % glutaraldehyde 0.1 M cacodylate buffer or half this concentration) for 1 hour. Fungus was brought through 2 washes of cacodylate buffer and left in 1% OsO_4 for 1 hour. After 2 further washes, zoospores were attached to glass coverslips, dehydrated in a graded acetone series and critical-point dried. Cover slips were then mounted on aluminium stubs, sputter coated with gold and observed on a Cambridge Stereoscan 240 SEM.

3.6.3.2 TEM

Various samples of sporulating mycelium, and motile and encysted zoospores were prepared according to Burr and Beakes (1994), but with varied fixative and buffer concentrations. Samples were examined using a Philips 301 electron microscope.

3.6.3.3 Immunocytochemistry

Zoospore cysts and germlings of EUS (PA1, 33P), RSD (24P) and *S. parasitica* (TP41) isolates were probed with fluorescein isothiocyanate (FITC)-labelled antibodies against a range of Oomycete fungi (*A. invaderis* [polyclonals, PAbs], *L. giganteum*, *P. cinnamomi* and *S. parasitica* [all monoclonals, MAbs]) and particular lectins as listed in Figure 3.6.1.

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Zoospore preparations were fixed for 30 minutes by mixing with an equal volume of double strength fixative to give a final concentration of 4% formaldehyde in 50 mM PIPES buffer. Following PIPES buffer washing, these were given two rinses in PBS (phosphate buffered saline) before being finally suspended in double distilled water. 10 μl aliquots were dispensed into multiwell slides (Flow Labs Inc.) and dried down. These were stained with antibodies and lectins as outlined by Burr and Beakes (1994), mounted in DAKO antifade mountant and examined under a Leica DMRB research microscope and pictures recorded photographically using Tmax 1600 ASA film uprated to 3200. Dr Beakes continued this work using samples of glutaraldehyde-fixed (2% in 50 mM PIPES) zoospores and cysts of the mosquito parasite, *Lagenidium giganteum,* provided by Peterson and Kerwin.

3.6.3 Outputs

3.6.3.1 SEM

Scanning EM photos show that the primary zoospore cysts form strong clusters comprising variable numbers of cysts (usually over 20). Although papillae can be observed on some cysts, these are not as pronounced or consistent as in the truly papillate *Aphanomyces* species and probably develop prior to release of secondary zoospores (Plate 3.6.1). In concordance with other members of the genus *Aphanomyces* (Scott, 1961), SEM photographs given here show that *A. invaderis* secondary zoospores do not posses cyst coat ornamentation.

3.6.3.2 TEM

The zoospores and cysts of *A. invaderis* have a rather atypical fine structure and their cyst coat lacks the electron dense (encystment vesicle derived) outer wall layer typical of saprolegniacean Oomycetes (Plates 3.6.2 and 3.6.3)

K bodies were not identified in secondary zoospores and zoospore cysts, although only a few well-fixed samples were observed. K bodies were also difficult to locate in primary cysts but a few samples showed putative K bodies with different substructure to that observed in *A. astaci, A. laevis* and saprophytic *Aphanomyces*, WSA. Peripheral vesicles were also less apparant in *A. invaderis* than other species examined.

Plate 3.6.4 shows a secondary cyst of saprophytic *Aphanomyces*, WSA, with an obvious outer wall and K body.

3.6.3.3 Immunocytochemistry

A summary of the parts of the zoospores and germlings to which particular antibodies and lectins reacted is given in Figure 3.6.2 and 3.6.3.

Polyclonal antibodies raised against mycelium of *A. invaderis* cross-reacted with the zoospore, cyst and germling surfaces of all species tested. In contrast the monoclonal antibodies raised against zoospore and cyst surfaces of *L. giganteum* and *S. parasitica* showed no significant cross-reactivity with either *Aphanomyces* or *Lagenidium*. However, the zoospores of *A. invaderis* did show a strong cross-reactivity with the MAb Vsv-1 which had been raised against the plant pathogen *P. cinnamomi*. There was also some cross-reaction with the MAb Lpv-1.

3.6.4 Contribution of outputs

K bodies are not found associated with kinetosomes in zoospores of the Oomycete order Peronosporales, which includes the *Phytophthora*. Given the ability of *A. invaderis* zoospores to cross-react with *P. cinnamomi* MAbs, the lack of a dense outer cell wall, and the initial apparent lack of K bodies, the possibility of a taxonomic relationship with the Peronosporales was being considered. However, putative K bodies were subsequently observed in primary cysts of *A. invaderis*, and these appeared to have a distinct substructure. This is consistent with the work of Powell *et al* (1985) which showed that different species of *Aphanomyces* can have structurally different K bodies, and can

therefore act as taxonomic markers. However, further work is needed to characterise the organelle shown in Plate 3.6.4(a). Due to its appearance in a mature primary cyst, it could be a K1 body or a K2 body, the former of which is known to be morphologically variable depending on stage of development (Holloway and Heath, 1977). However, clear differences were observed between the microbodies of *A. invaderis* and those of saprophytic *Aphanomyces* WSA, *A. laevis* (isolate unknown) and *A. astaci* (isolate unknown) all of which showed numerous and distinct K bodies in various zoospore stages.

Plate 3.6.1

(a) Scanning electron micrograph of *A. invaderis* (33P) showing cluster of at least 12 encysted primary zoospores. Cyst diameter = 5.6 - 5.7 μm

(b) Scanning electron micrograph of saprophytic *Aphanomyces* (TF54) showing cluster of at least 8 encysted primary zoospores. Cyst diameter = 4.9 - 5.8 μm

Plate 3.6.2

Transmission electron micrograph of *A. invaderis* (33P) showing a section through a cluster of at least 35 encysted primary zoospores (1 cm = 1.9 μm)

Plate 3.6.3

Transmission electron micrograph of *A. invaderis* (33P) showing a section through an encysted primary zoospore (1 cm = 270 nm)

Plate 3.6.4

Transmission electron micrograph of saprophytic *Aphanomyces* (WSA) showing a section through an encysted seconadary zoospore (1 cm = 345 nm)

Plate 3.6.5

Transmission electron micrographs showing variable structure of K bodies in:

(a) top - *A. invaderis* (33P) primary cyst (1 cm = 154 nm)

(b) left, bottom - saprophytic *Aphanomyces* (WSA) secondary cyst (1 cm = 147 nm)

(c) right, bottom - *Aphanomyces laevis* primary cyst (1 cm = 265 nm)

Figure 3.6.1

Figure 3.6.2 (a)

Figure 3.6.2 (b)

Figure 3.6.2 (b)

Figure 3.6.3

3.7 GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

A manuscript prepared from this work entitled "Characterisation of *Aphanomyces invaderis* using electrophoretic and Western blot analysis" has been submitted to Diseases of Aquatic Organisms

3.7.1 Introduction

This study attempts to compare the EUS, RSD and MG pathogenic *Aphanomyces* isolates and a variety of other Oomycete fungi as listed in Appendix 1, in terms of their sodium dodecyl sulphate polyacylamide gel electrophoresis (SDS-PAGE) profiles; and to characterise strain-specific bands using various stains, lectins and polyclonal antibodies.

Callinan *et al* (1995) compared Philippines EUS and Australian RSD isolates and other *Aphanomyces* species in terms of their SDS-PAGE banding patterns. Although these were not clear for all isolates, EUS and RSD isolates showed some similarities in banding patterns, and appeared distinct from *A. cochloides, A. laevis* and *A. euteiches.*

Other studies have employed protein electrophoresis as a means of establishing interspecific and intraspecific relationships between Oomycete fungi (Kaosiri and Zentmyer, 1980; Erselius and Shaw, 1982; Bielenin *et al*, 1988; Brasier *et al,* 1993; Zhang *et al,* 1994; Latorre *et al*, 1995). Hansen (1987) considered protein electrophoresis to be one of the most valuable tools used in the speciation of *Phytophthora* fungi as the banding pattern obtained on polyacrylamide gels represents the diverse products of 50 or more genes. Even where *Phytophthora megasperma* isolates were indistinguishable by classical taxonomic means, protein banding patterns were capable of separating an alfalfa-pathogenic group from other legume and Douglas fir isolates (Hansen *et al*, 1987).

Peduzzi and Bizzozero (1977) suggested that serological tests using polyclonal antibodies may provide a useful criterion for the identification of saprolegniacean species. Although lack of specificity has been a problem in subsequent studies using polyclonal antisera against Oomycetes, serological profiling has been demonstrated in several cases (Krywienczyk and Dorworth, 1980; Bullis *et al*, 1990; White *et al*, 1994).

Lectins have been used in the intensive study of the surface components of Oomycete zoospores, cysts and germlings (Hardham, 1989; Lehnen and Powell, 1993; Burr and Beakes, 1994) from which, evidence in support of particular taxonomic groupings have been obtained (Beakes *et al,* 1994). However, no studies have been found on the use lectins in Western blots to distinguish fungi, as described here.

3.7.2 Activities

3.7.2.1 Preparation of fungal extracts

A list of the fungi used in the present study are given in Appendix 1. To obtain protein-rich fungal extracts, 45 ml zoospore suspensions were produced in Petri dishes as described in Section 3.1.2.5(b) and these were added to an equal volume of double strength GPY broth. Germlings were allowed to develop for 1-3 days at 22°C depending on the growth rate of the isolate, so that thin mycelium mats formed on the bottom of each Petri dish. Growth medium was decanted and the samples washed in 500 ml sterile distilled water. Samples were filtered through sterile Whatman 541 filter paper and excess water removed using dry sterile filter paper. Fungal mats were ground in liquid nitrogen using a pestle and mortar. The resulting powder was then homogenized in 1 ml Wood's (1988) extraction buffer (85 mM Tris HCl, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.198 g/l ascorbic acid and 1 g/l glycerol at pH 7.5) with the addition of 5 µM phenylmethylsulphoxylfluoride (PMSF) (Sigma). The homogenate was centrifuged twice at 13,000 g for 5 min and the protein concentration of the extracts were estimated by ultraspectrophotometric readings at $OD_{280.}$ These were adjusted to 5 mg/ml with sample buffer and the extracts were frozen at -70°C. Prior to adding sample buffer, a 100 µl aliquot was taken of each extract and stored at -70°C for subsequent protein digestion. This involved

incubating the aliquots with 100 µl of a 10 µl/ml solution of proteinase K (Sigma) for 1 hour at 60°C before diluting in sample buffer.

3.7.2.2 Preparation of extracellular products (ECP)

Media (500 mls GPY) in which the fungus had been cultured were collected, placed in dialysis tubing with a molecular weight cutoff of 10 kDa (Fisons) and concentrated using polyethylene glycerol (8 kDa, Sigma). Concentrates were dialyzed using three changes of two litres phosphate buffer saline (PBS: 0.02 M $NaH_2PO_4.2H_2O$, 0.02 M $Na_2HPO_4.2H_2O$, 0.15 M NaCl, pH 7.2) over 24 hours at 4°C.

3.7.2.3 SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970) using precast acrylamide separating gels (4-20 %) (BioRad). The gels were subjected to electrophoresis for 45 min at 200 V, then stained with either in 0.1 % (w/v) Coomassie blue R250 (Sigma), silver stain (BioRad) or Schiffs reagent (BDH).

3.7.2.4 Electroelution of 10 kDa band

An *A. invaderis*-specific band (from isolate PA7) of molecular weight 10 kDa was excised from a 12 % (w/v) polyacrylamide slab gel and electroeluted using a Hoefer electroeluter at 100 V for 1 hour according to manufacturers specifications.

3.7.2.5 Immunization schedule

Three New Zealand White rabbits, obtained from the animal house, University of Stirling, were immunized with mycelium extracts from either (a) a saprophytic *Aphanomyces* isolate F3SA, (b) *A. invaderis* PA7 or (c) the electroeluted band from PA7. The extracts (300 µg protein) were mixed 1:1 with Freunds complete adjuvant (Sigma) and 1.5 ml was delivered subcutaneously (SC) into two sites. The rabbits received two further SC booster injections of fungus extract in Freunds incomplete adjuvant 4 and 8 weeks later. A final injection of 1 ml fungus extract in sterile saline was given intravenously on week 12. The rabbits were then bled out by cardiac puncture 10 days later.

3.7.2.6 Western blotting

The polyclonal antisera were used to screen each isolate for antigenic bands by Western blot analysis. Samples were applied to each lane and subjected to electrophoresis as described above. The samples were transferred from the gel to a sheet of nitrocellulose membrane by a wet blotting system (Hoefer) at 50 V for 1 h. Following transfer, the nitrocellulose membrane was washed with two changes of high salt wash buffer (HSW: 0.02 M Tris, 0.5 M NaCl, 0.1 % Tween-20, 0.01 % methiolate, pH 7.8) and blocked for 2 hours with 1.0 % (w/v) bovine serum albumin in distilled water. The membrane was then washed twice in HSW and antisera (diluted 1/100 in PBS) were applied to the nitrocellulose membrane and incubated for 1 hour at 4°C. After two washes in HSW, goat anti-rabbit IgG-Horseradish Peroxidase (HRP) conjugate (SAPU: Scottish Antibody Production Unit, Carluke, UK) (diluted 1/100 in 0.5 % (w/v) casein in PBS) was applied to the membrane and left for a further hour. Unbound conjugate was removed from the membrane by washing twice in HSW followed by one wash with Tris buffer saline (TBS: 10 mM Tris, 0.15 M NaCl, pH 7.5). The blot was developed with 4-chloro-1-naphthol (BioRad) and the reaction stopped with distilled water.

Lectins were used to examine carbohydrate moieties in each of the fungal extracts. For these studies the nitrocellulose membranes were incubated for 1 hour with lectins labelled with biotin (Sigma) (see Table 1) diluted to 20 µg/ml in low salt wash buffer (LSW: 0.02 M Tris, 0.038 M NaCl, 0.05 % Tween-20, 0.01 % methiolate, pH 7.4) in place of the rabbit polyclonal antisera. The blots were washed and incubated for 1 hour with streptavadin-peroxidase (SAPU) diluted 1/100 in LSW buffer. Finally, the membranes were washed five times with HSW buffer and the reaction developed as described above.

3.7.2.7 Immunohistochemistry (IHC)

The technique used was based on the method of Adams and Marin de Mateo (1994). Fixed blocks of muscle from snakehead fish experimentally infected with *A. invaderis* (TA1) during experiments described in Section 3.2, were embedded in paraffin wax and slides of 5 µm cross-sections were

prepared. The tissue sections were dewaxed, encircled with a wax PAP pen (BDH) and fixed for 10 min with methanol containing 10% v/v hydrogen peroxide to bleach endogenous peroxidases. The slides were washed three times with TBS. Normal goat serum diluted in TBS (10% v/v) was added to the slides which were then incubated for a further 20 min. The serum was poured off, the slides were placed in a moist chamber and each of the three rabbit sera (1/100 in TBS) were added to the sections for 1 hour at 20°C. Normal rabbit serum was used as a negative control. The slides were washed as above. Goat anti-rabbit-HRP conjugate (1/50 in TBS) was added to the slides for 1 hour and the slides washed as previously described. To visualize the reaction, slides were incubated for 10 min with 3'3 diaminobenzidine tetrahyrochloride (DAB) (Sigma) in the presence of hydrogen peroxide (H_2O_2) [100µl of 1% H_2O_2 to 0.5 ml (1.5 mg/ml DAB) and 5 ml TBS]. The reaction was stopped by immersing the slides in tap water. The slides were counterstained with haematoxylin for 3-4 min then dehydrated and mounted. Positive tissue appeared brown in colour under a light microscope.

3.7.2.8 Indirect fluorescent antibody technique (IFAT)

IFAT was carried out on TA1 tissue sections according to Neelam *et al* (1995) using 1/100 dilutions of the rabbit antisera. Normal rabbit serum was used as the negative control. A 1/100 dilution of fluorescein isothiocyanate (FITC)-donkey anti-rabbit IgG (SAPU) was used as the secondary antibody.

3.7.3 Outputs

A list of the isolates tested by SDS-PAGE and Western blot analysis is given in Appendix 1. Given the volume of data generated, only selected results are illustrated here.

3.7.3.1 SDS-PAGE

Coomassie blue stained SDS-PAGE gels gave very similar banding patterns for all the EUS, RSD and MG isolates (Figure 3.7.1) and these were distinct from all other fungi tested. Bands shown by these isolates consistently visualized using Coomassie blue occurred at 48, 56 and 61 kDa. However, banding patterns were generally rather faint, therefore the more sensitive silver stain was used to further highlight bands. Using silver stain, the intensity and number of bands visualized were greater for the EUS, RSD and MG pathogens compared with the other fungi. In order to compare isolates from different gels a pictorial representation of the silver stain bands was constructed (Figure 3.7.2a). Bands specific to all the EUS, RSD and MG isolates were located at approximately 10, 84, 195 and 240 kDa. The 10 kDa band was electroeluted and its presence in the resulting sample verified using SDS-PAGE (Figure 3.7.2b). This sample was used to prepare the third polyclonal antiserum (αband). While the bands above remained constant, molecular weights of other bands were inconsistent when gels were stained with the silver stain reagent, even between different gels run using the same fungal extracts. Silver staining also showed similarities between other fungal isolates, with clear groups being identified among some of the saprophytic *Aphanomyces* (TF5, TF41, F3SA, SSA and T1SA), two of the *Achlya* isolates (S2AC and AC2) and two *Saprolegnia* isolates (TF29 and TF31).

Silver-stained proteinase K-treated samples and Schiffs-stained gels revealed high levels of the low molecular weight carbohydrate around 10 kDa in *A. invaderis* (as shown for PA7 in Figure 3.7.3a, lanes 1 and 2 respectively) and at approximately 5 and 14 kDa in most of the saprophytic *Aphanomyces* (as shown for F3SA in Figure 3.7.3b, lanes 1 and 2). These bands can also be identified on the untreated silver-stained gels (Figure 3.7.2a). Another carbohydrate band of note revealed by Schiffs staining was the 100 kDa band which was apparent on EUS (Figure 3.7.3a, lane 2), RSD and MG isolates (data not shown) but not on any of the other fungi.

3.7.3.2 Western blotting (i) Lectins

Figure 3.7.3 also shows the bands of PA7 and F3SA recognised by each lectin. The bands described above could not be positively identified by lectin staining studies, although the 100 kDa band visualized using Schiffs stain may equate with a band of similar size revealed by the lectin LEA on EUS, RSD and MG isolates. The relative affinities of each lectin for the fungal carbohydrates varied, as indicated by the time taken for bands to develop in 4-chloro-1-naphthol. This ranged from 10 sec for Con A to 14 hours for LEA.

There was again remarkable consistency between EUS, RSD and MG isolates in terms of the bands revealed by lectin-binding on the Western blots. Figure 3.7.4a illustrates this consistency with regard to HGA. The lectin UEA-1 gave a very similar banding pattern to HGA. Out of the nine lectins tested, only ECA revealed any differences between *A. invaderis* isolates with 36/1P and 10D showing possible additional bands (Figure 3.7.4b, lanes 5 and 6). For all the EUS, RSD and MG isolates a band at approximately 45 kDa was recognised by Con A, ECA, HGA, BS-1, LEA and UEA-1. WGA recognised a region between 33 and 123 kDa, producing an area of continuous staining.

For F3SA and a few apparently similar saprophytes, 2 bands were generally recognised at 55 and 90 kDa for all lectins tested except LEA. Similar banding patterns were obtained with GMA, BS-1, UEA-1 and AHA among the saprophytes.

3.7.3.3 (ii) Polyclonal antisera

Western blot analyses showing the response of the three polyclonal antisera against fungal extracts are presented in Figure 3.7.5. The anti-saprophyte (αF3SA) and anti-*A. invaderis* (αPA7) sera showed a high degree of cross-reactivity with all isolates tested, with both antisera recognising similar bands on a given isolate (Figure 3.7.5a and b). However, the αF3SA serum recognised a band at 45 kDa on PA7 and a band at 42 kDa on F3SA which the αPA7 serum only faintly recognised. There was also a substantial amount of staining of low molecular weight material in the F3SA extract by the αF3SA serum which was not recognised by the αPA7 serum. Both the αF3SA and αPA7 sera recognised the 10 kDa band of PA7 and were also able to recognise this band in the pathogenic MG isolate NJM9030 (RSD isolates were not tested with these antisera).

Antiserum raised against the PA7 electroeluted band (αband) recognised only two bands found solely in extracts of fungi from the EUS, RSD or MG group (Figure 3.7.5c). However neither band appeared at the molecular weight of the original 10 kDa band, but instead at around 50 kDa.

The reaction of the polyclonal antisera with fungal ECP is shown by Western blots in Figure 3.7.6. There was little response of either the αF3SA or the αPA7 sera with the ECP from the saprophyte F3SA (Figure 3.7.6a), however, both antisera reacted strongly with the ECP of the *A. invaderis* isolate PA7 (Figure 3.7.6b). Six major bands were observed at about 20, 35, 45, 50, 60 and 85 kDa in the PA7 ECP. Anti-PA7 band serum did not react with ECP of either PA7 or F3SA (Figure 3.7.6c).

3.7.3.4 Histochemical analysis

Fungal hyphae in tissues of snakehead fish infected with *A. invaderis* isolate TA1 were positively labelled with both αF3SA and αPA7 when the secondary antibody was conjugated with either HRP (Figure 3.7.7a and b respectively) or FITC (Figure 3.7.8a and b), with the αF3SA serum eliciting the stronger response of the two. Hyphae were only very faintly labelled with αPA7 band serum (Figures 3.7.7c and 3.7.8c), while no reaction occurred with the negative control (Figures 3.7.7d and 3.7.8d).

3.7.4 Contribution of outputs

EUS, RSD and MG pathogenic *Aphanomyces* isolates were shown to be very similar in terms of their protein and carbohydrate components, as indicated by SDS-PAGE banding patterns visualised using various stains, lectins and polyclonal antibodies. Furthermore, these techniques distinguished this group of pathogens from all the other Oomycete fungi that were previously shown to be non-pathogenic to EUS-susceptible fish (Section 3.2). The results are consistent with other studies in this report which also indicate that the EUS, RSD and MG isolates constitute a single species.

Protein profiles, as revealed by Coomassie blue and silver staining, were complex and difficult to reproduce consistently. However, it was possible to identify specific bands that could act as taxonomic markers for *A. invaderis*, although they differed between the two stains. It may be the case that the three bands identified in the Coomassie blue-stained gels of *A. invaderis* equate with the three major bands in EUS and RSD samples that are shown between the 43 and 87 kDa reference markers in

Callinan *et al* (1995). The latter workers, however, grew their fungi for 15 d at 30°C, whereas in the present study, mycelium was grown from zoospores for a maximum of 3 d at 22°C to ensure a high proportion of protein-rich growing tips. This difference in the age of the samples would affect the biochemical composition of the cells, and thus may be reflected in the different SDS-PAGE profiles. Chen *et al* (1991) warned that in the case of *Pythium,* soluble protein profiles were affected by storage and culture conditions. This is also an important consideration when comparing different fungi of greatly varying growth rates. In the present study, growth times were adjusted to between 1-3 days so as to produce similar yields for each fungus. Despite this adjustment, the slower-growing EUS, MG and RSD isolates still showed more intense coloration than the other fungi with both Coomassie blue and silver stain.

Silver stain reacts with carbohydrate as well as protein and in order to highlight the carbohydrate bands an initial protein digest treatment was performed on each sample. This treatment revealed very few bands, suggesting that the majority of bands in non-treated silver-stained samples were associated with protein. Proteinaceous glycoconjugates would have been broken down by the proteinase K digest and the resulting products may constitute at least part of the low molecular weight bands observed in the samples. Sadowski and Powell (1990) used silver methenamine to show that the plasma membranes of *Aphanomyces euteiches* zoospores were rich in glycoproteins and thus the membranes may be the main source of this material in the mycelial extracts tested here. Schiffs, a general carbohydrate stain, also showed few bands, but these did reveal differences between the EUS, MG and RSD isolates and the other fungi.

Other workers have used isozyme profiles to characterise *Aphanomyces* species (Larsson, 1994) and other Oomycetes (Wang and LéJohn, 1974a; 1974b; 1974c; Beakes and Ford, 1983; Oudemans and Coffey, 1991). During the present study a preliminary attempt was made to obtain esterase profiles for some isolates. This was unsuccessful and as new fungal extracts had to be made, this work was abandoned due to lack of time.

Western blots using lectins gave a more sensitive analysis of the specific carbohydrate moieties in each sample, and provided a robust technique for distinguishing EUS, MG and RSD samples from other fungi. Only one lectin (ECA) showed any differences between the pathogens and may provide a useful means of strain-typing. The main difference was the extra major band shown by Philippine isolate 10D. Evidence from Section 3.10 shows that the pathogens form a very genetically homogenous group, although in this case also, the Philippine isolates showed a marginally different pattern in their polymorphic DNA bands.

Some lectins with different carbohydrate specificities revealed similar banding patterns, which may be explained in that each of the bands visualised were composed of complex polysaccharides with different residues accessible to several different lectins.

In their study of the lectin-binding properties of Oomycete propagules, Burr and Beakes, (1994) showed that Con A-binding material (mannose and glucose) was associated with the glycocalyx of zoospores of a variety of Oomycete species. The high reactivity of Con A with all the fungi tested here suggests that there are significant amounts of these saccharides in the mycelium of saprolegniaceous species as well.

The polyclonal antisera, αF3SA and αPA7, showed a great lack of specificity, with αF3SA reacting more strongly with PA7 than homologous antiserum. These antisera also reacted strongly with bands from *Achlya* and *Saprolegnia* samples (data not shown). This is consistent with the results of Bullis *et al* (1990; 1996), which showed that mouse anti-*Saprolegnia parasitica* polyclonal and monoclonal serum cross-reacted, albeit to a lesser extent, with a UM-*Aphanomyces* isolate used here (84-1240), and even some non-Oomycete fungi. However, the antisera prepared in this study was useful in highlighting antigenic fungal bands on Western blots. These were consistent for all EUS, MG and RSD isolates and distinct from other *Aphanomyces* spp tested. The 10 kDa band was clearly identified on

EUS, MG and RSD samples by both the αF3SA and the αPA7 rabbit antisera. This band was also identified by artificially and naturally infected snakehead fish sera (see Section 3.8). Thus, the 10 kDa band has been shown to be immunogenic in fish and rabbits. However, the antiserum prepared by injecting the electoeluted 10 kDa band into rabbits did not recognize the 10 kDa band by Western blot. Instead, two bands at around 50 kDa were recognised on EUS, MG and RSD samples. These bands were also faintly visible on silver-stained gels of the electroeluted 10 kDa band (Fig. 3.7.2b) and their appearance may be due to confirmational changes to the 10 kDa band as a result of electroelution. Nonetheless, these bands were specific to EUS, MG and RSD isolates as αband serum did not cross-react with other fungus samples.

The serum raised against mycelium extract from a saprophytic *Aphanomyces* (αF3SA) did not recognise ECP secreted by homologous fungus but did recognise the same secreted products from PA7 as αPA7. This suggests that *A. invaderis* isolate PA7 secreted greater quantities of ECP than the saprophyte F3SA, which may represent the release of proteolytic enzymes relevant in the pathogenesis of *A. invaderis*. The molecular weights of the bands in the ECP recognised by the antisera did not directly correspond to bands found in the mycelium extract. However, the antisera were raised against mycelium extract and therefore bands recognised in the ECP should correspond to components found in the mycelium extract. If this is indeed the case, the difference in molecular weight of the bands revealed between the two samples may be explained by ECP components being altered in some way, such as being cleaved on secretion. The fact that the αF3SA serum recognised the PA7 ECP again illustrates the non-specific nature of the antisera. However, the lack of any reaction with the αband serum suggests that the electroeluted band is not secreted into the ECP or was lost on dialysis during sample preparation.

In his study of histological staining techniques of *S. parasitica*- and *A. astaci*- infected tissue, Southgate (1983) found the latter to be significantly more difficult to stain, probably, he speculated, due to the smaller diameter of the hyphae which presented insufficient cell wall cellulose for most techniques to work. The only technique that demonstrated *A. astaci* hyphae in infected tissues was Grocott's silver stain, a long and involved procedure, and in conclusion, he strongly advised the development of alternative fluorescence antibody techniques. The rabbit antisera used in the present study proved a very effective diagnostic tool for identifying *A. invaderis* hyphae in fish tissue, particularly by IFAT, and compares favourably with Grocott's stain in terms of ease of use. It would be interesting to use this technique on sections of UM-affected fish tissue to compare reactivity of the invasive fungus involved in that disease with that of *A. invaderis*. A UM isolate was shown here to have very different protein and carbohydrate profiles from *A. invaderis,* but it is possible that this is not actually the true pathogen involved in that disease (Section 3.2).

Raising monoclonal antibodies against *A. invaderis* hyphal material would provide a means of developing a more specific probe for use in the immunohistochemical diagnosis of EUS. Lectins were used on sections of infected fish tissue in ths study, in an attempt to obtain a more specific stain for *A. invaderis*. This technique has been used for other fish diseases (Marin de Mateo *et al*, 1993) but the lectins tested here gave no discernible reactivity.

Table 3.7.1 **Lectins used in the study**

Lectin (*origin*)	Abbreviation	Carbohydrate specificity
Concanavalin A (*Canavalia ensiformis*)	Con A	terminal α-D-mannosyl and α-D-glucosyl residues
Wheat germ agglutinin (*Triticum vulgaris*)	WGA	N-acetyl-β-D-glucosaminyl residues and N-acetyl-β-D-glucosamine oligomers
Coral tree agglutinin (*Erythrina cristagalli*)	ECA	D-galactose and D-galactosides

Horse gram agglutinin (*Dolichos biflorus*)	HGA	terminal N-acetyl-α-D-galactosaminyl residues
Soybean agglutinin (*Glycine max*)	GMA	N-acetyl-α-D-galactosamine
(*Bandeiraea simpicifolia*)	BS-1	terminal α-D-galactosyl and N-acetyl-α-D-galactosaminyl residues
Tomato agglutinin (*Lycopersicon esculentum*)	LEA	N-acetyl-β-D-glucosamine oligomers
Gorse seed agglutinin (*Ulex europaeus*)	UEA-1	L-fucose
Peanut agglutinin (*Arachis hypoaea*)	AHA	D-galactose

Figure 3.7.1

Coomassie-stained SDS-PAGE gel (4-20%) of various isolates of the EUS pathogen (lanes 2 to 7) and RSD pathogen (lanes 8 and 9) showing almost identical polypeptide band patterns. Lanes: (1) and (10) are BioRad low molecular weight markers; (2) RF6; (3) G2PA; (4) PA7; (5) BH; (6) 36/1P; (7) 10D; (8) 4P and (9) 24P

Figure 3.7.2

(a) left - Pictorial representation of silver-stained SDS-PAGE gels (4-20%) of selected isolates

(b) right - Silver-stained electroeluted 10 kDa band (on 4-20% SDS-PAGE gel)

Figure 3.7.3

Polysaccharide banding patterns of SDS-PAGE gels (4-20%) (a) *Aphanomyces invaderis* isolate PA7 and (b) saprophytic *Aphanomyces* isolate F3SA. Lanes: (1) proteinase K-treated silver-stained gel; (2) Schiffs-stained gel; (3-11) Western blots stained with various lectins. BioRad low-range markers are indicated on the left of lanes (1-2) and BioRad broad-range markers are indicated on the right of the lanes (3-11).

Figure 3.7.4

Western blots of various isolates of the EUS pathogen (lanes 1 to 6) and the RSD pathogen (lanes 7 and 8) stained using (a) HGA and (b) ECA. Lanes: (1) RF6; (2) G2PA; (3) PA7; (4) BH; (5) 36/1P; (6) 10D; (7) 4P and (8) 24P

Figure 3.7.5

Western blot analyses showing the response of various polyclonal antisera with different fungal extracts. (a) rabbit anti-saprophyte (αF3SA); (b) rabbit anti-*A. invaderis* (αPA7); (c) rabbit anti-PA7 electroeluted band (αband). Lanes: (1) extract from *A. invaderis* strain PA7; (2) extract from saprophytic *Aphanomyces* F3SA; (3) extract from MG strain NJM9030; (4) extract from *Aphanomyces astaci* strain FDL458; (5) extract from UM strain 84-1240.

Figure 3.7.6

The response of polyclonal antisera with fungal extracellular products (ECP) by Western blot analysis. (a) rabbit anti-saprophyte (αF3SA); (b) rabbit anti-*A. invaderis* (αPA7); Lanes: (1) ECP from *A. invaderis* strain PA7; (2) ECP from saprophytic *Aphanomyces* F3SA.

Figure 3.7.7

The reaction of rabbit polyclonal antisera with tissues from snakehead fish infected with *A. invaderis* (strain TA1) by immunohistochemistry. The plates show the reactions obtained with: (a) rabbit anti-saprophyte (αF3SA); (b) rabbit anti-*A. invaderis* (αPA7); (c) rabbit anti-PA7 electroeluted band (αband) and d) negative control (normal rabbit serum). The magnification is 100-fold.

Figure 3.7.8

The reaction of rabbit polyclonal antisera with tissues infected with *A. invaderis* (strain TA1) by the indirect fluorescent antibody technique. The plates show the reactions obtained with: (a) rabbit anti-saprophyte (αF3SA); (b) rabbit anti-*A. invaderis* (αPA7); (c) rabbit anti-PA7 electroeluted band (αband) and (d) negative control (normal rabbit serum). The magnification is 100-fold.

3.8 IMMUNE RESPONSE OF SNAKEHEADS AND TROUT

Two manuscripts have been prepared from this work. The first entitled "The antibody response of snakehead, *Channa striata* Bloch, to *Aphanomyces invaderis* is in press in Fish and Shellfish Immunology, and is reprinted in Appendix 6. The second, entitled "The response of rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages against *Aphanomyces invaderis* (Thompson, K.D., Lilley, J.H., Chen S-C. & Adams. A) has been submitted to Veterinary Immunology and Immunopathology.

3.8.1 Activities and outputs

3.8.1.1 Challenge Experiments of Rainbow trout

The possibility of using rainbow trout (*Oncorhynchus mykiss*) as a species model to investigate the immune response of fish to *Aphanomyces invaderis* was examined and a challenge system devised to evaluate vaccination trials. Initial challenge experiments were carried out on 100g rainbow trout using mycelium implants (Roberts *et al*, 1994). Pathogenic strain PA7 was used as the *A. invaderis* challenge strain. The challenge was performed three times using the implants from which it would appear that the water temperature had to be above 15°C for the challenge to be successful. It was, however, difficult to ensure that a constant dose of fungus was give to each fish using this method. Spore suspensions administered according to Chinabut *et al*, (1995), produced a more effective and reliable method for challenging the fish and were therefore, used in the study. Spore suspensions of *A. invaderis* isolate PA7 was prepared as in Section 3.1.2.5(a). Mycelium mats were washed five times with sterile distilled water. They were then incubated overnight at 20°C in APW to produce a suspension of zoospores which were then enumerated using a haemocytometer. To establish the idea, challenge dose fish were injected with different concentrations of spores in 0.2 ml of suspension, administered intramuscularly (im) into the flank of the fish just below the anterior part of the dorsal fin (Table 3.8.1). Fish injected with 0.2 ml sterile saline (0.85% v/v) were used as negative controls. Muscle was sampled from around the injection site of the experimental rainbow trout and fixed in 10% (v/v) formalin. Samples were embedded in paraffin wax and slides of 5 μm cross-sections prepared. Serum was also sampled from the fish to determine the level of specific antibody produced using an enzyme-linked immunosorbant assay (ELISA) and Western blot analysis.

The success of the challenge was assessed both by the appearance of lesions at the injection site (Figure 3.8.1) and by histopathology using haematoxylin and eosin (H&E) and immunohistochemistry (IHC) (See Sections 3.2 & 3.7). The presence of fungus in muscle could be clearly seen in sections stained with IHC. Specific mortalities were, therefore, confirmed using IHC with rabbit anti-A. *invaderis* serum as in Section 3.7. Positive tissue appeared brown in colour under a light microscope.

As the dose of fungal spores increased the number of fish succumbing to the disease also increased. Lesions were seen to develop at the injection site of some fish challenged with higher spore concentrations, but it was also necessary to use IHC to determine the extent of fungus invasion present in muscle sections of the remaining fish.

The level of antibody produced by fish four weeks after challenging them with the spores was also determined (Table 3.8.1) using an ELISA. Briefly, ELISA plates were sensitised with 10 μg/ml of fungal extracts diluted in coating buffer and non-specific binding sites were blocked with 250 μl/well of 1% (w/v) bovine serum albumin (BSA) (Sigma). The fungal extracts were prepared as in Section 3.7. Fish sera diluted two fold in phosphate buffered saline (PBS) (0.02M) containing 1% BSA were added to the wells (100 μl/well). A negative control of PBS was also included. The plates were washed five times with high salt wash buffer (HSW) containing 0.02M Trizma base, 0.5M NaCl and 0.01%(v/v) Tween-20 (pH 7.8). Neat supernatant from an anti-rainbow trout IgM immunoglobulin monoclonal antibody (MAb) cell line (4C10) (Courtesy of Dr Ann Thuvander, Department of Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden) was

added for 60 min. After washing the plate five times with HSW, the plates were then incubated for 60 min at 20°C with goat anti-mouse IgG-Horseradish Peroxidase (HRP) (SAPU)(100\l/well of a 1/1000 dilution in PBS) and again washed 5 times with HSW. Chromogen /substrate [120ml of 43 mM tetraethyl benzidine dihydrochloride in 2 M acetic acid added to 12 ml of substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4 containing 0.33 % v/v H_2O_2)] was added to each well (100 µl/well) and plates were incubated for 5 min. The reaction was stopped with 50 µl/well of 2 M H_2SO_4 and plates were read spectrophotometrically at 450 nm against a blank of chromogen, substrate and stop solution. Optical densities exceeding or equal to three times the mean background absorbance were considered positive. Fish injected with 1x104 and 1x105 spores had significantly higher antibody titres compared to fish injected with 1x103 or 1x102 spores or control fish injected with PBS. The antibody titre of naïve rainbow trout four weeks after challenging with A. *invaderis* (PA7) spores (1x103) is shown in Table 3.8.2. The titre of antibody from challenged fish (7.3 ⇧2.2) was significantly higher than the control fish (3.8 ⇧1.5). The possibility of invading fungus *in situ* resulting in a higher antibody response compared to responses obtained with dead fungus or fungal extracts is discussed below in Section 3.8.1.5.

The size of the fish may be related to their susceptibility to invading fungus and the rate at which lesions develop. We found that 40g fish develop lesions after 14 days, while 300g trout develop lesions after 21 days. A MSc student is current examining the relationship of the size of the fish to their susceptibility to the disease.

3.8.1.2 Vaccination and specific antibody responses

Very few reports of a specific antibodies produced by fish to fungal pathogens exist and it is unknown whether such a response can immunologically protect the fish from fungal invasion. The antibody response of rainbow trout to A. *invaderis* was, therefore, studied. It was also intended to establish whether such an antibody response, if elicited, could protect the fish and prevent the grow of the fungus. Over the course of the study fish were immunised intraperitoneally (ip) with various preparations of both pathogenic (PA7) and saprophytic (F3SA) *Aphanomyces*. In the first experiment, fish (100g rainbow trout) were injected ip with 0.2 ml PA7 or F3SA homogenised in sterile saline, or with PBS as a control. The water temperature during this time was 16°C. Serum was sampled at weeks 3, 5, 7 and 9 post vaccination and specific antibody titres determined by ELISA. Small increases in antibody levels were observed, very few of which were significant (Table 3.8.3). The increases were not, however, due to a specific reaction since sera from fish immunised with PA7 mycelium reacted equally as well against saprophytic F3SA extract and serum from control fish also elicited a non-specific reaction against both PA7 and F3SA extract. Presumably the control fish had been previously exposed to environmental fungi which had antigens common to the two fungi tested here. Antibody titres of control fish, shown in Tables 3.8.1 and 3.8.2, also exhibited a non-specific immune response.

In an attempt to elicit a specific antibody response against pathogenic A. *invaderis* PA7, rainbow trout were immunised with various preparations of the fungus. Two groups of 100g fish were immunised with 0.2ml ip PA7 or F3SA mycelium homogenised as above, and a third group was injected with filtrate from the PA7 homogenate. A four group of fish was immunised with a PA7 extract, prepared as described above, while a fifth group received the extract in Freund incomplete adjuvant (FIA). Control fish injected with PBS were also included. The water temperature was maintained at 12°C over the 12 week period of the trial. There again appeared to be little specificity in the antibody response of these fish, with the control group having levels as high as those of the vaccinated fish (Table 3.8.4). Fish immunised with PA7 extract in FIA did, however, appear to have a significantly greater antibody titre at week 11 compared with the control group.

A third vaccination trial was carried out using 50g fish maintained at a water temperature of 18°C. The fish were vaccinated ip with either 0.2 ml of homogenised PA7 mycelium, extracellular products (ECP) of the fungus (Section 3.7) or with an electro-eluted preparation of the 10kDa

also described in Section 3.7. Control fish were again administered with PBS. All vaccinated were prepared in FIA. Both groups immunised with ECP or the 10kDa band had significantly higher antibody titres than that of the control group. Antibodies sampled from fish immunised with mycelium did not, however, differ to those of the control group (Table 3.8.5).

Sera from the experimental fish were screened by Western blot analysis to examine their reactivity profiles against fungal bands separated by SDS-PAGE (as in Section 3.7). Fungal samples on the gel were transferred to a nitrocellulose membrane which were washed twice with HSW then blocked with 1.0 % (w/v) BSA in distilled water. The membranes were again washed twice with HSW and fish sera (diluted 1/100 in PBS) were applied to the nitrocellulose membrane and incubated for 1 hour at 4°C, after which the membranes were washed and neat supernatant from MAb cell line (4C10) was added to the membrane for 60 min. After two washes in HSW, goat anti-mouse IgG-HRP conjugate (SAPU) (diluted 1/100 in 0.5 % (w/v) casein in PBS) was applied to the membrane and left for a further hour. Unbound conjugate was removed from the membrane by washing twice in HSW followed by one wash with TBS. The assay was developed with 4-chloro-1-naphthol (BioRad) and the reaction stopped with distilled water. Sera used from fish vaccinated in Trial 2 (Table 3.8.4) were used for the analysis and the bands recognised by the sera are detailed in Table (3.8.6). A faint band around 37 kDa was observed with the F3SA extract, while a faint band at around 55kDa was seen with the PA7 extract. This suggests that these fish must have been exposed to ubiquitous fungi present in the environment since they cross reacted with the two *Aphanomyces* strains examined here. The same two bands were recognised by sera from fish immunised with sonicated F3SA, but the reaction was stronger. For some reason no bands were observed with sera from fish vaccinated with the PA7 sonicate, however, bands were recognised when filtrate was used as the vaccinate with a doublet being recognised in PA7 extract. A strong reaction was observed with the band at 55kDa, while a band at 40kDa was less intense. The band at 37kDa in F3SA extract was again recognised by sera from PA7 extract vaccinated fish as was the doublet in the PA7 extract. This time the intensity of the reaction was equally distributed between the two bands. When PA7 extract was placed in FIA a strong reaction only occurred with the 55kDa band in PA7 extract, as well as the 37kDa band in the F3SA extract.

It was intended that vaccinated fish from the three trials be challenged with A. *invaderis* spores to assess whether a protective immune response had been elicited by the fish. Due to technical difficulties in maintaining rainbow trout at 18-20°C in our aquarium, we were unable to perform these challenges at Stirling over the course of the study. This analysis was, therefore, carried out on snakehead fish in Thailand (See Section 3.8.1.5).

3.8.1.3 Non-specific immune response of rainbow trout to *A. invaderis*

The results of the vaccination studies suggest that it is difficult to elicit a specific antibody response in fish using the preparations of fungus described above, since substantial cross reactivity occurs with control sera, presumably with other ubiquitous fungi. Histological examinations of infected tissue reveal a large inflammatory response produced by the non-specific immune defences of the fish against the invading fungus (Roberts *et al*, 1994). In an attempt to halt the invasion of fungus through the tissues of the fish, macrophages surround and envelop invading mycelium by forming granulomas (Chinabut *et al*, 1995). The significance of macrophages in the inflammatory response was, therefore, investigated as an alternative method for the fishes immune system to avoid infection by A. *invaderis*. Macrophage activity was examined *in situ* in the muscle of rainbow trout muscle after challenging the fish with PA7 spores and by culturing macrophages *in vitro* in the presence of fungal spores.

Macrophage Assays:

Macrophages were prepared from head kidney tissue according to Secombes (1990) by teasing the tissue through a 100 µm nylon gauge into Leibovitz-15 medium (L-15) (Sigma Chemical Co. Poole, Dorset, U.K.) containing 10 i.u. heparin ml-1, 1% penicillin /streptomycin (P/S) and 2%

foetal calf serum (FCS). The resulting cell suspension was layered onto a 34%/51% (v/v) Percoll gradient and centrifuged for 30 min at 400 xg at 4°C. The leukocytes at the interface were collected and washed with L-15 medium by centrifuging at 400 xg for 10 min. The cell pellet was resuspended in L-15 containing 1% P/S and the cell concentration was adjusted to 1.0 x 107 cells/ml. Macrophage monolayers were prepared by placing 0.4 ml of the cell suspension into chamber slides and allowing them to adhere for 2 hours at 15°C. After this time non-adherent cells were removed by washing the chambers three times with L-15 medium. The monolayers were used either to examine phagocytosis of spores by the macrophages or to examine the growth of mycelium in the presence of the macrophages. Fungal spores were stained with 0.1% (w/v) Congo red for 30 min, washed twice with sterile PBS by centrifuging for 10 min at 1500 xg, then adjusted to 1 x104 ml-1 using L-15 supplemented with P/S and 5% FCS. A 0.4ml aliquot of the suspension was added to the macrophage monolayer and incubated for 2 hours at 15°C. Medium without spores was added to some of the wells as a negative control. After the incubation, the macrophages were washed three times with PBS, then fixed and stained with RapiDiff (Siomedia laboratory suppliers, Romfield, Essex, UK) or by IHC.

The macrophages were able to phagocytosis the fungal spores. Congo red staining allowed easy identification of the spores within the macrophage. Figure 3.8.2 (a) shows a number of macrophages with engulfed spores. Some of the macrophages had clumped together to form giant cells. In Figure 3.8.2 (b) mycelium is seen to be growing from germinated cysts, with macrophages clumping around the growing mycelium. Staining with IHC using rabbit anti-PA7 polyclonal serum allows the growing mycelium to be seen (Figure 3.8.3). Macrophages have phagocytosed fungal spores which have then germinated within the macrophage. In some cases the macrophages have join together to form large giant cells in the presence of the fungus (Figure 3.8.4). Smaller giant cells were also noted in macrophage monolayers without spores, however.

When unstained spores were added to cultures of macrophages they encysted and germinated. Macrophages were seen to cluster around the tips of the growing mycelium (Figure 3.8.5). Different concentrations of spores were added to the macrophage monolayers. With lower concentrations of spores (1 x102 and 1 x103) no germination of spores or growth of mycelium in culture could be observed. Presumably the macrophages were able to destroy the spores which were present or prevent them from germinating. At concentrations of 1 x104 or higher, the macrophages were unable to kill all the spores and mycelium eventually overcame the culture. This resembles the situation observed *in vivo* where lower doses of spores injected im did not result in fungal infection.

Examination of macrophages by electron microscope:

Electron microscopy (EM) was used to examine the interaction between macrophages and fungus. The concentration of macrophages were adjusted to 1x106 cells/ml in L-15 with additives (see above). One ml aliquots of the macrophage suspension were placed in polystyrene culture tubes and 100 µl of fungal spores (1 x104 cells/ml) were added to the tubes and gently mixed. The tubes were incubated for 4, or 24 hours or 2 days at 15°C with intermittent shaking. The macrophages were then rinsed with cacodylate solution by centrifuging the tubes at 400 xg for 7 min at 4°C. The resulting pellets were fixed with 2% (v/v) glutaraldehyde diluted in cacodylate buffer for 1 hour and the tubes were again centrifuged. The pellets were dehydrated with acetone before embedding them in Epon 812 (Agar Scientific Ltd., Stanstead, U.K.). Thin sections of the pellets were prepared and stained with uranyl acetate and lead citrate. These were observed with a Philips EM301 electron microscope.

Figure 3.8.6 illustrates examples of the interactions which were observed between macrophages and fungus by EM:- (a) illustrates the structure of mycelium which possesses a thick cell wall, the outer surface of which is covered with microvilli. The centre of the mycelium is vacuolated; (b) shows a macrophage containing a phagocytosed spore in its cytoplasm which has, as yet, not

germinated; (c, d and e) illustrates macrophages which have attached onto the fungal mycelium, while some macrophages attached to mycelium appear damaged by the fungus (f) and (g); a damaged macrophage attached to a germinating spore is pictured in (h). The interaction between the macrophage and mycelium is shown in more detail in Figures 3.8.7 and Figure 3.8.8. In the former, two macrophages are seen in cross-section wrapped around a piece of mycelium; (b) and (c) highlight the junction where the two macrophages meet while (d) pictures the tip of the mycelium. The microvilli appear to be secreting vesicles into the cytoplasm of the macrophage. Perhaps they are secreting substances involved in the pathogenicity of the fungus such as proteolytic enzymes. Figure 3.8.8 shows different magnifications of the interaction between the mycelium and the macrophage (a and b); (c and d) emphasis the interface between the macrophage and the mycelium. It would appear that the cell wall of the mycelium is being digested by the macrophage. The fungal cell wall is much more defuse in (d) were it is in direct contact with the macrophage compared to (c) where it is at edge of the macrophage.

Macrophages response to the fungus in situ
Infiltration of macrophages at the site of injection in challenged rainbow trout began to occurred soon after injection as seen in H&E and IHC stained sections. Over the course of the challenge extensive destruction of muscle occurred. Necrosis of muscle fibres was extensive and myophagia was observed with macrophages phagocytosing damaged muscle fibres. Giant cell formation was widespread through the infected area. By day 28 many of the challenged fish exhibited muscle tissue regeneration.

Measurement of the innate immune response of rainbow trout during infection with A. invaderis
Attempts were made to examine the affect of growing mycelium on the non-specific immune responses of the fish during *A. invaderis* infection. Nitroblue tetrazolium (NBT) reduction by blood neutrophils performed according to Chen *et al* (1997) was examined in fish from the first challenge experiment outlined in Section 3.8.1.1. Group 1 fish vaccinated with saline possessed 23.8 ⇧17.4% positive NBT neutrophils, Group 2 fish vaccinated with F3SA mycelium had 27.2 ⇧7.2%, while fish vaccinated with PA7 mycelium had 19.6 ⇧17.7% positive NBT cells. The variation within the assay is very large, shown by the large SD obtained with each group. No significant difference was, therefore, observed between the groups.

3.8.1.4 Vaccination and Challenge Experiments of Snakehead fish in Thailand

Two vaccination trials were performed on snakehead fish, a species known to be susceptible to EUS in Thailand. In the first trial suspensions of secondary zoospores were prepared as described in Section 3.8.1.1 and were used as vaccinates by microwaving them for 10 s, after which time the zoospores were no longer motile. Both the zoospore suspensions and homogenised mycelium mats (used to produce the zoospores) were used to immunise the snakehead fish (500 g), obtained from Suphanburi Province, Thailand. The experiment was carried out at AAHRI, Kasetsart University Campus, Bangkok, where three groups of fish were used in the study. The first group (n=8) were non-vaccinated healthy control fish. The second group (n=5) received a primary im injection of microwaved PA7 spores (1 ml containing 5000 spores/ml), followed by an ip injection of microwaved PA7 spores mixed with homogenised mycelium (1 ml containing 5000 spores/ml mixed with 1 mat of mycelium) 10 days later. The third group, consisting of non-vaccinated healthy fish, were challenged with an im injection of motile spores (i.e. viable) from strain PA7 (1 ml containing 5000 spores/ml). The first two groups of fish were maintained at a water temperature of 27°C, while the third group was maintained at 20°C throughout the challenge period. Blood was collected from the caudal vein of fish on day 30 of the study. Blood samples were also collected from seven snakehead fish infected with EUS during a natural outbreak of the disease in Pichit Province, Thailand. These fish had also been sampled for fungal, viral and bacterial analysis, and both A. *invaderis* and rhabdoviruses were recovered (Kanchanakhan, 1996a).

Sera from the fish of each group were pooled and screened by Western blot analysis as in SEction 3.7. Fungal extracts (10 µl of 2 mg protein/ml solution and 10µl sample buffer) were applied to each lane of the gel, which was then subjected to electrophoresis for 45 min at 200 V. Fungal antigens were transferred from the gel to sheets of nitrocellulose membrane by a wet blotting system using 50 V for 60 min. The gels were stained with 0.1 % (w/v) Coomassie blue R250, while the nitrocellulose membranes were washed with two changes of Tris buffered saline with Tween-20 (TTBS). Non-specific binding sites were blocked by incubating the membranes with 1.0 % (w/v) BSA in distilled water for 2 h. They were washed twice in TTBS, before applying the fish sera [1/10 dilution in PBS (0.2M, pH 7.3)]. The nitrocellulose membranes were incubated for 60 min at 20°C, then were washed twice with TTBS. They were again incubated for 60 min at 20°C, this time with rabbit anti-snakehead IgM [diluted 1/100 in PBS] (courtesy of Jitkasem Changphong, AAHRI). They were washed twice with TTBS, then goat anti-rabbit IgG-HRP conjugate (SAPU) (diluted 1/100 in TTBS) was applied to the membranes for 1 hour. Unbound conjugate was removed by washing the membranes twice with TTBS, followed by one wash with PBS. The assay was developed by incubating with chromogen (6mg DAB) (Sigma) dissolved in 10 ml of substrate buffer (20mM Tris hydrochloride, 500 mM NaCl, 30 µl H_2O_2, pH 7.5) until bands appeared. The reaction was stopped by washing the membranes with distilled water for 10 min.

SDS-PAGE protein profiles of the extracted *Aphanomyces* mycelium were visualised with Coomassie blue stain (Figure 3.8.9). Similarities in the banding patterns were evident between the extracts EUS (PA7), MG (NJM9030) and RSD (3P) pathogenic isolates and these were markedly different to the profiles obtained with the saprophyte (F3SA) and *A. astaci* (FDL458).

The response of the snakehead anti-*A. invaderis* sera to the different *Aphanomyces* mycelium extracts by Western blot analysis reflected the similarities found between the protein profiles of the *Aphanomyces* strains by SDS-PAGE (Figure 3.8.10). Sera from healthy fish elicited a weak non-specific reaction with all mycelium extracts, particularly with bands present at around 40 and 55 kDa in the extracts of the pathogenic strains (PA7, NJM9030 and 3P) (Figure 3.8.10a, lanes 1, 3 and 4, respectively). Sera from fish immunised with the spores and mycelium from strain PA7 produced a stronger reaction with these extracts than was observed with healthy fish sera, particularly with the 40 and 55 kDa bands (Figure 3.8.10b). This sera also identified a band at 37 kDa, unique to the mycelium extracted from saprophytic *Aphanomyces* strain, F3SA (lane 2), while only an undefined region of staining between 36 and 80 kDa was observed with the sera against A. astaci (FDL458) (Figure 3.8.10b, lane 5). As well as recognising the bands mentioned above, antisera from both the experimentally challenged fish and fish naturally infected with *A. invaderis* (Figure 3.8.10c and 3.8.10d), identified a low molecular weight band at around 11 kDa, which was not detected by the immunised sera from group two fish immunised with heat killed fungi and which was not apparent on the profiles of saprophytic strain of *Aphanomyces* or with *A. astaci* (FDL458). The 37kDa band in the F3SA extract and the 55 and 40kDa bands in the PA7 extract recognised here by the snakehead sera appeared to be the same bands as recognised previously by the rainbow trout (see Section 3.8.1.2 and Table 3.8.6).

As mentioned in Section 3.8.1.2, the antibody titre of naïve rainbow trout measured by ELISA four weeks after challenging them with A. *invaderis* (PA7) spores (1x103) was significantly higher than control fish. A possible reason for this increase may be that the challenged fish were also producing antibodies to the 11 kDa band of the fungus growing *in vivo*. The snakehead fish vaccinated above with killed fungi did not produce antibodies to this band. Analysis of sera sampled from the rainbow trout still remains to be performed by Western blot to establish if they do in fact recognise the 11 kDa band.

In the second vaccination trial eighty two snakehead fish (≅ 200g) were obtained from a farm located in Suphanburi Province, Thailand. The fish were divided into two groups and maintained in 150 L tanks during the course of the vaccination. One group of forty fish were vaccinated ip with

a mixture of sonicated PA7 mycelium and spores. Forty control fish were injected with sterile PBS. The fish were transferred to 75 L tanks (eight fish per tank) with a static water system five weeks post vaccination. They were fed once daily with a commercial pellet diet and partial water changes were carried out daily. Aeration was continuously supplied through air stones. A suspension of A. *invaderis* (isolate PA7) secondary zoospores were prepared. Both vaccinated and non-vaccinated fish were injected im with 0.1 ml of the suspension on their left shoulder. The fish were maintained at 22°C and muscle sampled regularly throughout the trial. The antibody titres of the fish were determined prior to the challenge using an ELISA (Table 3.8.7). No significant differences were observed in these levels between the vaccinated and the non-vaccinated fish. Both groups of fish produced a bigger antibody response against the ECP of PA7 compared to PA7 extract. However, the response was non-specific as shown by the antibody titre values obtained for the control fish. Western blot analysis remains to be performed on the sera from vaccinated and non-vaccinated snakehead fish sampled at the end of the challenge.

Macrophage infiltration and the presence of fungus determined by IHC is shown in Table 3.8.8. No differences were evident in the inflammatory response between the vaccinated and the non-vaccinated fish, examined by histopathology. It was not possible to determine whether only macrophages had moved to the site of the growing fungus or if lymphocytes were also present in the H&E and IHC stained muscle sections. Possibly cytotoxic T-cells may have also been present in the sections from the vaccinated fish. These are cells which elicit a specific immune response (i.e. a memory response as a result of the vaccination) and which have been implicated in the immune response of mammals against fungal infections. It may be possible to determine the presence of lymphocytes by IHC using an rabbit anti-snakehead IgM polyclonal sera. Lymphocytes express IgM (or parts of the molecule on their surface) which can be identified by the rabbit polyclonal antisera. Unfortunately, this method does not allow the differentiation of subpopulations of lymphocyte. This is an interesting area for future research, however.

3.8.1.5 Challenge experiments and non-specific immune response of Tilapia (*Oreochromis nilotica*)

Tilapia were chosen as a fish model for species not susceptible to EUS. The reason why they are able to resist infection of A. *invaderis* could be due to a number of reasons. For example it may possibly have something to do with the ability of their immune response to non-specifically attack the fungus. Currently, little information is available for normal parameters of the non-specific immune responses of Tilapia. Normal range values for a variety of components of the innate response of tilapia were, therefore, examined and are shown in Table 9.3.8.

Tilapia (50g) were challenged with 0.2ml (1x103) PA7 spores im and maintained at 20°C. NBT activity was examined in these fish at 4, 8 and 14 days (Table 9.3.10), but no apparent difference between experimental and control fish was observed. Although muscle samples were taken over the course of the challenge they remain to be analysed. The MSc student is currently repeating this challenge.

3.8.1.6 Protease activity of *Aphanomyces*

Proteolytic activity of fungus has been extensively examined by several authors. This activity is undoubtedly important in the pathogenicity of A. *invaderis*. Azocasein hydrolysis described by Prestidge *et al* (1971) was used to examine the activity of proteases in ECP and mycelium homogenates. The hydrolysis was carried out at a variety of pH (4.5 - 6.5). Mycelium from F3SA contained higher activity than that of PA7 per weight of fungal mycelium tested. The proteolytic activity of the ECP from both fungi was higher than that observed of the corresponding mycelium preparation and this activity again increased with pH (Table 3.8.11). Attempts were made to characterise the different proteases and determine their molecular weight using PAGE. Various protein solutions were incorporated into agarose gel prior to performing PAGE. The gels were then incubated over night in buffers of varying pH and zones of clearing showed where protease activity had taken place. Only very faint bands were observed on the gel and the protocol remains

to be optimised. Had time allowed, further identification and purification of these enzymes would have been performed and their activity examined when administered to fish.

3.8.7 Contribution of outputs

The im challenge system used over the course of the study proved a very successful method for infecting rainbow trout with EUS. It was noted, however, that the viability and virulence of the spores appeared to differ between batches making the actual challenge dose difficult to standardise. It was also not a particularly suitable delivery method for evaluating vaccination trials and establishing if vaccinations could induce a protective immune response in fish since it does not represent a true model of infection for the disease. In actuality, the spores attach to the fishes skin and germinating hyphae gradually penetrate and invade the animal's muscle. The present system is a very vigorous challenge. Injection of thousands of spores into the muscle of the fish bombards the fishes defences, while lower spore doses did not induce infection. It is essential to develop a bath challenge of subsequent vaccination work.

Fungal spores need to breach the physical barriers of the fish before they can penetrate the fishes muscle tissue. It is believed that fungal invasion is aided by damage to the skin or scales by environmental factors such as water pH, salinity, viruses or even the fishes ability to produce mucus. Possible methods of inducing a mucus associated immune response, seems a more likely mode for conforming protection against the invading spore, thus preventing the spore from breaching the fish's skin. Degradation of muscle tissue can occur some distance from the growing fungi, indicating that the activity of ECPs is present. Preparations of individual components of the ECP such as proteases may also be appropriate vaccine candidates.

Antibody responses elicited against fungal preparations appeared on the whole to be non-specific. As mentioned above this probably resulted from fish having been previously exposed to fungi ubiquitous in the fishes environment. Significantly higher antibody titres were observed in naïve fish challenged with the fungus. It would appear that the growth of the fungus *in situ* induces antibodies which are not produced when fish are injected with dead fungal preparations. Challenged snakehead fish produced antibodies to an 10kDa band present on the growing fungus. When fish were injected with fungal preparations containing this band they were unable to recognise it, not even when they were vaccinated with purified electroeluted 10kDa band in FIA. The results, therefore, suggest that the 10 kDa band is not immunogenic to the fish when it is presented as a vaccinate in the form of mycelial extracts, but is immunogenic when the fungus is actually growing within the fish. Sera from rabbits immunised with the electroeluted 10kDa band did not recognise this band after immunisation, but did recognise a band at 55kDa (Section 3.7). We are unable to explain this observation, but possibly differences in the antigenicity of this band may be due to alterations in its structural confirmation during vaccine preparation compared to *in situ* fungal growth within the fish. These findings have serious implications for the development of vaccines against EUS. Possibly, protective antibodies are produced to antigens present on growing fungus, but it would be very difficult to use these as a vaccine if the fungus needs to be alive for a protective response to be elicited and if conformational changes occur as a result of processing the dead fungus. Once an oubreak of EUS has occurred in an area it generally reoccurs with similar severity over the next 2-3 years and whilst not disappearing, later occurs with reduced frequency. This may represent a development of resistance by remaining fish. Passive immunisation with sera from challenged fish should show if protection is conferred on the fish after a natural *A. invaderis* challenge.

Substantial macrophage activity occurs around the invading fungus and stimulation of this response may offer the fish an alternative route of protection from fungal invasion. Future studies should try to enhance the fish's inflammatory response against the fungus by using immunostimulators substances such as glucans and adjuvants. The inflammatory response observed in fish artificially challenged with the fungus closely resembled that seen during a natural challenge. One of the problems of using IHC to assessing the affects of vaccination on

fungal challenges is that the examination is restricted to the area of muscle sampled and higher levels of muscle degeneration and fungus may be found in regions of unsampled muscle, thus giving a bias view to the extent of the infection ie the technique is not quantitative, only qualitative.

Since work to date has shown that the antibody responses by fish against *A. invaderis* can be very non-specific, perhaps it is unfeasible to rely on antibody responses to protect the fish against the fungus. Snakehead fish vaccinated and challenged in Thailand did not show increased survival as a result of the vaccination. This may have been because the im challenge system used was too vigorous a method for the assessment. The more subtle bath challenge may indeed have shown protection. The inflammatory response looked similar for both vaccinated and the non-vaccinated fish, but possibly cytotoxic T cells were present in the immune response of the vaccinated fish. As mentioned above, this is a specific cellular response reliant on a memory response produced by vaccination and is known to be important to mammals in combating fungal infections. No cell markers are currently available for fish cytotoxic T cells, however, and we are therefore unable to identify them within a lymphocyte population. The only thing we may currently be able to identify is the infiltration of the total lymphocytes post vaccination using the rabbit anti-snakehead IgM polyclonal antisera.

As a final note, the fact that rainbow trout are susceptible to the disease has significant implications for the fish farming industry in Europe, especially with regard to the rate at which the disease is spreading. EUS is known to affect regions, such as the Himalayan valley regions of Nepal, where water temperatures are comparable to those found for trout culture.

Table 3.8.1: Determination of dose of *Aphanomyces invaderis* (PA7) spores for rainbow trout challenge.

Dose of spores per fish	Sample number	Specific mortalities (%)	Antibody titre log2+1	Visible lesions (%)	IHC (%)
1 x102	25	0	4.0⇧1.9	0	0
1 x103	25	8	2.6 ⇧2.0	0	48
1 x104	25	60	9.2 ⇧0.7	44	100
1 x105	25	84	8.2 ⇧2.4	76	100
PBS	25	0	5.2 ⇧2.5	0	0

Table 3.8.2: Antibody titre of rainbow trout four weeks after challenging with *Aphanomyces invaderis* (PA7) spores

Dose of spores per fish	Sample number	Specific mortalities (%)	Antibody titre log2+1	Visible lesions (%)	IHC (%)
1 x103	25	2	7.3 ⇧2.2	20	80
PBS	6	0	3.8 ⇧1.5	0	0

Table 3.8.3: Antibody titre of fish vaccinated with a pathogen (PA7) or a saprophytic strain (F3SA) of *Aphanomyces* assessed by an enzyme linked immunosorbant assay (ELISA)

		antibody titre (log2+1)*		
		immunising antigen		
coating antigen	weeks	PBS	F3SA	PA7
PA7 extract	3	2.5 ⇧1.6	1.5 ⇧0.9	2.0 ⇧0.7
	5	3.0 ⇧1.4	5.5 ⇧1.1	2.8 ⇧1.8
	7	2.2 ⇧1.6	5.0 ⇧1.9	3.0 ⇧2.0
	9	2.0 ⇧1.0	5.0 ⇧2.5	4.8 ⇧1.1
F3SA extract	3	3.0 ⇧1.2	2.5 ⇧1.6	3.5 ⇧1.5
	5	2.2 ⇧1.3	2.2 ⇧1.3	4.5 ⇧1.6
	7	3.0 ⇧1.9	5.2 ⇧1.6	5.2 ⇧0.4
	9	1.5 ⇧0.9	2.8 ⇧1.8	4.8 ⇧1.5

* n = 4

Table 3.8.4: Antibody response of fish vaccinated with a pathogenic (PA7) or a saprophytic strain (F3SA) of *Aphanomyces*

Immunising antigen	antibody titre (log2+1)*			
	week 3	week 5	week 8	week 11
PBS	2.8 ⇧2.4	4.0 ⇧0.9	4.5 ⇧0.7	4.0 ⇧0.0
F3SA sonicate	3.2 ⇧1.6	3.4 ⇧1.2	3.0 ⇧1.7	4.2 ⇧0.4
PA7 sonicate	3.6 ⇧1.9	3.2 ⇧1.8	4.2 ⇧1.7	2.8 ⇧2.2
PA7 filtrate	2.6 ⇧2.2	4.4 ⇧2.0	3.8 ⇧1.6	4.2 ⇧1.3
PA7 extract	4.0 ⇧2.0	4.0 ⇧1.7	4.6 ⇧2.1	4.5 ⇧1.8
PA7 extract in FIA	2.4 ⇧2.3	4.2 ⇧0.4	4.0 ⇧1.7	7.6 ⇧2.3

* n = 5

Table 3.8.5: Antibody titre of fish vaccinated with a variety of pathogenic *Aphanomyces invaderis* (PA7) preparations

immunising antigen	sample number	antibody titre (log2+1)
homogenised mycelium	6	8.0 ⇧3.5
ECP	6	7.6 ⇧1.2
10 kDa band	8	6.8 ⇧1.5
PBS	6	3.8 ⇧1.5

Table 3.8.6: MW of proteins recognised by antibodies of fish vaccinated with a pathogenic (PA7) or a saprophytic strain (F3SA) of *Aphanomyces*

Immunising antigen	kDa		
	F3SA extract	PA7 extract	10 kDa band
PBS	37 (faint)	55 (faint)	nb
F3SA sonicate	37	55	nb
PA7 sonicate	nb	nb	nb
PA7 filtrate	37	(strong), 40 (faint)	nb
PA7 extract	37	55, 40	nb
PA7 extract in FIA	37	55 (strong)	nb

* n = 5
nb: no band
FIA: Freund's complete adjuvant
kDa: kilodalton

Table 3.8.7: Determination of antibody titre of snakehead fish vaccinated with *A. invaderis* (PA7)

coating antigen	antibody titre (log2+1)*	
	non-vaccinated fish	vaccinated fish
PA7 extract	2.7 ⇧2.2	1.0 ⇧0.0
PA7 ECP	5.2 ⇧3.2	4.6 ⇧3.0
F3SA extract	4.6 ⇧1.8	1.2 ⇧0.7
F3SA ECP	3.6 ⇧2.3	4.6 ⇧1.3

* n = 16

Table 3.8.8: Progression of *Aphanomyces invaderis* (PA7) challenge in snakehead muscle

Sample day	Sample number	IHC (%)	Macrophage infiltration vaccinated fish	Macrophage infiltration non-vaccinated fish
0	3	-	none	none
1	3	-	very slight	very slight
3	3	+	yes	yes
6	3	+	yes	yes
11	5	+	intense	intense
18	5	+	intense	intense

IHC: Immunohistochemistry (presence of the fungus)
NB: remaining fish not accounted for in the table died over the course of the trial but the presence of fungus was not present, therefore excluded from trial

Table 3.8.9: Normal range values for an array of non-specific immune responses of Tilapia

Parameter	sample no.	normal range ⇧SD
Weight (g)	12	112.5 ⇧34.5
Serum:		
Haematocrit	12	34.2 ⇧3.5
RBC (x109 ml-1)	12	1.7 ⇧0.3
WBC (x107 ml-1)	12	11.3 ⇧4.1
Differential WBC count (%)		
thrombocyte	8	29.5 ⇧9.7
lymphocyte	8	64.1 ⇧10.2
neutrophil	8	6.0 ⇧1.6
macrophage	8	0.6 ⇧0.5
Lysozyme activity (change in OD510/min)	12	0.005 ⇧0.004
Complement activity	12	0.016 ⇧0.012
Serum protein (mgml-1)	12	2.34 ⇧0.34
Neutrophils:		
NBT positive cells (%)	12	10.1 ⇧8.9
phagocytic cells (%)	9	21.3 ⇧5.6
Macrophage:		
NBT activity (per 1x105)		
(PMA)	9	0.245 ⇧0.025
(no PMA)	9	0.154 ⇧0.018
phagocytic cells	12	27.6 ⇧11.0

WBC: white blood cell
RBC: red blood cell
OD: optical density
NBT: Nitroblue tetrazolium
PMA: Phorbol Myristate Acetate

Table 3.8.10: NBT activity of Tilapia head kidney macrophages from fish challenged with pathogenic (PA7) *Aphanomyces invaderis*

weeks	NBT activity *					
	F3SA		PA7		PBS	
	no PMA	PMA	no PMA	PMA	no PMA	PMA
4	0.17 ⇧0.04	0.39 ⇧0.18	0.17 ⇧0.03	0.26 ⇧0.06	0.15 ⇧0.04	0.36 ⇧0.16
8	0.11 ⇧0.08	0.35 ⇧0.18	0.17 ⇧0.06	0.32 ⇧0.16	0.17 ⇧0.05	0.31 ⇧0.15
14	0.15 ⇧0.05	0.34 ⇧0.05	0.15 ⇧0.05	0.29 ⇧0.11	0.14 ⇧0.06	0.30 ⇧0.14

*OD610 for 1x105 cells (n=4)

WBC: white blood cell
RBC: red blood cell
OD: optical density
NBT: Nitroblue tetrazolium
PMA: Phorbol Myristate Acetate

Table 3.8.11: Proteolytic activity of pathogenic (PA7) and saprophytic (F3SA) *Aphanomyces* assessed by the hydrolysis of azocasein at various pH

	pH*				
	4.5	5.0	5.5	6.0	6.5
PA7 extract	0.08 ⇧0.01	0.08 ⇧0.02	0.13 ⇧0.01	0.10 ⇧0.02	0.20 ⇧0.04
F3SA extract	0.23 ⇧0.01	0.24 ⇧0.01	0.23 ⇧0.03	0.24 ⇧0.02	0.26 ⇧0.02
PA7 ECP	0.19 ⇧0.04	0.22 ⇧0.03	0.25 ⇧0.01	0.28 ⇧0.01	0.20 ⇧0.04
F3SA ECP	0.33 ⇧0.04	0.36 ⇧0.04	0.38 ⇧0.05	0.46 ⇧0.06	0.50 ⇧0.06
No fungal extract	0.22 ⇧0.01	0.03 ⇧0.01	0.02 ⇧0.00	0.00 ⇧0.01	0.00 ⇧0.00

* Azocasein units = change in OD at 430nm over 60min / ml of solution

Figure 3.8.1

Rainbow trout (*Oncorhynchus mykiss*) artificially infected with A. *invaderis*

Figure 3.8.2

Phagocytosis of *A. invaderis* spores by rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages: (a) shows a number of macrophages which have engulfed Congo red stained spores and some have clumped together to form giant cells; (b) mycelium can be seen to be growing from germinated cysts with macrophages clumping around the growing mycelium

Figure 3.8.3

Immunohistochemsitry of A. *invaderis* germinated spores and mycelium after rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages had phagocytosed spores

Figure 3.8.4

Rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages join together around the fungus to form large giant cells

Figure 3.8.5

Rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages cluster around the tips of the growing mycelium cultured *in vitro*

Figure 3.8.6

Examples of interactions observed by electron microscopy between rainbow trout head kidney macrophages and A. *invaderis* when cultured *in vitro*:- (a) illustrates the structure of mycelium which possesses a thick cell wall, the outer surface of which is covered with microvilli. The centre of the mycelium is vacuolated; (b) shows a macrophage containing a phagocytosed spore in its cytoplasm which has, as yet, not germinated; (c, d and e) illustrates macrophages which have attached onto the fungal mycelium, while macrophages attached to mycelium appear damaged by the fungus (f) and (g); a damaged macrophage attached to a germinating spore is pictured in (h).

Figure 3.8.7

(a) cross section of two rainbow trout head kidney macrophages wrapped around a piece of A. *invaderis* mycelium; (b) and (c) the junction where the two macrophages meet; (d) the tip of the mycelium. The microvilli appear to be secreting vesicles into the cytoplasm of the macrophage.

Figure 3.8.8

(a and b) different magnifications of the interaction between the mycelium and a rainbow trout head kidney macrophage; (c and d) highlight the interface between the macrophage and the mycelium.

Figure 3.8.9

Separation of *Aphanomyces* mycelium extract by SDS-PAGE. The gel (4-20%) was stained with Coomassie blue. Lanes: (1) PA7; (2) F3SA; (3) NJM9030; (4) 3P; (5) FDL458. Approximately 20 µg protein was applied to each lane.

Figure 3.8.10

The response of snakehead anti-*Aphanomyces* (strain PA7) sera against extracts of mycelium from *Aphanomyces* by Western blot analysis. a) healthy fish; b) fish immunised with spores and mycelium from strain PA7; c) fish experimentally challenged with PA7; and d) fish naturally infected with *Aphanomyces* Lanes: (1) PA7; (2) F3SA; (3) NJM9030; (4) 3P; (5) FDL458

3.9 PYROLYSIS MASS SPECTROMETRY (PYMS)

A paper based on work described in this chapter, entitled "Characterisation of *Aphanomyces invaderis* using pyrolysis mass spectrometry (PyMS)" has been sumitted to Mycological Research for publication.

3.9.1 Introduction

Pyrolysis mass spectrometry (PyMS) is an analytical technique that can be used to obtain biochemical fingerprints of whole micro-organisms (Magee, 1993). Briefly, the complex organic material of the sample is thermally degraded (pyrolyzed) in an inert atmosphere. Curie point PyMS, as used here, employs a ferro-magnetic foil as a sample carrier which is heated and maintained at its Curie point by means of high-frequency alternating magnetic field. The resulting vapour or pyrolysate is bombarded with low-energy electrons which generate molecular and fragment ions. These are separated by a quadrupole mass spectrometer on the basis of their mass:charge ratio (m/z) and displayed in the form of quantitative mass spectra.

Pyrolysis followed by gas chromatography (PyGC) has been used for the identification and characterization of filamentous fungi, including *Aspergillus* (Stretton *et al,* 1976) and *Penicillium* (Söderström and Frisvad, 1984). However the use of mass spectrometry as a separation method following pyrolysis provides greater resolution (>150 variables) than PyGC (~40 variables) and has been increasingly used in bacterial systematics (Shute *et al,* 1985; Winstanley *et al,* 1992; Magee *et al,* 1993; Manchester *et al,* 1995). Fungal studies using PyMS have been mainly restricted to yeasts (Windig and Haverkamp, 1982; White *et al,* 1994). Weijman *et al* (1984) evaluated PyMS as a method of diagnozing potato-gangrene caused by *Phoma,* and Niemann *et al* (1991) used this technique to demonstrate different levels of lignin degradation in carnations by *Fusarium* and *Phialophora*. However there are no reports using PyMS as an aid to classification in filamentous fungi. The present study aimed to evaluate PyMS as a method of discriminating different levels of Oomycete taxa: between strains of *A. invaderis*, species of *Aphanomyces* and genera of Saprolegniaceae.

3.9.2 Activities

Forty five fungal isolates were tested as listed in Appendix 1. Fungal colonies were grown in Petri dishes of GPY liquid media (Appendix 2a). The same batch of GPY was used throughout the experiment. Asian isolates were grown at room temperature (20-24°C) and UK and USA isolates were grown at 12°C. For each isolate, three squares of mycelium, approximately 2mm^2, were cut from the edges of actively growing colonies and used to inoculate a Petri dish containing GPY media. Halfway through the total growth period, the resulting three mycelial mats were again cut into 2mm^2 squares. The total incubation time was calculated from known growth rates of the isolates to produce end wet weights of approximately 0.3g, and ranged between three days for the saprophytic *Aphanomyces* spp to eight days for *A. astaci*. Ideally, identical growth conditions should be adopted for each isolate, but in this case that would result in mycelium at very different stages of growth. The present regime was used so that each fungal culture was rich in actively growing hyphal tips.

The resulting mycelial mat was washed in sterile distilled water and filtered through cheesecloth. This was repeated four times. The mycelial mat was then homogenized in liquid nitrogen and stored at -20°C. Preliminary work had shown that unhomogenized, washed mycelium produced less consistent results, possibly because of a lack of uniformity in the age of hyphae within the fungal preparation. Duplicate preparations of four isolates (S1PA, PA7, 10P and WSA) were prepared to check the reproducibility of different preparations of the same isolate.

Thawed homogenated material formed a paste, a small amount of which was smeared thinly on to alloy foils (50% iron : 50% nickel). The foils were inserted into pyrolysis tubes and oven-dried for a few minutes. It was found that excessive drying often resulted in the sample dropping off the foil. Three replicate tubes were prepared from each fungal homogenate. The samples were loaded on a RAPyD 400 pyrolysis mass spectrometer (Horizon Instruments Ltd, Heathfield, East Sussex) and pyrolyzed for three seconds at a Curie point of 530°C. Preliminary runs were carried out to evaluate the amount of homogenated material required on the foil to give total ion counts of between three and ten million.

3.9.3 Outputs and contribution of outputs

Canonical variate analysis (CVA) was performed on the pyrolysis data and the average value for the three replicates was calculated. A 3-D representation of these results (Figure 3.9.1) shows that the majority of *Aphanomyces* species could be easily distinguished from a wide scatter of *Achlya* and *Saprolegnia* outgroup species. Further CVA using the *Aphanomyces* species alone (Figure 3.9.2) succeeded in discriminating EUS, MG and RSD pathogens from the saprophytic strains. The variation within the group of saprophytes is probably an indication that at least two species are involved.

A dendrogram produced by group-average hierarchal cluster analysis (HCA) (Figure 3.9.3) gave a more clear-cut distinction between EUS, MG and RSD pathogens and the saprophytes by forming two main groups separated at a similarity index of 61%. The first main group clustered the EUS pathogen with RSD and MG isolates. Other studies described in this report indicate that these fungi all represent a single species. The PyMS evidence tended to support this hypothesis, although two isolates (33P and 4P) clustered with the main group at a much lower similarity index. Also of interest was the proximity that the crayfish plague fungus, *Aphanomyces astaci,* showed to the EUS-RSD-MG group.

UM *Aphanomyces* strains were shown to be distinct from *A. invaderis,* and instead clustered with saprophytic *Aphanomyces* to form the second group. This is consistent with the suggestion by Dykstra *et al* (1989) that at least one of the UM-isolates is *Aphanomyces laevis.*

Duplicate cultures of one isolate (WSA) clustered together at only 91% similarity, indicating that any of the detailed relationships at this level or above were unreliable. Therefore PyMS probably lacks the sensitivity to resolve intraspecific differences in these fungi. Indeed the isolates representing some species, *A. astaci* and *A. laevis* in particular, failed to group convincingly. However in the clustering of the EUS-RSD-MG group of fungi and their separation from non-pathogenic *Aphanomyces* species, the system proved highly discriminatory.

Figure 3.9.1 - above

Canonical variate analysis (CVA) of pyrolysis mass spectra of all fungus isolates. Markers represent the average of three replicates.

Figure 3.9.2 - below

CVA of pyrolysis spectra of *Aphanomyces* species only. Markers represent the average of three replicates.

Figure 3.9.3

Hierarchal cluster analysis (HCA) dendrogram of same data set as in Figure 3.9.2. Includes *Aphanomyces invaderis* (EUS), MG-*Aphanomyces* (MG), RSD-*Aphanomyces* (RSD), *Aphanomyces astaci* (*astaci*), UM-*Aphanomyces* (UM) and various saprophytic *Aphanomyces* species (Sap.). S1PA$^{(2)}$, PA7$^{(2)}$, 10P$^{(2)}$ and WSA$^{(2)}$ are duplicate cultures of S1PA, PA7, 10P and WSA respectively.

3.10 RANDOM AMPLIFICATION OF POLYMORPHIC DNA (RAPD)

Three manuscripts have been prepared involving work outlined in this section. A short publication on the molecular characterisation of *A. invaderis* by RAPD entitled "Pan-Asian spread of single fungal clone results in large scale fish-kills" is currently in press in the Veterinary Record, and is reprinted in Appendix 7. Another publication entitled "RAPD evidence for the origin of crayfish plague in Britain" has been resubmitted to Aquaculture following referees comments. A third, further publication is currently under preparation; this details the molecular characterisation of *A. invaderis* using RAPD analysis described here, as well as restriction fragment length polymorphism (RFLP) and ribosomal RNA (rRNA) gene sequencing undertaken at the Veterinary School, Glasgow University. This last manuscript includes all the isolates listed in Tables 3.1.2 and 3.1.3.

3.10.1 Introduction

Recently molecular analysis, and the use of random amplification of polymorphic DNA (RAPD) in particular, has enabled workers to establish detailed, intraspecific relationships between organisms. In the case of the crayfish plague fungus, *Aphanomyces astaci*, Huang *et al* (1994) used RAPD analysis to categorise three groups of Swedish isolates, and show that new strains are being introduced and affecting indigenous crayfish populations. Diéguez-Uribeondo *et al* (1995) later showed that a new Spanish *A. astaci* isolate represented another, more distantly related group.

In the present study, a wide selection of EUS, MG and RSD isolates were used for RAPD analysis, along with UM isolates, *A. astaci* and several saprophytic *Aphanomyces* spp.

Given that no UK isolates of *A. astaci* have been previously characterised using RAPD-PCR, this study offered a valuable additional opportunity to type the two UK isolates obtained for the various comparative studies (FDL457 and FDL458) according to the groups established by Huang *et al* (1994). It was envisaged that this would provide evidence of the origin of the crayfish plague in Britain.

In another ODA project on the molecular biology of *A. invaderis* (details under separate cover), D. Hart the Veterinary School, Glasgow University used restriction fragment length polymorphism (RFLP) and rRNA gene sequencing to demonstrate the detailed interspecific relationships between most of the isolates listed in Table 3.1.2 and 3.1.3.

3.10.2 Activities

This work was carried out at the Department of Physiological Mycology, University of Uppsala, Sweden.

3.10.2.1 Fungi

The isolates used in this study are indicated in Appendix 1. In addition to the fungi used in several studies here, additional *A. astaci* isolates were obtained from the culture collection of the Department of Physiological Mycology, Uppsala. These comprised J1, PI and Kv (representing RAPD groups A, B and C respectively, as described by Huang *et al,* 1994) and Pc (group D, Diéguez-Uribeondo *et al,* 1995).

3.10.2.2 DNA preparation

Genomic DNA was extracted using the following procedure for all isolates except *Aphanomyces astaci.* About 50 mg of mycelium grown in GPY broth was homogenised in 11 ml lysis buffer (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 2 % SDS). Proteinase K was added to a final concentration of 1 mg/ml and incubated overnight at 37°C with shaking. The sample was then chilled on ice for 10 min. Five millilitres of saturated NaCl was added to the tube which was mixed and then chilled for another 5 min. Precipitated protein was pelleted by centrifugation at 2000 g for 15 min at 4°C. The supernatant was transferred to a fresh tube and centrifuged again to ensure removal of the

precipitate. RNase A was added to a final concentration of 20 μg/ml and the tube incubated for 30 min at 37°C. Two volumes of 100 % ethanol was added to the sample which was then mixed and stored at -20°C overnight. The sample was centrifuged at 2000 g for 15 min at 4°C. The resulting DNA pellet was washed with 10 ml ice-cold 75 % ethanol and centrifuged again for 5 min. The pellet was vacuum-dried and resuspended in 200 μl dH_2O.

DNA preparations of *A. astaci* isolates were made from cultures grown in PG-1 broth using the Nucleon II kit by following the supplied procedure for filamentous fungi. Briefly, mycelium was ground in liquid nitrogen and scraped into 2 ml lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% SDS). Three microlitres of 10 mg/ml RNase A was added and the sample incubated at 37°C for 30 min. Then 1.5 ml 5M sodium perchlorate was added to the sample which was mixed for 15 min and incubated at 65°C for 25 min. Ice-cold chloroform (5.5 ml) was then added and the solution mixed for 10 min and centrifuged at 800 g for 1 min. About 800 μl of a silica suspension was added and the tube centrifuged at 1400 g for 3 min. The clear DNA-containing phase was recovered to which equal volume of 99 % cold ethanol was added and the resulting precipitate was centrifuged at 5000 g for 5 min. The DNA pellet was washed in 70 % ethanol and centrifuged again. The final pellet was vacuum-dried and resuspended in 100 μl TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA).

3.10.2.3 RAPD-PCR

Each 50 ml reaction tube contained 5-50 ng genomic DNA, 20 pmol primer, 5 μl 10X USB buffer (Amersham), 1500 mM $MgCl_2$, 200 mM of each dNTP and 1.5 units of AmpliTaq DNA polymerase. Fourteen random 10-mer primers were tested (A3, A4, A6, A7, A10, A12, A18, A19, A20, B1, B2, B4, B5 and B10; Operon Technologies). PCR reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 using the same temperature cycle as Huang *et al* (1994). Amplification products were separated on 1.4% agarose gels stained with ethidium bromide and visualised under UV illumination.

3.10.2.4 Cluster analysis

The presence or absence of fragments given by each isolate was marked by hand, and the data analysed using the "Dice" similarity measure in the statistics package, SPSS for Windows Release 601 (SPSS, 1988). This is a binary matching coefficient that gives double weight for matching bands. It is identical to the formula of Nei and Li (1979), which is used in most RAPD studies of fungi, including those of Huang *et al*, 1994 and Diéguez-Uribeondo *et al*, 1995 on *A. astaci*. Nei and Li's similarity coefficient (F) is given as:

$$F= 2n_{XY}/(n_X + n_Y)$$

where n_X and n_Y are the numbers of fragments in samples X and Y respectively and n_{XY} is the number of fragments shared by the two samples.

3.10.3 Outputs

3.10.3.1 An average of 80.7 bands per isolate which was used to calculate similarity coefficients (F). The mean similarity coefficient (F ± SD) comparing all the EUS, MG and RSD isolates was 0.95 ± 0.03 (Figure 3.10.1). The six saprophytic *Aphanomyces* spp, four Swedish *A. astaci* isolates and two UM-*Aphanomyces* isolates in combination gave an average similarity coefficient of 0.14 ± 0.05 compared with the *A. invaderis* isolates. A dendrogram generated from this data is given in Figure 3.10.2. Two sample gels showing polymorphism fragments generated using two random primers are given in Figure 3.10.3.

3.10.3.2 Crayfish plague

When the *A. astaci* isolates are analysed separately, an average of 56.7 bands per isolate was used to calculate similarity coefficients (Figure 3.10.1 and 3.10.4). The British isolates FDL457 and FDL458 were found to be almost identical, with an F value of 0.98. In comparison to the other strains the British isolates most resembled PI, the strain representing group B, with an

average similarity coefficient of 0.90. Average F values between the British isolates and group A, C and D fungi were 0.58, 0.72 and 0.34 respectively.

3.10.4 Contribution of outputs

Given that RAPDs have been shown to be very sensitive in distinguishing strains of Oomycete fungi (Huang *et al*, 1994; Diéguez-Uribeondo *et al*, 1995; Cooke *et al*, 1996), the results of this study indicate an extreme lack of genetic diversity between all the EUS, MG and RSD isolates, which may then be considered "clonets" by the definition given by Tibayrenc (1996). Figure 3.10.2 shows that the Philippine isolates may show some slight variation from the other isolates but that this is not great enough to be considered significant.

These results correspond with separate RFLP and rRNA analyses which also failed to show any differences between any of the EUS, MG and RSD isolates (Hart, pers. comm.). As the isolates are shown to be con-specific, it is recommended that the name *Aphanomyces invaderis*, formally described by Willoughby *et al* (1995) is used for all the EUS, MG and RSD ulcerative disease isolates.

The genetic homogeneity between all the *A. invaderis* isolates may be associated with observations that it lacks any sexual reproductive structures (Willoughby *et al*, 1995). However, RAPD studies on *A. astaci*, which is similarly asexual, yielded four distinguishable groups from 15 European isolates (Huang *et al*, 1994; Diéguez-Uribeondo *et al*, 1995). These showed an average between-group similarity of 0.25 ± 0.08, and the average within-group similarity (0.84 ± 0.11) was also lower than for the *A. invaderis* isolates (data recalculated from Diéguez-Uribeondo *et al*, 1995). This indicates that there have been several introductions of *A. astaci* to Europe over a number of years whereas *A. invaderis* has achieved its colonisation of Asia in one relatively rapid episode. This correlates with the accounts of the spread of EUS outbreaks described in Section 1.

<u>Crayfish plague</u>

Despite its long history in most of Europe, the crayfish plague was only first reported in Britain in 1981 (Alderman, 1993). This coincided with the wave of imports of signal crayfish, *P. leniusculus*, which began in 1976. Although this species is known to act as a vector for the disease (Alderman *et al*, 1990), no direct evidence could be supplied to link the events. Indeed it was conjectured that the outbreaks may simply be a recrudescence of a disease that had been present in Britain for as long as in mainland Europe. However, the indication here that English stocks of *A. astaci* belong to a group only isolated since 1970 and which originate from imported signal crayfish in Sweden, provides strong evidence that the disease was introduced to Britain with similar shipments.

The Spanish strain of *A. astaci* (group D) is known to be very different physiologically from other strains and may also differ in terms of pathogenicity (Diéguez-Uribeondo *et al*, 1995). Therefore this raises the possibility of further introductions of new, more pathogenic strains of the fungus and mitigates against a relaxation in restrictions on the movement of crayfish.

Figure 3.10.1

Similarity matrix of *Aphanomyces* isolates calculated from RAPD band patterns

Figure 3.10.2

Dendrogram constructed from RAPD data showing extreme homogeneity of EUS, MG and RSD isolates

Figure 3.10.3

RAPD band profiles using primers (a) top - A19 and (b) bottom - A7 (Operon Technologies). Markers denote 1200, 800 and 400 base-pairs.

Lanes for (a) correspond to (1) TA1, (2) RF6, (3) RF8, (4) S3PA, (5) G2PA, (6) PA1, (7) PA4, (8) 96PA, (9) BR, (10) BH, (11) 36/1P, (12) 10D, (13) 30P, (14) 33P, (15) 34P, (16) 4P, (17) 10P, (18) 24P, (19) NJM9030, (20) NJM9201, (21) 84-1240,(22) TF41, (23) FDL457, (24) PC.

Lanes for (b) are the same except that (22) is F3SA

Figure 3.10.4

RAPD band profiles using primers (a) A10 and (b) B01 (Operon Technologies). Centre markers denote 1200, 800 and 400 base-pairs. Lanes 1 and 2 are UK isolates FDL457 and FDL458. Lanes A, B, C and D are isolates J1, Pl, Kv and Pc representing *A. astaci* groups A, B, C and D.

3.11 INFORMATION DISSEMINATION ACTIVITIES

3.11.1 Conferences

3.11.1.1 An oral presentation entitled "The aggressive *Aphanomyces* pathogen of Asian freshwater and estuarine fishes" was given at the Sixth International Marine Mycology Symposium, Portsmouth in July 1995.

3.11.1.2 A poster presentation entitled "Comparative studies of the EUS *Aphanomyces* and other fungi associated with fish diseases" was given at the World Aquaculture Society Conference, Bangkok in February 1996.

3.11.1.3 Another poster presentation entitled "Immunocytological study of zoospore and cyst surface characteristics in fish and invertebrate pathogenic Oomycete "fungi"" in cooperation with G.W. Beakes, A.R. Hardham, J. Kerwin and E. Peterson was given at the Symposium on Fungal Physiology and Biochemistry, Nottingham in April 1997.

3.11.2 Training

3.11.2.1 JHL provided training in fish mycology to two regional scientists from Philippines and Vietnam, during a trip to Thailand in February 1996.

3.11.2.2 Lectures and practicals were held by KDT and JHL for students on the Aquatic Pathobiology MSc at Stirling University. This included supervising two SE Asia Aquatic Disease Control Project - funded students with their final projects, involving studies on *A. invaderis*.

3.11.2.3 In March 1997, JHL joined a team from AAHRI, NACA and NSW-Fisheries (Australia) that presented two sessions of training seminars to Punjabi and Sindhi fisheries officers and fish farmers at Lahore and Karachi, during an EUS mission to Pakistan (Plate 3.11.1). A report of the mission will be supplied to ODA under separate cover.

Plate 3.11.1

Mission to investigate EUS outbreaks in Pakistan 9-19 March 1997

(a) Training seminars and practicals were given at Department of Fisheries offices in Lahore and Karachi

(b) A number of farms, hatcheries and canal sites were visited to assess the extent of EUS outbreaks

4. SUMMARY OF OUTPUT CONTRIBUTIONS

4.1 General discussion

This study has clearly established that it is not the case that multiple opportunistic fungal species are responsible for the development of EUS lesions in fish; but rather that the specific fungus described by Willoughby *et al* (1995) as *Aphanomyces invaderis* is the cause. Comparative molecular, morphological, biochemical and behavioural studies described here have shown that MG and RSD isolates represent the same fungus. Any variation in morphology described by different workers (Table 3.1.1) may be due to culture conditions or observational differences. The present studies, combined with restriction fragment length polymorphism (RFLP) analyses and rRNA gene sequence data generated by a parallel ODA funded project based at Glasgow University, together confirm that taxonomically *A. invaderis* can be placed within the genus *Aphanomyces*, and, out of the species tested, it is most closely related to the crayfish plague fungus, *A. astaci*. Its unusual cell wall structure and ability to cross-react with anti-*Phytophthora* monoclonal antibodies warrant further investigation.

The extreme genetic homogeneity between all the *A. invaderis* isolates indicate that it is the agent that has spread across Asia resulting in new outbreaks of EUS. The spread in some areas can be accounted for by flood events like the serious inundations in Bangladesh in 1988. However, most outbreaks of EUS can only be explained by the massive cross-border movement of fish for the aquaculture or ornamental fish industries. Indeed, one isolate tested here (NJM9201) was obtained in Japan from a shipment of dwarf gouramies imported from Singapore (Hatai, 1994). Potential dangers extend beyond Asia as trials have demonstrated that *A. invaderis* can also produce severe pathological changes in several European salmonid species.

As this study indicates, the unrestricted trade in aquatic animals is already having a major negative impact on aquaculture, fisheries and indigenous aquatic biological diversity through the transmission of disease. Several major programmes involving the Food and Agriculture Organisation (FAO) and Office International des Epizooties (OIE) are being undertaken to develop effective regional health certification and quarantine guidelines, and this study lends support to the importance and urgency of these initiatives.

At present, neither *Aphanomyces invaderis* nor *Aphanomyces piscicida* are listed in the International Mycological Institute's (IMI) Index of Fungi; nor are they kept in any of the major international fungal culture collections. As discussed in Section 3.1.1.3, *A. piscicida* does not conform to the International Code of Botanical Nomenclature as any taxon of the rank of family or below requires a Latin description (Korf, 1995). As the description of *A. invaderis* by Willoughby *et al* (1995) adheres to this standard, we recommend adoption of this name for all the EUS, MG and RSD isolates. A holotype specimen (RF6) is presently being maintained at the Freshwater Biological Association, Windermere, England as well as at the Institute of Aquaculture, University of Stirling, Scotland.

Given that the same pathogenic fungus is responsible for EUS, MG and RSD, there is a strong argument for adopting the same name for all of these diseases. Of the names presently used, mycotic granulomatosis (MG), first proposed by Miyazaki and Egusa (1972), is considered the most appropriate as it describes the defining feature of the disease. Although the name EUS is widely known in SE and South Asia, it has been used to describe a number of unrelated disease conditions, and adoption of a more specific name may avoid some of the confusion that has resulted. In addition, the description of the disease given by Roberts *et al* (1994) (See Section 1.1) is not wholly accurate as work in Australia has indicated that outbreaks there do not require a "complex aetiology"; and, although the disease has predominated in tropical and subtropical areas, it is known to be capable of affecting cold water fish like rainbow trout in Japan and *Tor* sp in the Himalayan valley regions of Nepal. Any new name or definition for EUS would, however, have to be deliberated by a consultation of regional experts.

The relationship of EUS to UM remains unclear. The *Aphanomyces* fungus considered by Dykstra *et al* (1986) to be an invasive pathogen of UM, is shown here to be quite unlike *A. invaderis*. However, there has been no evidence of its ability to grow in internal tissues of fish (Section 3.2; Noga, 1993), and it is probable that another, as yet uncharacterised invasive pathogen is involved in UM.

Roberts *et al* (1993) (and Noga, 1993, in the case of UM) have commented that dead fungus is seen in the lesion of fish and sporulating mycelium is never seen, implying that no mechanism of fish to fish transmission could be found and that there may be some environmental source of infections. The infectivity studies described in Section 3.2 do not contradict this hypothesis. However, Callinan (pers. comm.) has shown SEM photographs of artificially infected fish clearly demonstrating the emergence of the fungus through the skin and its subsequent sporulation. This, along with the fact that *A. invaderis* has not been isolated from water bodies not experiencing an active EUS outbreak; and the obvious difficulties *A. invaderis* would have competing with vigorous saprophytic fungi outside a fish host; all suggest that diseased fish, and not environmental sources, are the main source of infective material for EUS. Further studies are required to show the length of *time A. invaderis* motile zoospore can remain infective after sporulation, and how long a quarantined EUS-affected pond remains infective without further introduction of *A. invaderis*.

4.2 Main conclusions

4.2.1 A single specific fungus, recently named *Aphanomyces invaderis,* is responsible for the mycotic granulomas characteristic of epizootic ulcerative syndrome (EUS).

4.2.2 *A. invaderis* represents part of the same clonal lineage as *Aphanomyces* isolates from Australian redspot disease (RSD) and from Japanese mycotic granulomatosis (MG) (also known as *Aphanomyces piscicida*). Given that *A. invaderis* has been given a formal published description, this name is adopted here to describe all these fungi. There is therefore a strong argument for adopting the same name for EUS, MG and RSD; and of these, mycotic granulomatosis (MG) is considered the most appropriate.

4.2.3 Fungal isolates from American ulcerative mycosis (UM), crayfish plague, saprolegniasis and saprophytic infections of aquatic animals are distinct species from *A. invaderis* and unable to reproduce EUS. However, there is a possibility that the true UM pathogen is not among the isolates so far characterised.

4.2.4 *Aphanomyces invaderis* is sexually sterile; slow-growing; thermolabile; incapable of growing on SDA, CMA and MEA media; and highly susceptible to several chemical treatments. Zoospores show particular chemotactic behaviour; are capable of limited polyplanetism, even in the presence of nutrient media; and germinate indirectly and have an abbreviated lifecycle in low nutrient backgrounds. Primary cysts lack a thick outer coat and appear to have a distinctive K-body. Zoospores and cysts have distinctive lectin-binding characteristics and cross-react with a specific anti-*Phytophthora* MAb. Mycelial extracts can be distinguished by means of electrophoretic banding profiles and pyrolysis mass spectrometry.

4.2.5 Striped snakeheads and rainbow trout infected with *A. invaderis* produce non-specific antibodies, but it is not known whether these are protective. Temperature and number of zoospores are important factors governing the success of the fishes cellular response.

5. RECOMMENDED FUTURE STUDIES

Given that the importance of the fungal aetiology of EUS has only been realised in recent years there are still many avenues of research to pursue:

5.1 Treatment of EUS

5.1.1 A priority for further work is clearly to investigate methods of control of this damaging disease. Now that that *Aphanomyces invaderis* has been established as the essential aetiological agent for EUS infection, studies can be targeted at treating this fungus, if possible in infected fish, but probably more feasibly, by devising strategic water treatments to prevent infection of fish.

5.1.2 There are currently several untested treatments for EUS being recommended. The relative merits, or otherwise, of agricultural, slaked and quick lime need to be assessed. Accepted fungicidal agents are sometimes applied (potassium permanganate, salt, malachite green) but optimal dosages need to be assessed. Other remedies are used in local circumstances and rarely publicised (eg homeopathic drugs: Mitra and Varshney, 1990; ash: De, 1991; neem leaves and turmeric: Anon, 1994; banana: Sarkar, 1995). These should be evaluated.

5.2 Fungal ecology, physiology and developmental morphology

Questions on the following aspects of the biology of *A. invaderis* are also important to a fuller understanding of EUS.

5.2.1 The natural ecology of the fungus: its ability to persist outside a fish host and any other possible habitats or substrata which it can colonise and with which it can be transmitted.

5.2.2 Zoospore physiology: identify chemical attractants produced by fish, study mechanism of chemoattraction in zoospores, identify factors involved in sporulation with a view to arresting this process.

5.2.3 Developmental morphology of zoospores: adaptations as a fish-pathogen.

5.2.4 Comparative ultrastructure and immunocytochemistry: to investigate the taxonomical relationship of *A. invaderis* to other fungi.

5.2.5 Characterisation of the ECP: what enzymes are involved, genetic control pathways (is secretion constitutive or induced?), its role in pathogenesis and fungal nutrition.

5.2.6 Susceptibility to parasitism as a form of biological control. The ability of particular bacteria (Hatai and Willoughby, 1988; Petersen *et al*, 1994; Bly *et al*, 1997) and fungal parasites (Willoughby and Roberts, 1992) to kill *Saprolegnia*, has been studied in this regard.

5.3 Fish immune systems and protective mechanisms involving the skin

5.3.1 Further work is required to establish the feasibility of inducing protective immunity in susceptible fish. The presence of a specific humoral response to the fungus in naturally and artificially infected snakeheads has been demonstrated here, and a passive immmunization experiment would test the ability of serum from such fish to confer immunity in other fish. This would give an indication of the potential for the possible future development of vaccines. The importance of the fishes cellular response in combating infection by *A. invaderis* has been shown in histopathological studies of resistant common carp (Wada *et al*, 1996), and by *in vitro* macrophage studies described here. The possibility of enhancing cellular defences in susceptible fish by applying glucan or adjuvant preparations requires exploration.

5.3.2 Fish mucus also contains substances with antimicrobial activity (Magarinos *et al*, 1995) and the importance of this mechanism in the resistance of fish to infection should to investigated.

5.3.3 A study of the sequential histological changes in the integument of susceptible fish during challenge experiments, involving *A. invaderis* and other agents that could induce dermatitis, would provide information on the interaction between the fish and the pathogen at the time of infection.

5.4 Identification of other pathogens/risk factors

5.4.1 Given that the fishes skin needs to be breached before *A. invaderis* can penetrate, other factors are clearly involved in the disease process. Acid water has been identified in some outbreaks in Australia and elsewhere as being capable of inducing infection (Callinan *et al,* 1995; 1996), and low temperature is known to immunocompromise the fish and allow the disease to progress fully (Chinabut *et al*, 1995). However, in the absence of acid water, EUS outbreaks do occur, and there is a possibility that biological agents such as viruses (Kanchanakhan, 1996b) or parasites (Subasinghe, 1993), may have an involvement in the disease process in some areas. Further studies are required to evaluate of the importance of these as risk factors in the disease process.

5.4.2 A full epidemiological survey of EUS outbreaks in particular areas would give an indication of the relative importance of the great number of factors reportedly involved in the disease process, and identify further risk factors.

5.5 Effective diagnosis and monitoring future spread

5.5.1 As indicated in Section 1.5, reports of new ulcerative diseases and mycoses of fish continue to occur. It is known that clonal lineages of other pathogenic Oomycete fungi have established a pan-global distribution (Goodwin *et al,* 1994), and given the recent spread of EUS, it is probable that without effective restrictions on the movement of fish, *A. invaderis* will do the same. For such restrictions to be implemented, specific diagnostic techniques should be developed and informed quarantine guidelines drawn up. As knowledge of histological procedures is more widespread and probably more reliable than expertise in fungal isolation and characterisation, the diagnosis of EUS should primarily rely on histology; and the development of a MAb probe specific for *A. invaderis* hyphae would provide a valuable immunohistochemical stain for use in this regard.

5.5.2 It would also be desirable to use an *A. invaderis*-specific probe to establish the true extent of the area colonised by the fungus, so that particular measures can be taken for fish within these areas. Given the extreme similarity in the pathology of UM to EUS, it would be of particular interest to ensure that the invasive fungal pathogen of UM is isolated and compared with *A. invaderis*.

5.6 Planned follow-up activities

5.6.1 A follow-up 3.5 year collaborative project between AAHRI and Stirling University, timetabled to start in July 1997, has been proposed to the ODA. It is entitled "Applied studies on *Aphanomyces invaderis*, the fungal pathogen of epizootic ulcerative syndrome (EUS): its ecology, immunology and pathogenicity in relation to other disease agents and an assessment of various treatment regimes". It aims to address many of the recommended future studies indicated above. It is proposed that an *A. invaderis*-specific molecular probe, developed in Glasgow University is used to study the natural ecology of the fungus, and its prevalence in different habitats. Optimal and lethal conditions for sporulation will be examined to aid the design of strategic treatments. A number of treatment compounds will be screened using *in vitro* techniques developed in the present study, and candidate treatments will be tested in tank and pond trials. A tank-based immersion challenge model will be developed to investigate fungal pathogenicity in relation to other disease agents. A PhD student will be recruited to assess the potential of inducing EUS resistance in fish and use immunological techniques to study the pathogenicity of *A. invaderis*. A training component of the project will include the initiation of short research projects in regional institutes to investigate local aspects of EUS outbreaks.

5.6.2 The ODA SE Asia Aquatic Disease Control Project, based at AAHRI, will fund an EUS/fish mycology training workshop in February 1998 to disseminate new information on the diagnosis and control of EUS.

5.6.3 The Network of Aquaculture Centres in Asia-Pacific (NACA) and Australian Centre for International Agriculture Research (ACIAR) are organising training activities for Pakistani fisheries officers, in Pakistan and at AAHRI, as a follow-up to the mission to investigate EUS outbreaks in March 1997 (see Section 3.11).

5.6.4 A project proposal to increase expertise in veterinary epidemiology in Asia is being prepared by Dr J. Turnbull, Stirling University. The initiation of epidemiological studies on EUS is a component of this project.

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APPENDIX ONE

List of fungal isolates used in comparative studies

(a) EUS, RSD and MG pathogenic *Aphanomyces* isolates

	Isolate	Path-ogen-icity	Growth data	Chem-ical suscep-tibility	EM	Immun-ocyto-chem-istry	SDS-PAGE	West-ern blots	PyMS	RAPD
Section		**3.2**	**3.3**	**3.4**	**3.6**	**3.6**	**3.7**	**3.7**	**3.9**	**3.10**
EUS - Thailand	TA1	X	X	X			X		X	X
	RF6	X	X	X			X	X	X	X
	RF8	X	X	X			X		X	X
	S1PA	X	X	X			X		X	
	S3PA	X	X	X					X	X
	G2PA	X	X	X			X	X	X	X
	PA1	X	X	X		X			X	X
	PA3	X	X	X						
	PA4	X	X	X			X			X
	PA5	X	X	X			X			
	PA7	X	X	X			X	X	X	
	PA8	X	X		X					
	PA10	X	X	X			X			
	96PA								X	X
EUS - Bangladesh	BR	X	X	X			X		X	X
	BH	X	X	X			X	X	X	X
	BS	X	X	X			X		X	
EUS - Indonesia	36/1P	X	X	X			X	X	X	X
EUS - Philippines	30P	X	X	X						X
	33P	X	X	X	X	X	X		X	X
	34P	X	X	X					X	X
	10D	X	X	X			X	X	X	X
RSD - Australia	3P	X	X	X			X		X	
	4P	X	X	X			X	X	X	X
	10P	X	X	X			X		X	X
	24P	X	X	X		X	X	X	X	X
MG - Japan	NJM9030	X	X	X			X	X	X	X
	NJM9201									X

(b) Non-Asian pathogens and saprophytic *Aphanomyces, Achlya, Saprolegnia* and *Phytophthora* spp.

Fungus species	Isolate	Path-ogen-icity	Growth data	Chem-ical suscept-ibility	EM	Immun-ocyto-chem-istry	SDS-PAGE	West-ern blots	PyMS	RAPD
Chapter		**3**	**4**	**5**	**7**	**7**	**8**	**8**	**10**	**11**
UM-*Aphanomyces*	84-1240	X	X	X	X		X	X	X	X
	84-1249								X	
	84-1282								X	X
Aphanomyces astaci	J1									X
	PL									X
	KV									X
	PC									X
	FDL457	X	X	X			X		X	X
	FDL458	X	X	X			X	X	X	X
Saprophytic *Aphanomyces* spp.	TF5	X	X	X			X	X	X	
	TF33	X	X	X			X	X	X	
	TF41	X	X	X			X	X	X	X
	TF54	X	X	X						X
	T1SA	X	X	X			X	X		
	A2SA	X	X	X					X	
	F3SA	X	X	X	X	X	X	X	X	X
	SSA	X	X	X			X	X	X	
	WSA	X	X	X	X	X	X	X	X	X
	SA11	X	X	X			X		X	X
Aphanomyces laevis	ASEAN1	X	X	X	X		X	X	X	
	ASEAN3	X	X	X					X	X
Achlya sp	S2AC	X	X	X			X		X	
	AC2	X	X	X			X			
	AC5	X	X							
	AC10	X	X	X						
Achlya diffusa	W2BAC	X	X	X			X		X	
Saprolegnia sp	TF20S	X	X						X	
	TF23	X	X	X						
	TF24	X	X							
	TF25	X	X	X						
	TF26	X	X							
	TF29	X	X	X			X		X	
S. diclina	TF27	X	X	X						
	TF31	X	X	X			X			
	E3	X	X	X					X	
S. australis	*S. aust*	X	X	X			X		X	
S. ferax	P32	X	X	X			X		X	
S. parasitica	TP41	X	X	X		X	X		X	
Phytophthora cinnamomi						X				

APPENDIX TWO

(a) Formulae for media

GP (glucose-peptone) broth

3 g/l	glucose (BDH)
1 g/l	peptone (bacteriological, Oxoid)
0.128 g/l	$MgSO_4.7H_2O$ (Hopkins & Williams, Chadwell Heath, Essex)
0.014 g/l	KH_2PO_4 (BDH)
29.3 mg/l	$CaCl_2.2H_2O$ (Sigma)
2.4 mg/l	$FeCl_3.6H_2O$ (Sigma)
1.8 mg/l	$MnCl_2.4H_2O$ (BDH)
3.9 mg/l	$CuSO_4.5H_2O$ (BDH)
0.4 mg/l	$ZnSO_4.7H_2O$ (BDH)

GPY (glucose-peptone-yeast) broth

as GP broth with:

0.5 g/l yeast (BDH)

GP agar

as GP broth with:

12 g/l technical agar (Oxoid No. 3)

GPY agar

as GPY broth with:

12 g/l technical agar (Oxoid No. 3)

GP-PenOx broth

prepare GP broth and after autoclaving and cooling to 50°C add:

100 units/ml	penicillin-K (Sigma)
10 μg/ml	oxolinic acid (Sigma)

GP-PenStrep agar

prepare GP agar and after autoclaving and cooling to 50°C add:

100 units/ml	penicillin-K (Sigma)
10 μg/ml	streptomycin sulphate (Sigma)

concentrated assay broth (50 ml)

4.5 g	glucose (BDH)
1.5 g	peptone (bacteriological, Oxoid)
0.19 g	$MgSO_4.7H_2O$ (Hopkins & Williams, Chadwell Heath, Essex)
0.02 g	KH_2PO_4 (BDH)
44 mg	$CaCl_2.2H_2O$ (Sigma)
3.6 mg	$FeCl_3.6H_2O$ (Sigma)
2.7 mg	$MnCl_2.4H_2O$ (BDH)
5.9 mg	$CuSO_4.5H_2O$ (BDH)
0.6 mg	$ZnSO_4.7H_2O$ (BDH)

Autoclave glucose separately to prevent carmelisation. After autoclaving and cooling to 50°C add:

15 mg (15000 units)	penicillin-K (Sigma)
15 mg	oxolinic acid (Sigma)

Add 1ml of concentrated assay broth to Petri dish with 29ml of test solution.

PG-1 broth

3 g/l	glucose (BDH)
6 g/l	peptone (bacteriological, Oxoid)
0.17 g/l	$MgCl_2.6H_2O$ (BDH)
0.15 g/l	$CaCl_2.2H_2O$ (BDH)
0.37 g/l	KCl (BDH)
0.02 g/l	$FeCl_3.6H_2O$ (Sigma)
0.044 g/l	Na_2 EDTA (Sigma)

Buffer with 13 mM sodium phosphate (BDH). Adjust pH to 6.3. Autoclave the glucose and sodium phosphate buffer separately from the other ingredients.

sodium phosphate buffer

make up stocks of:

31.2 g/l	solution A - $NaH_2PO_4.2H_2O$ (BDH) - store at 4°C
71.7 g/l	solution B - $Na_2HPO_4.12H_2O$ (BDH) - store at room temperature

407.5 ml solution A, 92.5 ml solution B and 500 ml distilled water are combined to make 1000 ml phosphate buffer (100 mM). 130 ml of this buffer is used in 1000 ml PG-1.

PG-1 agar

as PG-1 broth with:

12 g/l	technical agar (Oxoid No. 3)

V8 broth

5%	V8 juice (Campbell Grocery Products Ltd)
0.2%	$CaCO_3$ (BDH)

Adjust pH to 6.1

APW (autoclaved pond water)

Pond or lake water known to support fungal growth is filtered through Whatman 541 filter paper. One part pond water is combined to two parts distilled water and autoclaved.

(b) Water quality of lake/pond water used in APW

Stirling/Newcastle - Airthrey Loch water

(monthly sampling between 20/1/94 - 20/1/95 at surface, 2m and 4m depth, from Kelly and Smith, 1996)

	mean	range
Loch area	6.9 ha	
Loch mean depth	1.85	up to 4.5 m
Macrophyte cover	-	up to 25%
Temperature	9.3	2.0 - 20.0 °C
Dissolved oxygen	10.7	8.1 - 13.5 mg/l
pH	-	6.85 (Feb) - 9.54 (May)
Secchi disc	1.4	0.8 - 2.3 m
Total phosphorus	0.61	34.1 (May) - 83.1 (July) μg/l
Dissolved reactive phosphorus	2.2	not detectable - 9.8 (Jan) μg/l
Total silica	0.90	0.07 - 3.00 (Jan) mg/l
Molybdate reactive silicate	0.53	0.05 - 1.99 (Jan) mg/l
Chlorophyll *a*	34.0	2.7 - 102.9 (Jan) μg/l

(monthly sampling between 1/2/95 - 31/7/95, from L.A. Kelly (pers. comm.)

	mean	range
Alkalinity	1.0	0.9-1.16 meq/l
Total hardness ($CaCO_3$)	49	42-55 mg/l
Conductivity	232	216-250 uS/cm

Bangkok - National Inland Fisheries Instiute (NIFI) pond water
(analysis of surface water done at NIFI at 11:00am 3/3/97)

Temperature	29 °C
Alkalinity	161 mg/l
Hardness	413 mg/l
Dissolved oxygen	3.8 mg/l
pH	7.55
NO_2	0.58 mg/l
NO_3	1.30 mg/l
ammonia	0.06 mg/l
ortho PO_4	1.00 mg/l
total PO_4	1.24 mg/l

high ammonia and high temp and pH mean that proportionof unionised ammionia would be high

Hålsjön Lake near Uppsala

	Oct 1973	Nov 1974	Autumn 1978	Autumn 1979
pH	8.6	7.3		
NH_4 -N	0.022	0.085	0.030	0.088 mg/l
NO_2-N	0.004	0.004	0.016	0.023 mg/l
NO_3-N	0.202	0.195	0.346	0.259 mg/l
organic N	0.40	0.43	0.813	0.745 mg/l
total N	0.63	0.71	1.48	1.12 mg/l
PO_4-P	0.009	0.005	0.046	0.065 mg/l
general P	0.033	0.017	0.079	0.033 mg/l
total P	0.042	0.019	0.125	0.98 mg/l
COD	38	24 mg/l		
conductivity	164	163		

APPENDIX THREE

Histology staining procedures

H&E (haemotoxylin and eosin) (Clark, 1981)

i) Dewax and bring sections to distilled water	
ii) Stain in alum haemotoxylin	3 min
iii) Differentiate in acid alcohol	
iv) Blue in tap water	
v) Wash in running tap water	
vi) Counterstain with 1% eosin	1-3 min

Grocott silver stain (Grocott, 1955)

Using this technique, care must be taken that all glassware is washed well in distilled water and slides are handled only with plastic forceps otherwise extraneous silver staining will occur.

i) Dewax and bring sections to distilled water	
ii) 5% chromic acid	60 min
iii) Wash in tap water	
iv)1% sodium bisulphite	1 min
v) Running tap water	5 min
vi) Rinse 3 times in distilled water	
vii) Stain in the silver solution in the dark at 50°C	30-60 min

Stock methenamine solution: 3g methenamine (hexamine) dissolved in 95 ml distilled water bringing the volume to 100ml. Add 5 ml of a 5% aqueous solution of silver nitrate. Any white precipitation is redissolved with shaking. To 25 ml of this stock is added 25 ml distilled water and 2 ml of a 5% solution of borax just before using.

viii) Rinse 3 times in distilled water	
ix) Tone in 0.1% gold chloride	3-5 min
x) Rinse in distilled water	
xi) 2% sodium thiosulphate	2 min
xii) Running tap water	

A counterstain may be applied. Fungus should be a deep black, the inner parts of the hyphaemay be a red-rose colour. Background colour depends on any counterstain used.

PAS (periodic acid - Schiffs) (Hotchkiss, 1948)

i) Dewax and bring sections to distilled water	
ii) 1% periodic acid	10 min
iii) Running tap water	5 min
iv) Schiffs reagent	10 min
v) 3 rinses in 0.5% sodium metabisulphite	3 x 2 min
vi) Running water	5 min

APPENDIX FOUR

Paper one - Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi

APPENDIX FIVE

Paper two - Comparative effects of various antibiotics, fungicides and disinfectants on *Aphanomyces invaderis* and other saprolegniaceous fungi

APPENDIX SIX

Paper three - The antibody response of snakehead, *Channa striata* Bloch, to *Aphanomyces invaderis*

APPENDIX SEVEN

Paper four - Pan-Asian spread of single fungal clone results in large scale fish-kills