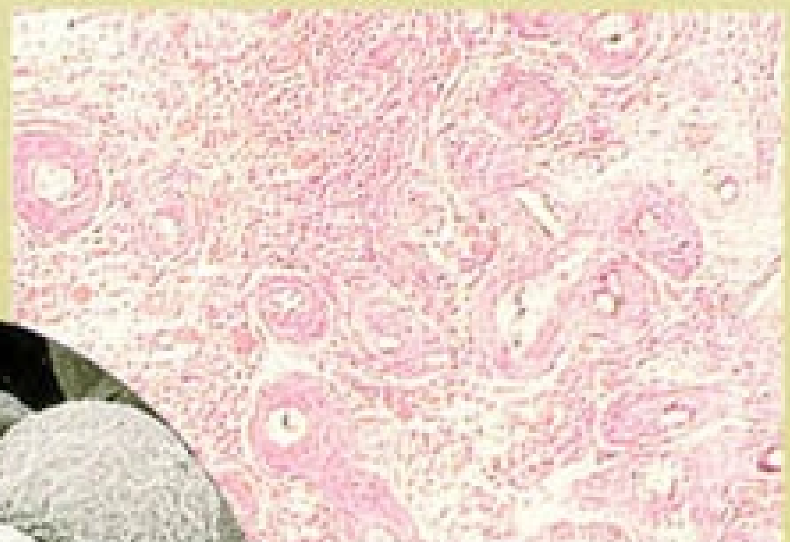
EPIZOOTIC ULCERATIVE SYNDROME (EUS) TECHNICAL HANDBOOK



Aquatic Animal Health Research Institute
Department of Fisheries
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Bangkok

Some of the many fish species affected by EUS

Mastacembelus

Colisa

Indian major carp

DFID Department for International Development

Epizootic Ulcerative Syndrome (EUS) Technical Handbook

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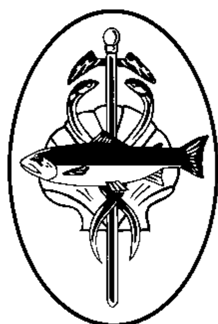

Published by:

The Aquatic Animal Health Research Institute (AAHRI)
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Jatujak
Bangkok 10900
Thailand.
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For reference, this publication should be cited as follows:

Lilley, J.H., R.B. Callinan, S. Chinabut, S. Kanchanakhan, I.H. MacRae and M.J. Phillips (1998) Epizootic Ulcerative Syndrome (EUS) Technical Handbook. The Aquatic Animal Health Research Institute, Bangkok. 88 pp.

ISBN 974-7604-58-2

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As it is intended that this handbook will be accessible on the internet, the home pages of some of the participating organisations are given below:

ACIAR	http://www.aciar.gov.au/
AAHRI	http://www.agri-aqua.ait.ac.th/aahri/
NACA	http://naca.fisheries.go.th/
SEAADCP	http://www.agri-aqua.ait.ac.th/aahri/

Other home pages of interest:

NSW Fisheries	http://www.fisheries.nsw.gov.au/
ACIAR	http://www.aciar.gov.au
IoA, Stirling	http://www.stir.ac.uk/aqua
DFID	http://www.dfid.gov.uk/

Preface

It is our pleasure to introduce this handbook on epizootic ulcerative syndrome (EUS), which aims to provide scientists and fish health workers with background information on this important disease, as well as practical recommendations for its diagnosis and control. There have been many research developments since the publication of a previous AAHRI-NACA review of EUS in 1992, and the present handbook provides a thoroughly updated and expanded analysis of the topic.

EUS is an international problem that has been studied independently, and collaboratively, by many different workers. The early occurrences of mycotic granulomatosis (MG) in Japan and red spot disease (RSD) in Australia over 25 years ago are now considered to have been outbreaks of the disease subsequently designated EUS. Reference to the early work on MG and RSD has therefore proved to be important in understanding the later EUS outbreaks in Southeast and South Asia. This handbook incorporates information from all these sources, bringing together, with acknowledgements, the work of many scientists across a wide range of specialist fields.

A number of different agencies have supported work on EUS. The initial outbreaks in Southeast Asia were investigated by a survey team funded by FAO, and the name, epizootic ulcerative syndrome, was later proposed at an FAO-convened Consultation of Experts in Bangkok in 1986. NACA has also been integrally involved in studies on EUS and much of the data on environmental parameters associated with outbreaks was generated by NACA's Regional Research Programme on Ulcerative Syndrome in Fish and the Environment. The Department for International Development (DFID) of the United Kingdom (formerly ODA), and the Australian Centre for International Agriculture Research (ACIAR), subsequently funded major research projects on the disease. Both organisations, through the Fisheries Programme of ACIAR and the DFID South East Asia Aquatic Animal Disease Control Project, provided support in the production of this handbook.

The spread of EUS may be due partly to the large-scale movement of fish within the Asia-Pacific region, and, as suggested in this handbook, the potential for further spread is high. Consequently, the risk of introducing EUS should be a matter of concern for countries that are, as yet, unaffected. The need for development of effective strategies to reduce risks associated with the spread of important aquatic animal pathogens is now widely recognsed, and in Asia is being addressed through a cooperative FAO/NACA/OIE Regional Programme for the Development of Technical Guidelines on Quarantine and Health Certification and Establishment of Information Systems for the Responsible Movement of Live Aquatic Animals in Asia. We would like to stress the value of cooperation among countries in Asia and others with an interest in controlling aquatic animal disease and promoting sustainable aquaculture development.

This handbook is one of a series of publications on important diseases of aquatic animals in Asian aquaculture published by the Aquatic Animal Health Research Institute (AAHRI) of the Department of Fisheries of Thailand. No doubt, future studies will enable the development of rapid diagnostic techniques for detecting *Aphanomyces invadans*, provide a better understanding of the various component causes of EUS in different outbreaks and introduce further means of controlling outbreaks. Other interesting areas to be studied include the epidemiological investigation of EUS outbreaks in areas on the "frontier" of the disease and the comparison with ulcerative mycosis outbreaks in other regions, which could provide further information on the origin and spread of this important disease. We look forward to further collaborative projects of this nature in the future.

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ACIAR Fisheries Programme Coordinator

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Acknowledgements

This publication was prepared in collaboration between the Aquatic Animal Health Research Institute (AAHRI), Bangkok; the Institute of Aquaculture (IoA), Stirling University, UK; NSW Fisheries, Australia; the Network of Aquaculture Centres in Asia-Pacific (NACA), Bangkok; and the South East Asia Aquatic Disease Control Project (SEAADCP), Bangkok. The authors wish to acknowledge the support of the United Kingdom's Department for International Development (DFID) and the Australian Centre for International Agriculture Research (ACIAR). The costs of publication of the review were borne by ACIAR. The Epidemiology sections of this review were modified from information provided by Dr C. Baldock (Ausvet Animal Health Services, Australia); Annex 2 was provided by Mr Graeme Fraser (NSW Agriculture, Australia); and the diagrams of *Aphanomyces invadans* are reproduced with kind permission of Dr L.G. Willoughby (Freshwater Biological Association, UK). The assistance of these researchers is gratefully acknowledged. The publication was reviewed and/or received inputs from the following scientists: Prof R.J. Roberts (Stirling, UK); Dr Kim Thompson (IoA, Stirling, UK); Dr Ruth Campbell (IoA, Stirling); Mr Jes Sammut (University of New South Wales); Dr Melba B. Reantaso, Ms Susan Lumanlan-Mayo, Mr Jose Paclibare, Ms Elena Catap and Ms Hazel Matias (Bureau of Fisheries and Aquatic Resources, Philippines); Dr Akhmad Rukyani, Mr Dayat Basiawan and Mr Taukhid (Research Institute for Freshwater Fisheries, Indonesia); Dr C V Mohan (College of Fisheries, Mangalore, India); Mr R.R. Dhital (Fisheries Development Division, Nepal); Mr Masud Hossain Khan (Bangladesh Fisheries Research Institute, Bangladesh); Miss Werawan Chin-aksorn (Suphanburi Fisheries Station, Thailand); Mr Douangkham Singhanouvong (Department of Livestock and Fisheries, Ministry of Agriculture and Forestry, Lao PDR); Mr K. Subramaniam (Brackishwater Research Station, Malaysia); Mr Ing Kim Leang (Department of Fisheries, Cambodia); Mr U Tin Myo Zaw (Department of Fisheries, Myanmar); and Mr Zafran (Gondol Research Station for Coastal Fisheries, Bali, Indonesia).

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Introduction

Epizootic ulcerative syndrome (EUS), was defined at a DFID Regional Seminar in Bangkok in 1994 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 necrotising ulcerative lesions typically leading to a granulomatous response" (Roberts *et al.*, 1994a). However, research since that time, discussed in the aetiology and epidemiology sections of this handbook, suggest a complex aetiology is not necessarily involved in all cases. Reference to a specific fungal pathogen (*Aphanomyces invadans*) could also now be included in the case definition for EUS. With these developments, EUS could be considered to be characterised beyond the level of a syndrome, however the name "epizootic ulcerative syndrome" is well known among fish health workers, and will continue to be used for the purposes of this booklet.

A previous review, published by AAHRI and NACA in 1992, brought together much of the literature on the subject published in national and international articles, reports and conference proceedings. It is intended that the present publication will have additional practical applications to assist fish health workers in the diagnosis and control of EUS. In particular, there is a substantial annex section, which includes information on fungal and viral isolation and identification, an outline for outbreak investigations, and EUS reporting datasheets. It should be emphasised that there are a large number of different ulcerative fish conditions, and a positive EUS diagnosis can be made only by histological confirmation of particular distinctive features described here on page 31. Therefore, it is hoped that this handbook will also encourage fish health workers to investigate other ulcerative conditions, if a diagnosis proves to be EUS negative.

History

For over 25 years, outbreaks of an ulcerative disease, characterised histologically by mycotic granulomas, have affected freshwater and estuarine fishes over much of Asia and Australia. The disease has been given various names, but is most commonly known as mycotic granulomatosis (MG) in Japan, red spot disease (RSD) in Australia, and epizootic ulcerative syndrome (EUS) in Southeast and South Asia. MG, RSD and EUS have, in the past, been described separately as distinct conditions, however recent studies have shown that the same pathogenic *Aphanomyces* fungus is involved in each case (see Aetiology section on "Fungi") and it is now apparent that an account of the history of EUS would be incomplete without consideration of outbreaks in Japan and Australia.

Mycotic granulomatosis (MG)

The first report of an EUS-like condition came in summer 1971, in farmed ayu (*Plecoglossus altivelis*) in Oita Prefecture, Japan (Egusa and Masuda, 1971). The characteristic lesion, a granulomatous response to invasive hyphae, was described and the disease was named mycotic granulomatosis (Miyazaki and Egusa, 1972). It rapidly spread to several other Prefectures and affected various species of fish, predominantly cultured ayu and goldfish *(Carassius carassius auratus)*; and wild Formosan snakehead *(Channa maculata)*, crucian carp *(Carassius auratus),* bluegill *(Lepomis macrochirus)* and grey mullet (*Mugil cephalus*) (Miyazaki and Egusa, 1972; 1973a; b; c). Significantly, common carp *(Cyprinus carpio)* were not affected. Hatai *et al.* (1977) isolated the invasive Oomycete fungus from affected fish and subsequently called it *Aphanomyces piscicida* (Hatai, 1980). *A. piscicida* is now known to be con-specific with the EUS pathogen, *Aphanomyces invadans* (Lilley *et al.*, 1997a; b). Although serious MG epizootics have not been reported in Japan since 1973, outbreaks have continued to occur periodically. Recently Hatai *et al.* (1994) reported a similar disease in imports of ornamental dwarf gourami (*Colisa lalia*) from Singapore, again shown to involve the same *Aphanomyces* pathogen (Lilley *et al.*, 1997a).

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 affect freshwater and estuarine fish in coastal rivers in New South Wales (Callinan *et al.*, 1989), Northern Territory (Pearce, 1990) and Western Australia (Callinan, 1994a).

An *Aphanomyces* fungus was recovered from diseased fish by Fraser *et al.* (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 (1992) showed that RSD outbreaks in estuarine fish in the Clarence river, NSW were associated with lower catchment rainfall and Callinan *et al.* (1995a) related this to runoff from acid sulfate 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, RSD was subsequently induced 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 (Callinan *et al.,* 1996; Callinan, 1997). As with *A. piscicida*, the pathogenic RSD-*Aphanomyces* has been shown to be the same species as the EUS pathogen, *A. invadans* (Callinan *et al.*, 1995a; Lilley *et al.*, 1997a; b).

Epizootic ulcerative syndrome (EUS)

Following the outbreaks of MG and RSD, there was a progressive spread westwards across Asia of a syndrome associated with dermal ulceration and involving large scale mortalities in a number of freshwater and estuarine fish species. The syndrome 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 (Figure 1), although not all have been positively confirmed as EUS according to procedures described in the Diagnosis section of this handbook.

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.* (1986) and confirmed as pathologically identical to EUS.

In 1980 outbreaks of an epizootic haemorrhagic condition occurred in Java, Indonesia affecting primarily cultured cyprinid and clariid 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 (pers. comm.) isolated *A. invadans* 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 there have been no records of high EUS losses to this industry.

Although there were reports of high mortality rates in fish in southern peninsular Malaysia in 1979 (Shariff and Law, 1980, described by Roberts *et al.*, 1986), the first reported typical EUS outbreaks were in December 1980, in rice-field fishes in northern Malaysia (Jothy, 1981). 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 farmed snakeheads and rice-field fish. 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. Recently (December 1996), EUS was experienced in NE, central and southern provinces (S. Kanchanakhan, unpublished). The isolation of the pathogenic fungus, *A. invadans,* from EUS-affected snakeheads in Suphanburi province was described by Roberts *et al.* (1993).

Myanmar, Lao PDR and Cambodia, first reported major outbreaks of EUS in 1983 or 1984 (Lilley *et al.*, 1992). Subsequent epizootics were less extensive (*e.g.* 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. In 1996, diseased snakeheads from Laos were confirmed at AAHRI, Bangkok as suffering from EUS.

Several accounts of EUS-affected fish have also come from Vietnam, China and Hong Kong although these are still not confirmed. 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). Clariid 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 up to 70% of snakeheads (*Channa maculata*) in late summer in Hong Kong since 1988.

Laguna de Bay in the Philippines, 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 and Gacutan, 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). Mullet, goatfish (*Upeneus bensai*), croaker (*Johnius* sp.), *Psettodes* sp. and *Scanthophagus argus* in a lagoon in Cagayan Province

suffered an outbreak in 1990 which was confirmed as EUS by histological examination (Reantaso, 1991; S. Chinabut, unpublished). The occurrence of EUS in these brackishwater and marine species provided an explanation as to how the condition may have spread between the islands. The severity of outbreaks has decreased since 1993. Several *A. invadans* isolates were recovered from EUS-affected fish in the Philippines (Paclibare *et al.,* 1994).

A major outbreak of EUS in freshwater and estuarine fish 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 Southeast Asia in shipments of infected fish, possibly ornamental angel fish (*Pterophyllum scalare*), some of which were ulcerated and suffered high 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.).

Over the past 10 years, EUS has had a serious effect on fisheries throughout mainland South Asia, 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 spread rapidly throughout the country, seemingly 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 large-scale mortalities due to the disease were probably restricted to fingerlings (Roberts *et al.,* 1989). EUS prevalences subsequently declined, but there are reports that, as from 1995, the severity of outbreaks is increasing in Bangladesh (G.U. Ahmed, unpublished report). In January 1993, *A. invadans* cultures were isolated from farmed Indian major carp (*Labeo rohita*) in NW Bangladesh and wild fish in the productive flood plain area of NE Bangladesh.

Outbreaks of EUS in India have been comprehensively reviewed (Zoological Society of Assam, 1988; Jhingran and Das, 1990; National Workshop on Ulcerative Disease Syndrome in Fish, 1990; Kumar *et al.*, 1991; ICSF, 1992; Das and Das, 1993; Mohan and Shankar, 1994). The NE Indian states were the first to report losses in May 1988. The disease appeared to 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). *Aphanomyces* isolates consistent with *A. invadans* have been recovered from EUS-affected fish in southern India (I. Karunasagar, pers. comm.).

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;

Shresta, 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 country to be affected most recently by EUS was Pakistan, where EUS was confirmed in snakeheads from Punjab Province in April 1996, and in *Cirrhinus mrigal* from Sindh Province in January 1998 (DFID, 1998). The blotched snakehead or mud murrel (*Channa punctata*) was the most commonly affected species; with *Puntius* spp., *Labeo rohita* and *Cirrhinus reba* 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 (AAHRI, ACIAR, IoA and NACA, 1997). Reported losses have not been high in the Punjab, possibly due to the extensive use of tube-well water for fish farms and elevated salinities (AAHRI, ACIAR, IoA and NACA, 1997), but EUS is now well established in parts of the Indus river, and given its apparent rapid spread across the country (DFID, 1998), there are fears of potentially serious future impacts to fisheries and aquaculture development.

Other similar diseases

Mention is made here of other similar ulcerative fish diseases, although their relationship with EUS is presently unknown.

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 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 develop lesions consistent with UM (Noga, 1993; Lilley and Roberts, 1997). Fish have developed UM when lesion material is used as an inoculum, suggesting that some other, unidentified agent, possibly another fungus, 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 prevalence in these species was markedly lower than in menhaden (Levine *et al.*, 1990a). In menhaden, a larger proportion of age-0 fish were shown to be affected than age-1 fish (Levine *et al.,* 1990b). Levine *et al.* (1990b) 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, with infection rates of menhaden up to 100% (Levine *et al.,* 1990b).

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

Cod ulcer disease

Munday (1985) reported the presence of severely ulcerated red cod *(Pseudophycis bachus)* 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.

Figure 1. Map showing the spread of EUS across the Asia-Pacific region.
Dates indicate the time of the first serious outbreak.
(There is some doubt about outbreaks marked with asterices).

Species affected

More than 100 fish species 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 the pathogenic *Aphanomyces* fungus from tissues underlying ulcers. Table 1 lists these confirmed cases, 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 *A. invadans* and found them to be refractory to infection. Wada *et al.* (1996) and Shariffpour (1997) experimentally injected common carp (*Cyprinus carpio*) with zoospores of *Aphanomyces* from MG and EUS outbreaks respectively, and demonstrated that fungal growth was suppressed by an intense inflammatory response.

Some authors have commented that the most severely affected species in natural outbreaks are generally bottom dwellers (Llobrera and Gacutan, 1987; Chondar and Rao, 1996) or possess air-breathing organs (Roberts *et al.,* 1994b), but examination of Table 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 or age may be significant in other species. For example, Indian major carp, suffer high mortalities as fingerlings (Roberts *et al.,* 1989) but larger fish, although appearing ulcerated, are not reported as dying in large numbers (AAHRI, ACIAR, IoA and NACA, 1997).

Some of the EUS-susceptible species listed in Table 1 have a wide geographical distribution, beyond the current limits of EUS outbreaks. For example, several snakehead and clariid catfish species occur in Africa and central Asia. This suggests that there is potential for further spread of the disease to these areas. However, it should be noted that optimal temperatures for vegetative growth *in vitro* for *A. invadans* are in the range 20-30°C (Fraser *et a.l,* 1992; Lilley and Roberts, 1997) and, probably for this reason, natural outbreaks to date have been limited to latitudes between 35°N and 35°S. Experimental injection challenges of native European and American fish species have shown that the pathogenic fungus, *A. invadans,* is capable of causing lesions in rainbow trout at 18°C (Thompson *et al.*, in press), but is less infective in stickleback (*Gasterosteus aculeatus)* and roach (*Rutilus rutilus*) at 11-16°C (Khan *et al.,* 1998).

Table 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)

ϕ The two genera *Channa* and *Ophicephalus* were united as *Channa* by Myers and Shapovalov (1931, cited by Clark, 1991)

Ψ Ornamental fish imported from Singapore

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.* (1995b)
2 Callinan (unpublished)
3 Catap (pers. comm.)
4 Chinabut *et al.* (1995)
5 Chinabut (unpublished)
6 Chowdhury & Chinabut (pers. comm.)
7 DFID (1998)
8 Fraser *et al.* (1992)
9 Ahmed & Hoque (submitted)
10 Hanjavanit *et al.* (1997)
11 Hatai (1994)
12 Kanchanakhan (1996a)
13 Khan (pers. comm.)
14 Lilley and Roberts (1997)
15 Miyazaki (1994)
16 Mohan and Shankar (1995)
17 Pearce (1990)
18 Reantaso (1991); S. Chinabut (unpublished)
19 Roberts *et al.* (1989)
20 Thompson *et al.* (in press)
21 Vishwanath *et al.* (1997)
22 Vishwanath *et al.* (1998)
23 Viswanath *et al.* (1997)

Table 1 Species susceptible to EUS (or MG† or RSD‡)

Latin name (common name)	Jap†	Aus‡	Ino	Tha	Lao	Mya	Phi	Ban	Ind	Pak	Sco
Acanthopagrus australis (yellowfin bream)		8									
Anabas testudineus (climbing perch)							18				
Bidyanus bidyanus (silver perch)		2									
Carassius auratus (crucian carp)	15										
Carassius carassius auratus (gold fish)	15										
Channa maculata (=Ophicephalus maculatus) (Formosan snakehead)	15										
Channa marulia (=Ophicephalus marulius) (river murrel - India)								5			
Channa micropeltes (=Ophicephalus micropeltes) (red snakehead)				5							
Channa pleurophthalmus (=Ophicephalus pleurophthalmus) (snakehead)	10$^{\phi}$										
Channa punctata (=Ophicephalus punctatus) (mud murrel - India)								14		12	
Channa sp. (*=Ophicephalus* sp.) (snakehead)								19	21,22		
Channa striata (=Ophicephalus striatus) (striped snakehead)				4*,14	5	5	1		16		
Catla catla (catla)								6,9			
Cirrhina mrigala (mrigal)								9,13,19		7	
Clarias batrachus (walking catfish)							1				
Clarias gariepinus (African catfish)							2*				
Colisa lalia (dwarf gourami)	11$^{\psi}$										
Epinephelus sp. (grouper)									23		
Esomus sp. (flying barb)									21,22		
Etroplus sp. (chromide)									21,22		
Fluta alba (swamp eel)				5							
Glossogobius giurus (bar-eyed goby)							1	9			
Glossogobius sp. (goby)									21,22		
Heteropneustes fossilis (stinging catfish)									21		
Johnius sp. (croaker fish)							18				
Labeo rohita (rohu)								9,14,19			
Lepomis macrochirus (bluegill)	15										
Liza diadema (mullet)		17									
Macquaria ambigua (golden perch)		2									
Mastacembelus armatus (armed spiny eel)								5			
Mastacembelus pancalus (guchi - Bangladeshi)								5			
Mugil cephalus (grey mullet)	15	8					1		16		

Table 1 (Cont'd) Species susceptible to EUS (or MG† or RSD‡)

Latin name (common name)	Jap†	Aus‡	Ino	Tha	Lao	Mya	Phi	Ban	Ind	Pak	Sco
Mugil sp. (mullet)									21,22		
Mystus sp. (catfish)									22		
Notopterus notopterus (grey featherback)				5							
Oncorhynchus mykiss (rainbow trout) - marginally susceptible	11*										20*
Osphronemus goramy (pla raet - Thai)				5							
Oxyeleotris marmoratus (sand goby)			14								
Oxyeleotris sp (gudgeon)											
Platycephalus fuscus (dusky flathead)		2									
Platycephalus sp. (flathead)									22		
Plecoglossus altivelis (ayu)	11,15										
Psettodes sp. (spiny turbot)							18				
Puntius gonionotus (silver barb)				5				13			
Puntius sophore (punti - Bangladeshi)								9		5	
Puntius sp (puntius)								19	16,22		
Rhodeus ocellatus (tairiku-baratanago - Japanese)	11*										
Rohtee sp (keti - Bangladeshi)						5					
Scardinius erythrophthalmus (rudd) - marginally susceptible	11*										
Scatophagus argus (spotted scat)							18				
Scatophagus sp. (scat)									21,22		
Sillago ciliata (sand whiting)		8,3*									
Sillago sp. (sillago)									21,22		
Terapon sp. (therapon)									21,22		
Trichogaster pectoralis (snakeskin gourami)				5							
Trichogaster trichopterus (3-spot gourami)	10^{ψ}	3*		14							
Tridentiger obscurus obscurus (Japanese trident goby)	15										
Upeneus bensai (goatfish)							18				
Valamugil sp. (mullet)									21,22		
Wallago attu (wallago)									22		
Xenentodon cancila (round-tailed garfish)								14			

Socio-economics

The most severe impact of EUS has probably been on small-scale, mixed-species fisheries and aquaculture activities in rice-fields and rural waterways. It is estimated that 250 million families in the Asian-Pacific region depend on rice as a main crop and much of the incidental fish harvests from these paddies are an important part of the families' diet (Macintosh, 1986). It should be noted that the chief months for harvesting rice paddy fish are from September to February, the period when most ulcerative disease episodes occur. In these circumstances, any figure on the financial cost of EUS may underestimate the full impact of the disease to these communities.

Estimates of the economic value of fish losses to commercial fish traders are given in Table 2. These figures do not, however, take into account indirect socio-economic costs due to market rejection of harvested ulcerated fish, or in some cases, even unaffected fish. In the 1980s, in some communities, a widespread, but unfounded, fear of disease transmission to consumers led to a drastic decrease in market demand for all food fish. Confidence in freshwater fish farming, particularly among potential investors and financial agencies, was badly affected.

In the Philippines, the average daily income of fishers (approximately US$4) declined to US$1.50 during disease outbreaks in Laguna de Bay due to the rejection of affected fish (ADB/NACA, 1991). Bangladesh suffered severe losses from EUS in 1988 and 1989, and extensive local media coverage about the disease fuelled the public's fear of health risks from fish consumption, resulting in initial price reductions of up to 75% and high losses to fish traders. Nepal has no marine fish resources and therefore relies heavily on EUS-susceptible species. It was reported that 15-20% of total fish production was lost in Nepal during initial EUS outbreaks (ADB/NACA, 1991). The occurrence of EUS in cultured major carp fingerlings gave rise to fears of a potentially crippling effect on the expansion of carp culture in the subcontinent region. Bhaumik *et al.* (1991) reported that 73% of the culture ponds in West Bengal were affected at that time, and most of these were reported to have lost between 30-40% of their stock. In their report giving details of losses to inland fishworkers in Kerala, the ICSF (1992) quote the official figure of Rs 20 million, but commented that newspapers reported losses up to ten times this figure.

The EUS pandemic has demonstrated to national authorities the ability of fish disease to cause major financial losses, and as a result, one positive impact of EUS has been the increased funding allocated to fish disease research and diagnostic facilities in Asia by governments and international organisations.

Table 2 Estimated economic losses from fish mortalities due to EUS

COUNTRY	YEAR	ECONOMIC LOSS		REFERENCE
		LOCAL CURRENCY	US$ (approx.)	
Eastern Australia	annually	Aust $ 1 million	700,000	Callinan *et al.* (1996)
Indonesia	1980-83	-	119,000	ADB/NACA (1991)
	1984-87	-	116,000	ADB/NACA (1991)
Thailand	1982-83	B 200 million	5.5 million	Tonguthai (1985)
	1983-93	-	100 million	Chinabut (1994)
Bangladesh	1988	Tk 118 million	2.8 million	Barua (1990)
	1989	Tk 88.2 million	2 million	Barua (1990)
Sri Lanka	1988-89	Rs 1 million	20,000	ADB/NACA (1991)
	upto 1993	Rs 20-40 million	4-800,000	Balasuriya (1994)
Eastern Nepal	1989-90	Rs 30 million	550,000	ADB/NACA (1991)
India - Bihar	1990	Rs 4.8 million	150,000	Das (1994)
- Orissa	1989-91	Rs 3 million	95,000	Das (1994)
- Kerala	1991-92	Rs 20 million	625,000	Das (1994)
Pakistan - Punjab	1996	-	300,000	AAHRI, ACIAR, IoA and NACA, 1997)

Public health

Prior to the initial EUS outbreaks, most countries in the region had not experienced a fish disease epizootic on such a large scale and, not surprisingly, there has been a great deal of local apprehension as to the consequences of consuming diseased fish or using affected waters for domestic or agricultural purposes. The concurrent deaths of ducks, cattle and other animals were attributed to the occurrence of EUS. There is however, no scientific evidence that the disease itself causes any human or animal illness. Rahman *et al.* (1988) were unable to induce any disease symptoms in ducks fed EUS-infected fish or even injected with *Aeromonas hydrophila* cultures. Therefore it is important to take public educational measures and allay the natural fears of farmers, fishers and consumers about any wider effects of EUS. However, it must be stressed that good hygiene practices should be adhered to. In particular, dead fish should not be collected for sale or consumption, not because of ulcerative disease as such, but because bacteria or toxins present in decomposing, EUS-affected fish may cause human illness.

The uncontrolled use of chemotherapeutants to treat EUS or other diseases in intensive culture systems is also a matter of public health concern. Chloramphenicol for instance, is used in treating typhoid in humans and there is a risk that the build up of bacterial resistance in treated fish (Poonsuk *et al.,* 1983) may be transferred to humans. Of greater concern to farmers is the possibility of severe allergic reactions affecting farm workers in contact with the drug. There is also the danger that consumers may be exposed to drug residues in marketed fish that had been hurriedly harvested before the recommended withdrawal period had been completed. Although these are issues that affect aquaculture in general, the occurrence of EUS has underlined the need to develop appropriate guidelines and legislation to protect farmers and consumers against the indiscriminate use of chemotherapeutants.

Aetiology

Diseased fish, particularly those with cutaneous ulcers, are vulnerable to infection by opportunistic pathogens and, in long standing cases, it is often difficult to identify the cause of the initial lesion. Given the wide geographical area, and the diverse range of habitats in which EUS-affected fish occur, a particularly diverse mix of microbiological agents have been recovered from affected fish. Some of these agents may significantly contribute to a disease complex in a particular outbreak, but it is important to distinguish them from the factor (or factors) essential in all EUS outbreaks. A description of fungi, viruses, bacteria and parasites found associated with EUS lesions is given here, along with comment on their importance in EUS outbreaks.

Fungi

Recent work has confirmed that a single species of *Aphanomyces* "fungus"[1] is a necessary cause[2] of EUS, *i.e.* it occurs in all outbreaks, and in some outbreaks (*e.g.* in Australian estuaries), may be the only biological factor required for the disease to occur.

The pathogenic *Aphanomyces* fungus

Fungi have been known to be involved in the aetiology of EUS in Southeast Asia since the initial outbreaks in Thailand. Limsuwan and Chinabut (1983) described a "severe chronic granulomatous mycosis" in histological sections of affected fish. However, the dominance of saprophytic fungal contaminants on the surface of EUS lesions led to the identification of *Achlya* and *Saprolegnia* spp. from affected fish (Pichyangkura and Bodhalamik, 1983; Limsuwan and Chinabut, 1983). These were soon recognised as secondary agents (Tonguthai, 1985), but it was also assumed that this may be the case for all mycotic involvement in EUS.

As described in the History section, before the first appearance of EUS in Southeast Asia, the pathogenic *Aphanomyces piscicida* had been isolated from MG-affected fish in Japan (Hatai *et al.,* 1977), but MG had not yet been recognised as synonymous with EUS. An *Aphanomyces* fungus was subsequently obtained from RSD outbreaks in Australia in 1989 (Fraser *et al.,* 1992) and, independently, from EUS outbreaks in Thailand in 1991-1992

[1] The genus *Aphanomyces* is contained within the family Saprolegniaceae and the class Oomycetes, and it should be noted here that the Oomycetes are no longer regarded as true fungi, but rather fungal-like protists. They are now often 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). They are, however, still commonly referred to as fungi and this term will be used for the purpose of this review.

[2] For a definition of "necessary cause" see Epidemiology section.

(Roberts *et al.,* 1993). These isolates were shown to be capable of reproducing typical EUS lesions when injected below the dermis of susceptible fish. All of these pathogenic MG, RSD and EUS isolates were shown to be slow-growing and thermo-labile in culture. Similar isolates have also been obtained from the Philippines, Indonesia, Bangladesh (Lilley and Roberts, 1997) and India (I. Karunasagar, pers. comm.). Recently, pathogenic *Aphanomyces* cultures from most of these countries have been compared directly, and shown by means of protein banding profiles (Callinan *et al.,* 1995b; Lilley *et al.,* 1997b), growth characteristics (Lilley and Roberts, 1997) and chemical susceptibility (Lilley and Inglis, 1997) to be all the same species. Genetic fingerprinting techniques have also been used to show that the various isolates were genetically all very similar (Lilley *et al.,* 1997a). This is proof that the isolates are not long-term residents in each locality, as would be expected of opportunistic fungi. Instead, they are part of one fungal strain that has colonised much of Asia and Australia in a matter of decades, and resulted in the spread of EUS.

The pathogenic *Aphanomyces* has been named variously as *Aphanomyces piscicida* (Hatai, 1980), *Aphanomyces invaderis* (Willoughby *et al.,* 1995) and ERA (EUS-related *Aphanomyces* sp.: Lumanlan-Mayo *et al.,* 1997). As isolates in each case have been shown to be conspecific, however, one species name is required to describe all these isolates. As *A. invadans* is the only valid taxon name according to the International Code of Botanical Nomenclature (ICBN), this is the name that will be adopted here.

A. invadans is known to grow fastest in culture at temperatures between 26-30°C (Hatai and Egusa, 1978; Fraser *et al.*, 1992; Lilley and Roberts, 1997), and has been shown to grow in snakehead muscle tissue between 19-31°C (Chinabut *et al.,* 1995). However, further investigation has revealed that snakeheads are able to recover from *A. invadans* infection at higher temperatures (26, 30°C), but are unable to prevent fungal invasion and eventually succumb to the disease at lower temperatures (19°C) (Chinabut *et al.,* 1995). The humoral and cellular immune response of fish are known to be supressed at low temperatures (Avtalion *et al.*, 1980; Bly and Clem, 1991), which may explain why mortalities from EUS occur when water temperatures are low. Naturally and artificially infected snakeheads have been shown to produce an antibody response against *A. invadans* (Thompson *et al.*, 1997), and the cellular macrophage response is also considered to be important in enabling fish to resist infection (Wada *et al.,* 1996).

A summary of the various published descriptions of the characteristics of *A. invadans* from EUS, MG and RSD outbreaks is given in Table 3. Techniques for isolating *A. invadans* from fish and water, and identifying candidate cultures to the genus *Aphanomyces* are given in the Annex. As with other saprolegniacean fungi, *A. invadans,* is aseptate and produces two zoospore forms, the secondary form being free-swimming and laterally biflagellate. No sexual reproductive structures have been observed in any of the isolates from EUS, MG or RSD outbreaks. The lack of sexual structures is considered to be a particularly common phenomenon among the more pathogenic members of the Saprolegniaceae (Alderman and Polglase, 1988).

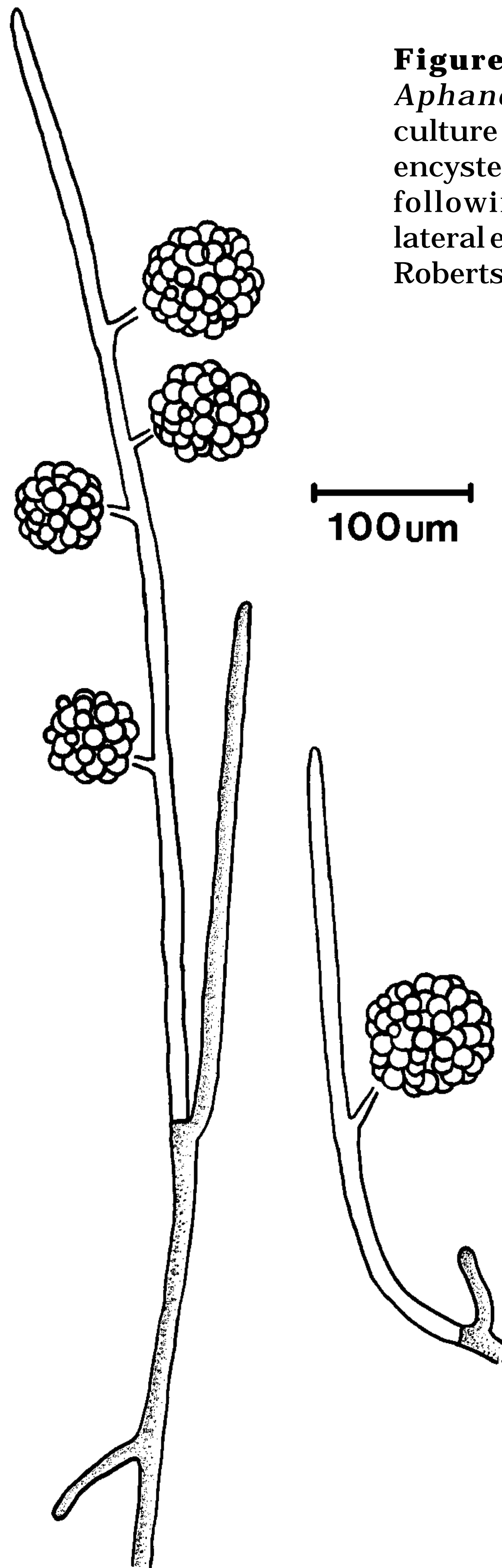


Figure 2 Sporulating *Aphanomyces invadans* culture showing clusters of encysted primary zoospores following discharge from lateral evacuation tubes (from Roberts *et al.* 1993)

Table 3 Characteristics of *Aphanomyces invadans* in culture

Characteristic	Description
Hyphal diameter	Variable. Wider in fish tissue (12-30 µm) than in artificial culture (5-20 µm on GPY agar). In culture, hyphae have rounded tips and branch almost at right angles to the main axis.
Radial growth	Grows at temperatures between 5-36°C, and salinity below 10ppt NaCl.
	No growth on cornmeal agar, malt extract agar or Sabouraud dextrose agar.
	On GPY agar (mm per 24h): 0.8 at 10°C 1.9 at 14°C 2.8 at 18°C 3.9 at 22°C 4.6 at 26°C 4.6 at 30°C 3.4 at 34°C no growth at 37°C
Oogonia	Not observed
Zoosporangia	Equal diameter to mycelium (about 10 µm)
Zoosporangial type	Terminal or intercalary. 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)
Zoosporangial renewal	Sympodial branching below empty sporangium
1° zoospore	Single row connected by thin strand of cytoplasm
1° zoospore cyst clusters	Achlyoid. Usually 30-50 1° zoospore cysts
1° zoospore cyst	Usually 6-10 µm diameter
2° zoospore	Motile, subspherical, biflagellate about 6 µm in diameter
	Released within 12 hours of sporangial development at 22°C.
	No sporulation above 2ppt NaCl
2° zoospore cyst	About 6.5 µm in diameter, sometimes "giant cysts" produced up to 27 µm in diameter
	Demonstrates limited polyplanetism (repeated zoospore emergence, encystment and re-emergence) in the presence of nutrient media

Involvement of other saprophytic fungi

Lilley and Roberts (1997) ruled out the possibility that multiple opportunistic fungal species are responsible for the mycotic granulomas typical of EUS, by showing that a number of saprophytic *Saprolegnia, Achlya* and *Aphanomyces* spp. from EUS-affected areas were incapable of sustained growth in snakeheads, even when injected directly into muscle tissue. Nonetheless, saprophytic *Saprolegnia, Achlya* and *Aphanomyces* spp. are commonly observed on the surface of EUS lesions (Pichyangkura and Tangtrongpiros, 1985; Willoughby and Lilley, 1992; Qureshi *et al.*, 1995), and may contribute to the disease as opportunistic wound parasites.

Reports of saprophytic *Aphanomyces* spp. acting as wound parasites on fish are not uncommon (Shanor and Saslow, 1944; Hoshina *et al.,* 1960; Srivastava, 1979; Ogbonna and Alabi, 1991; Khulbe *et al.,* 1995). *Aphanomyces* spp. have also been reported from freshwater dolphins (Fowles, 1976) and soft shell turtles (Valairatana and Willoughby, 1994), but these isolates can all be easily distinguished from *A. invadans* in terms of pathogenic and growth characteristics, and should not be confused with the EUS pathogen.

Viruses

Prior to recent mycological findings, viruses were considered to be the most likely necessary infectious cause of EUS. Several species of viruses have been isolated from EUS outbreaks and varying intepretations have been made of the pathogenic significance of these isolates. Evidence to date suggests that at least one of these species may be involved in some EUS outbreaks, particularly in Thailand, by predisposing fish to infection by *A. invadans.*

History of isolation of EUS-associated viruses

Following the 1982-3 EUS outbreak in Thailand, virus-like particles were demonstrated in various tissues of affected fish (Rattanaphani *et al.*, 1983; Wattanavijarn *et al.*, 1983a; b; 1984). These workers subsequently isolated the so-called snakehead rhabdovirus (SHRV), which was shown to be serologically distinct from other fish rhabdoviruses (Ahne *et al.*, 1988; Kasornchandra *et al.,* 1992). Between 1985-1989 a major sampling programme of over 200 fishes in 8 EUS-affected countries was undertaken, and as a result, 6 rhabdovirus isolates were obtained from Thailand, Myanmar, Sri Lanka and Australia (Frerichs *et al.,* 1986; 1989a; Roberts *et al.* 1989; Lilley and Frerichs, 1994). These isolates, named ulcerative disease rhabdovirus (UDRV), were shown to represent another species that was distinct from SHRV (Kasornchandra *et al.,* 1992) and other fish-pathogenic rhabdoviruses (Frerichs *et al.,* 1989b). Significantly, during this sampling programme, no viruses were obtained from Bangladesh, Lao PDR, Malaysia, Indonesia or the Philippines. Later virological surveys of northeast India (Boonyaratpalin, 1989a) and Pakistan (AAHRI, ACIAR, IoA and NACA, 1997) also yielded no viral isolates.

No further isolates of UDRV have been obtained since 1989, but sampling studies in Thailand have yielded an increasing number of isolates showing morphological and electrophoretic similarities to SHRV. Two such isolates were obtained in 1992, nine in 1994, and nine in 1996 (Kanchanakhan, 1996b). A further two virus isolates were obtained in 1997, but await characterisation (Kanchanakhan, unpublished data).

Aside from the rhabdoviruses, several birnaviruses and a single reovirus have also been isolated from ulcerated fish. Among the birnaviruses, sand goby virus (SGV) from Thailand, and a more recent isolate from Singapore, have both been shown to be distinct from the IPNV reference strains (Hedrick *et al.*, 1986; Subramaniam *et al.*, 1993). Two other birnavirus isolates were considered to be more similar to known IPNV strains: these comprised snakehead virus (SHV) from Thailand, and another isolate that was further identified as the Sp serotype of IPNV (Saitanu *et al.,* 1986; Wattanavijarn *et al.,* 1988). A reovirus, isolated from a diseased snakehead in 1992 (Frerichs, 1995), also appears to be a new, distinct viral strain or species (Riji John, 1997).

The heterogeneity of viral isolations and the low recovery rate of viruses led some workers to the conclusion that these were adventitious agents which would as likely have been isolated from healthy fish (Frerichs, 1995). Kanchanakhan (1996b) has recently revived interest in viruses by demonstrating that rhabdoviruses can be more readily isolated from fish specimens collected during the early period of outbreaks in Thailand. Viruses could not be obtained during the middle, late and recovery phases of outbreaks. In artificial challenge studies using a rhabdovirus strain isolated in Thailand in 1994 (T9412), the virus was reisolated from 100% of snakehead fish 3 days p.i. (post-injection), decreasing to less than 25% of fish 30 days p.i., at 20°C, suggesting that the virus was being partially or entirely eliminated by the host defence system (Kanchanakhan, 1996b). Successful virus isolation also requires that only freshly killed fish are sampled, and that tissue extracts are prepared immediately thereafter. The advised procedure for virus isolation is given in Annex 6.

Pathogenicity of EUS-associated viruses

Pathogenicity trials with most EUS-associated viruses have usually demostrated little more than scale damage or occasional development of minor skin lesions. Frerichs *et al.* (1993) were unable to show any consistent lesion in snakeheads immersed or injected i.p. (intra peritoneally) with an isolate of UDRV. Of the birnaviruses, only SHV has been tested in challenge studies. Saitanu *et al.* (1986) reported that i.p. injections of SHV resulted in scale damage in 80% of small snakeheads, but not at all in larger fish. Riji John (1997) demonstrated that the reovirus was not pathogenic to juvenile snakeheads in injection challenges.

More recent work by Kanchanakhan (1996b) showed that rhabdovirus strain T9412 can result in substantial lesions in striped snakeheads, particularly using challenges by i.m. (intra muscular) injection. The virulence of T9412 was shown to be dependent on temperature, fish species and fish age. All

snakehead fry died when challenged at 20°C, but no mortality was recorded at 29°C, or in other species of fish (including EUS-susceptible fish) at either temperature.

If viruses have a role in the pathogenicity of EUS, their most likely effect is to cause skin lesions sufficient to allow entry of the fungus, *A. invadans*. Kanchanakhan (1996b) subjected snakehead juveniles to i.m. injections of T9412 rhabdovirus or L15 medium, followed by bath challenges with *A. invadans* spores at 20°C. EUS was induced in 100% of fish given rhabdovirus infections and only 35% in fish given control L15 injections. This provides some evidence that T9412 may help to predispose fish to infection by *A. invadans*, but co-immersion challenges with the virus as well as the fungus are required to demonstrate that this can occur under more natural conditions.

Parasites

Several metazoan (*Dactylogyrus* sp., *Gyrodactylus* sp.) and protozoan (*Chilodonella* sp., *Trichodina* sp., *Costia* sp., *Henneguya* sp., *Ichthyophthirius* sp.) parasites were identified from 273 EUS infected fish during the 1982-3 epizootic in Thailand (Reungprach *et al.,* 1983) Several fish examined before the second outbreak, and thought to be at an early stage of the disease, showed tiny red spots on the skin. Examination revealed a large number of *Epistylis* sp. protozoans (Tonguthai, 1985).

In Australia, Callinan and Keep (1989) and Pearce (1990) found protozoan and metazoan parasites present on some affected fish, but concluded that no parasite species was intimately associated with lesions and there was no evidence to suggest that parasites initiate ulcers. In their survey of affected countries in southeast Asia, Roberts *et al.* (1986) found that diseased fish carried no more than the expected parasite load for wild rice paddy or riverine fish.

It therefore appears unlikely that any parasite acts either as a pathogen or a vector for a pathogen of EUS. However, parasites may at times induce stress in fish and predispose them to infection. For example, Subasinghe (1993) demonstrated a clear association between parasite burden of *Trichodina* sp. on gills and susceptibility of striped snakeheads to EUS infection. It is also possible that external parasites may, in some circumstances, induce mild skin lesions which would allow propagules of the fungal pathogen, *Aphanomyces invadans,* to attach and infect the fish host.

Bacteria

Available evidence suggests that bacteria may be important, but not essential, at two distinct stages in the pathogenesis of EUS.

1. Current evidence indicates *Aphanomyces invadans* must attach to the dermis before it can invade underlying tissues. Cutaneous bacterial infections (*e.g. Flexibacter*) may predispose fish to EUS by inducing skin lesions which provide an entry for the fungus (Figure 3).

It is possible that cutaneous bacterial infections may damage areas of epidermis and expose dermis, thereby allowing *A. invadans* to attach and invade underlying tissues. However, to date there are no reports confirming bacterial involvement in such a process, suggesting this is not a common means of EUS lesion induction. Although some workers have suggested that bacteria such as *Vibrio anguillarum* (Rodgers and Burke, 1981) or nocardioform bacteria (Chakrabarty and Dastidar, 1991) are necessary causes of EUS, several studies (Callinan and Keep, 1989; Boonyaratpalin, 1989b; Pearce, 1990) have failed to consistently associate any bacterial species with all, or even a large proportion of, ulcers on affected fish, suggesting bacteria are not necessary causes. This suggestion is supported by the observation that bacteria are only rarely visible in histological sections of EUS ulcers.

2. There is strong evidence that many EUS-affected fish die as a result of septicaemias caused by opportunist bacterial pathogens. It is likely that these bacteria first colonise the surface of established ulcers and then invade the bloodstream to induce lethal septicaemia (Figure 3).

   Aeromonas spp., notably *A. hydrophila* (Llobrera and Gacutan, 1987; Pal and Pradhan, 1990), can often be isolated from ulcers or internal organs of EUS-affected fish. Some of these *A. hydrophila* strains have been characterised as virulent (Torres *et al.*, 1990; Suthi, 1991; Karunasagar *et al.*, 1995) or cytotoxic (Yadav *et al.*, 1992).

Environmental Factors

Current findings indicate that normal skin defences must be compromised in some way before *Aphanomyces invadans* can attach to the skin and invade underlying tissues. Given that EUS outbreaks are usually seasonally recurrent, it is likely that a number of biotic and/or abiotic factors, influenced by seasonal changes, play a role in lesion induction and/or in the availability of infective forms of the fungus.

Several studies have examined possible associations between EUS outbreaks and changes in seasonal factors and water quality variables.

Temperature

Both low and high temperatures appear to influence outbreak occurrence and it is likely that these influences at least partially explain the seasonally recurrent pattern of EUS outbreaks.

Low temperatures appear to influence the severity of EUS lesions, and hence the severity of an outbreak, by impairing the ability of individual fish to contain and inactivate the invasive fungus. Chinabut *et al.* (1995) injected striped snakehead with *A. invadans* zoospores and showed that the inflammatory response was less pronounced, fungal invasion was more extensive, and mortality rates were higher, in fish kept at 19°C compared with fish kept at 26°C and 31°C.

Field studies also suggest that low temperatures are an important determinant for some, but not all, EUS outbreaks. Rodgers and Burke (1981) associated maximum EUS prevalence in estuarine fish populations with seasonal aggregations of fish stressed by low or rapidly changing water temperatures and rapid or prolonged depressions of salinity. Some EUS outbreaks in freshwater fish in Asia have occurred during periods of declining and/or unstable temperatures. During 1988 and 1989, outbreaks at sites in Bangladesh, China, India and Lao PDR occurred during months in which the mean daily temperature was below the annual mean daily temperature (Phillips and Keddie, 1990). However, outbreaks in the Philippines and Thailand have also been recorded in warmer months (Phillips and Keddie, 1990) suggesting there is no consistent relationship between EUS outbreaks and low temperatures. Diurnal temperature fluctuations of 10°C were recorded during outbreaks in both Bangladesh and the Philippines (Phillips and Keddie, 1990).

Studies in the Philippines (Lumanlan-Mayo *et al.*, 1997) suggested that outbreaks in rice-fish plots will not occur when maximum diurnal water temperatures remain at >30°C. It is likely that the causative fungus is substantially inactive at these temperatures. *A. invadans* hyphae grow only poorly at temperatures above 31°C and do not grow at 37°C (Hatai and Egusa,

1978; Fraser *et al.*, 1992; Roberts *et al.*, 1993). Zoospores are more sensitive than hyphae to temperature effects and zoospore production is inhibited at 35°C (Campbell, unpublished).

Rainfall and related water quality variables

EUS outbreaks in estuarine fish in Australia follow major rainfall events in the lower catchment (Virgona, 1992; Callinan *et al.*, 1995). It is likely that these events influence EUS occurrence in at least 3 ways.

1. The influx of fresh water into the estuary reduces salinity at outbreak sites to < 2 ppt (Rodgers and Burke, 1981; Costa and Wijeyaratne, 1989; Virgona, 1992), thereby allowing *A.invadans* to sporulate (Fraser *et al.*, 1992).
2. Acidified runoff water from acid sulfate soil areas in the coastal floodplain flows into the estuary (Sammut *et al.*, 1996). Fish sublethally exposed to this water develop areas of epidermal necrosis. *A. invadans* zoospores attach to, and invade, dermis exposed when this necrotic epidermis sloughs, thereby initiating EUS lesions (Callinan, 1997).
3. Organic matter, carried into the estuary with runoff water from the coastal floodplain, is broken down by microbial agents in the days following the major rainfall event, thereby reducing dissolved oxygen concentrations to <1 ppm for several days (Callinan, 1997). Fish sublethally exposed to this water may develop areas of epidermal necrosis (Plumb *et al.*, 1976) and underlying dermis may be colonised as above by *A. invadans* propagules.

Detailed environmental monitoring programs have linked EUS outbreaks in freshwater fish in Asia with rainfall events and associated low and/or decreasing water temperatures, alkalinity, hardness and chloride concentrations (Phillips and Keddie, 1990; Bondad-Reantaso *et al.*, 1992; Catap unpublished). However, in a study of EUS outbreaks in 4 ponds in Indonesia (Bastiawan unpublished), there was no consistent relationship between outbreak occurrence and rainfall, water temperature, hardness, alkalinity or any other measured water quality variable. Similarly, in a study of EUS in the Philippines, Palisoc and Aralar (1995) found that while outbreaks in Laguna Lake were associated with temperature, depth, Secchi disc transparency, alkalinity and chloride, outbreaks in Lake Naujan were associated with temperature only.

Flooding

Floods are thought to spread infection by aiding the spread of infected fish and the causal fungus. It is suggested that floods in Bangladesh in 1988 resulted in the rapid spread of EUS in that country.

Site characteristics

Source of infection

An EUS outbreak can occur only when susceptible fish, infective forms of the fungus and suitable environmental conditions are present at the site. Ahmed and Rab (1995) associated EUS outbreaks in Bangladesh with farming of susceptible fish species in ponds which had previously been derelict, or ponds which had been treated with piscicides to remove predators and other undesirable fish prior to stocking. Their findings indicate that the fungus must have survived in these ponds, either within surviving infected fish or in the environment, possibly as an encysted spore. Outbreaks in silver perch *Bidyanus bidyanus* in freshwater ponds in Australia are always associated with the presence of wild EUS-susceptible fish in the ponds or in the ponds' water supply (Callinan and Rowland, unpublished). These wild fish are a likely source of fungal propagules.

Soil or sediment characteristics

As noted above, EUS outbreaks in estuarine fish are often associated with recent acidified runoff from acid sulfate soil areas. It is also possible that soil and/or sediment characteristics influence outbreak occurrence in freshwater ponds, although no definite associations have yet been identified. Macintosh and Phillips (1986) found that sediments at many outbreak sites were slightly acidic and had low calcium content. They suggested that such soils would account for the poorly buffered acidic water and high levels of aluminium and iron in water samples from such sites. Ahmed and Rab (1995) noted an association between EUS outbreaks and ponds having reddish sandy soils, and suggested the associated relatively high turbidities in these ponds may have been stressful to fish.

Conclusion

Taken together, the available evidence suggests that a diverse group of biotic and abiotic agents, including viruses, bacteria, cutaneous ectoparasites, low pH and low dissolved oxygen concentrations, may initiate skin lesions in freshwater and estuarine fish and that these non-specific lesions are subsequently colonised by *A. invadans*. It is therefore unlikely that any specific environmental determinant is always associated with EUS outbreaks in freshwater or estuarine fish. It is more likely that environmental determinants will vary from outbreak to outbreak, depending on the agent initiating the non-specific skin lesions, the aquatic environment at the site and the fish populations at risk. Further studies are needed to identify these relationships in more detail.

Diagnosis

Correct diagnosis of EUS is important to avoid confusions with other ulcerative conditions. Positive diagnosis of EUS is made by demonstrating the presence of mycotic granulomas in histological section and isolating *Aphanomyces invadans* from internal tissues. Techniques for the isolation and characterisation of *A. invadans* are described in the Annex. The following gives some information on clinical signs and histopathology of EUS. Table 4 summarises these general findings. A glossary of technical terms is given at the end of this handbook.

Clinical signs

Studies on the pathology of EUS in Asia have tended to focus on the striped snakehead (*Channa striata*) as this is the species most commonly and most severely affected. However, significant differences with other species have been noted. In general, lesions on EUS-affected fish can be separated into 3 groups, on the basis of gross appearance (Viswanath *et al.*, 1997).

Clinical signs in the early stage of the disease are similar. Appetite is reduced or absent and fish become lethargic, either floating just beneath the surface or swimming with the head out of the water.

Gross pathology

Pinhead-sized, red spots develop on the body surface, head and fins, caudal peduncle, dorsum or operculum with no noticeable haemorrhages or ulcers. In the early stages these may simply be areas of acute dermatitis forming rosacea.

The intermediate stage lesions are represented by small (2-4 cm) dermal ulcers, with associated loss of scales, haemorrhage and oedema. Roberts *et al.* (1989) noted that in *Puntius* spp., gouramies and other midwater fish, ulcers are particularly dark and usually circular; often only one large superficial lesion occurs on the flank or dorsum. Most species, other than snakeheads and mullet, will die at this stage.

The advanced stage lesions appear on other parts of the fishes body and expand into large necrotic open ulcers; resulting eventually in death. Some affected species, *e.g.* striped snakeheads, can survive with much more severe, chronic lesions that may have completely destroyed the caudal peduncle or eroded deep into the cranium or abdominal cavity sometimes exposing the swim bladder. Head tissue erosion is a particularly common feature of diseased striped snakeheads and specimens have been found with exposed optic nerves or loosened articular bones such as maxillae and mandibles.

Table 4 Progressive diagnostic symptoms of EUS

EUS-affected fish in India (from Viswanath *et al.*, 1997)	EUS of mullet (*Mugil cephalus*) in Australia (from Callinan *et al.*, 1989)
Type I. Early lesions. Pinhead sized red spots on the body surface. No noticeable haemorrhage or ulceration. Skin around the spots is normal with no discolouration. Sections show focal inflammatory changes. There are several nodular structures in the epidermis, sometimes associated with fungal hyphae. Dermis and skeletal muscle are normal, without evidence of fungal invasion.	Erythematous dermatitis: Yellow skin with irregular reddening. Scales fractured. Usually <1 cm in diameter. 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.
	Intermediate-type dermatitis: Approximately 1 cm in diameter. Epidermis absent over lesion, though sometimes evidence of regeneration, scales usually retained. Mild to moderate chronic active dermatitis with some fungal hyphae and granulomas within skeletal muscle. Often muscle necrosis.
Type II. Moderately advanced lesions. Approximately 2-4 cm in diameter, raised, circular, discoloured areas on the body surface. These areas are soft with relatively intact skin and scales. In sections, mycotic granulomas seen in epidermis, dermis and skeletal musculature, associated with numerous, non-septate fungal hyphae. Significant necrotising dermatitis and myositis due to fungal invasion. In most of these lesions, scales and epidermis not completely lost.	Necrotising dermatitis: yellowish-grey to red, ovoid domed areas (1-4 cm diameter). Epidermis and scales usually absent, dermis swollen and macerated. Few hyphae trailed from lesion (not cotton-wool-like). Moderate to severe, locally extensive, necrotising, granulomatous dermatitis. Large number of sparsely-branching, aseptate hyphae (12-18 μm in diameter) usually within granulomas extending to skeletal muscle. Severe floccular degeneration of muscle.
Type III. Advanced lesions. Circular or oval, open dermal ulcers, extending into the skeletal musculature. Characterised by large haemorrhagic and necrotic open ulcers on the body surface, devoid of epidermis and scales, with loss of dermis at the site of the ulcer. In most cases the underlying musculature is exposed and largely replaced by fungal granulomas and host inflammatory tissue. Considerable myofibrillar necrosis. Fungal hyphae extend in all directions from the focus of the dermal ulcer. Necrotic muscle fibres and fungal hyphae often found within granulomas.	Dermal ulcer: About 1-4 cm in diameter. Margins sharply defined. Skeletal muscle exposed up to 1 cm 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.

Diseased striped snakeheads, with moderately advanced lesions, placed in improved water quality conditions often recover. Similarly, lesions on estuarine fish such as mullet appear to heal quickly when fish move into brackish or marine environments. Healing ulcers are characterised by a conspicuous dark colour caused by increased numbers of melanophores.

Histopathology

A general description of the typical histopathological developments that occur in EUS-diseased fish is given here with reference to some observations in particular species.

The early skin lesions of some samples have been observed and found to be principally areas of epithelial necrosis with surrounding oedema, haemorrhaging of the underlying dermis and some inflammatory cell infiltration. It has not been possible to confirm fungal involvement in most of these early samples but a few have harboured a small number of hyphae. The presence of fungal hyphae was demonstrated in the epidermis of some early stages of infected fish from India (Viswanath *et al.*, 1997). Similarly Roberts *et al.* (1989) were able to study early lesions during an EUS outbreak in a captive population of Indian major carp. They observed an acute necrotising myopathy, more severe than is usually seen in wild fishes, spread over a wide area below the active skin lesion. The epidermis at the margins of the ulcer itself was degenerate and thickened, and contained only a very small number of fungal hyphae enclosed within an epithelioid capsule. The blood vessels of the dermis were very hyperaemic and some had a collar of lymphoid or myeloid cells which might be associated with virus infection although no viral inclusion bodies were detected.

Subsequent pathological developments in all infected fish species involve significant degenerative changes in skin and muscle tissue with minimal disruption of internal organs.

In advanced lesions there is massive necrotising granulomatous mycosis of the underlying muscle fibres, involving the distinctive branching aseptate, invasive fungal mycelium. Large numbers of bacteria may be present on the surface of some advanced lesions. With advancing age of the lesion, fungal cells become progressively enveloped by thick sheaths of host epithelioid cells, and some areas may show evidence of myophagia and healing. In some advanced lesions, fungal hyphae can be seen invading the abdominal viscera, which would almost certainly be the ultimate cause of death. A large number of mycotic granulomas have been demonstrated in the kidney, liver and digestive tract of several fishes including spiny eels, *Cirrhinus mrigal*, *Colisa lalia*, *Channa* sp., *Puntius* sp., *Esomus* sp., *Mugil* sp., *Valamugil* sp., *Therapon* sp., *Glossogobius* sp. and *Sillago* sp. (Chinabut, 1990; Ahmed and Hoque, 1998; Wada *et al.,* 1994; Viswanath *et al.,* 1998). Wada *et al.* (1994) also found mycotic granumlomas in the abdominal adipose tissue, pancreas, gonad, spleen, central nervous system and heart of dwarf gourami; and Vishwanath *et al.* (1998) further demonstrated fungus penetrating the oesophagus and spinal cord of mullet and intermuscular bones of *Puntius*.

The internal organs of diseased fish, other than those invaded by fungal hyphae show only mild histopathological changes which, Roberts *et al.* (1986) pointed out, may sometimes be the result of background pathology. Palisoc (1990) observed minimal tissue disruption in the kidney of striped snakeheads in terms of an increased number of melanomacrophage centres, haemosiderin pigments and few mitotic figures. Spleen sections showed a marked increase of white pulp production and the heart, liver and gills underwent mild histopathological changes, but none were observed in the stomach and intestine. Other kidney samples have shown tubular, vacuolar degeneration with granular occlusion and haematopoetic tissue degeneration or focal proliferation. Chinabut (1990) demonstrated that these features were consistently more severe in armed spiny eels. Pancreas samples occasionally show acinar necrosis (Callinan *et al.,* 1989) with eosinophil and inflammatory cell infiltration. In the liver, mild focal hepatic cellular degeneration may also occur in advance of bacterial/fungal involvement. The only consistent haematological change in diseased fish is a significantly lower level of haemoglobin as a result of extra- and intra-vascular destruction of red blood cells (Tangtrongpiros *et al.,* 1985).

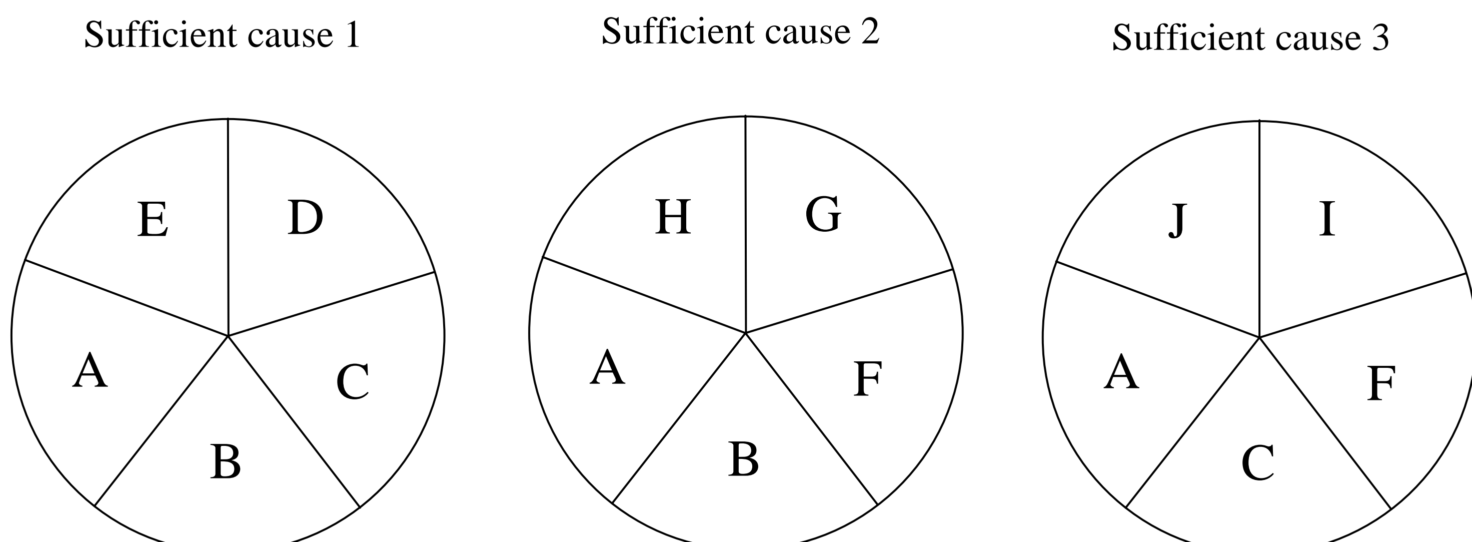
Epidemiology

Epidemiology is the study of the distribution and determinants (*i.e.* causes) of disease in populations. Epidemiologists typically take a wide view of causal factors, defining them as 'any event, condition or characteristic that plays an essential role in producing an occurrence of disease'. By contrast, many pathologists and microbiologists may consider, for example, a particular infectious agent to be the cause of a disease, and may relegate all other contributions to "contributing" or "predisposing" factors.

For most diseases, including EUS, there is strong evidence that outbreaks occur only when a number of causal factors combine. Many of the causal factors that have been identified or suggested, on the basis of reasonable evidence, for EUS may be represented in a causal web (Figure 3). Note that there are several levels within the web and that a number of factors may act at the same level (but not necessarily at the same time or intensity). Note also that, for EUS to occur, combinations of causal factors must ultimately lead to exposure of dermis, attachment to it by *A. invadans*, and subsequent invasion by the fungus of dermis and muscle. The resulting mycotic granulomatous dermatitis and myositis are, by definition, EUS.

The multifactorial nature of EUS causation can also be represented using the concepts of necessary cause, component cause and sufficient cause. Each combination of various causal factors ('component causes') which together cause a disease is known collectively as a 'sufficient cause' for that disease (Figure 4). It is important to recognise that, under different circumstances, different combinations of 'component causes' may constitute sufficient cause for a disease. Moreover, all sufficient causes for a particular disease have in common at least one component cause, known as a 'necessary cause'. This necessary cause must always be present for that disease to occur.

Figure 4. Schematic representation of the sufficient cause of a multifactorial disease. Note that factor A is the only component cause common to all sufficient causes, and is therefore the only necessary cause.



For EUS, recent studies have suggested there are a number of sufficient causes, each made up of its component causes (Figure 5). Note that each of these sufficient causes includes, amongst its component causes, the only currently recognised necessary cause, *A. invadans* propagules.

Figure 5. Sufficient causes for EUS established experimentally.

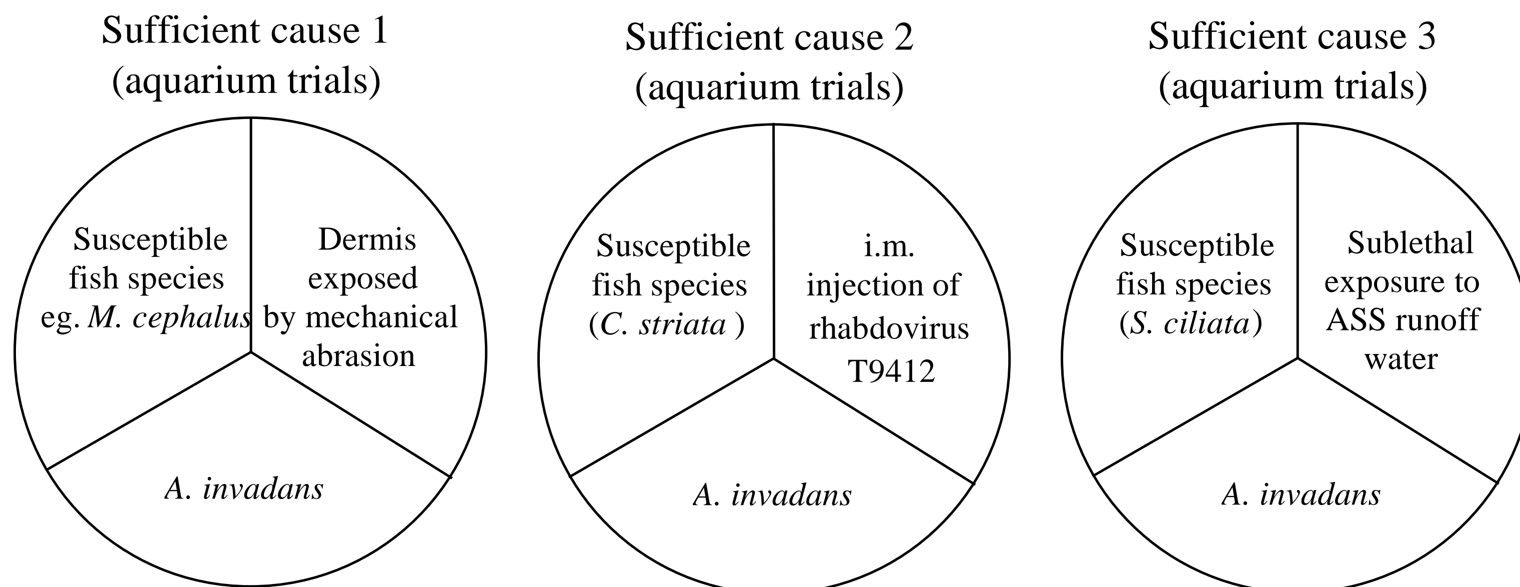
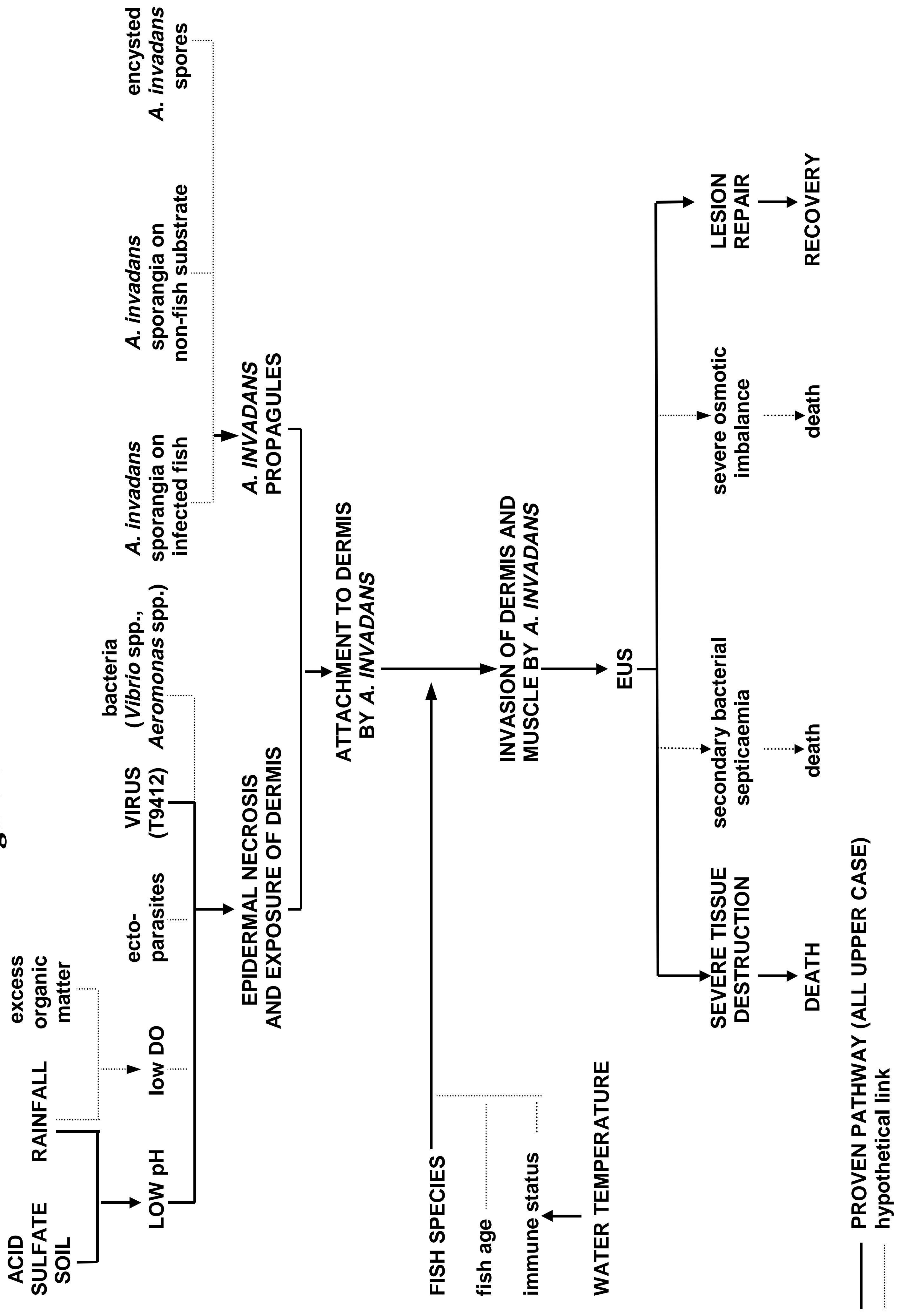


Figure 3 EUS Causal Web



Control of EUS

Now that research on EUS has conclusively identified some causal factors, rationally based control measures can be developed and implemented.

Prevention

Given that EUS occurs, and is often most damaging, in wild fish stocks, it can be very difficult to control outbreaks within a local area. Therefore, where EUS is not endemic, the most effective means of control would be to prevent the disease entering the country, or zone within the country (*e.g.* an EUS-free island), in the first place. Known EUS-susceptible fish are common in Africa and central Asia, and potentially susceptible fish occur in most other countries that are presently EUS-free. Quarantine and health certification guidelines for the movement of live fish between countries or regions are currently being proposed (Humphrey *et al.* 1997) and these may prove to be an effective means of preventing the spread of EUS to new areas. Publication of standard diagnostic techniques for important fish diseases such as EUS, and the development of pathogen-host databases will further assist implementation of these guidelines.

For areas where EUS is presently considered endemic, prevention programs should include the following activities :

- Eradication
- Exclusion
- Management
- Surveillance
- Treatment

Eradication

The aim here is to eradicate *A. invadans* from an already infected site (*e.g.* farm or pond). Although little is known about how the fungus survives between outbreaks, available evidence suggests that infection may persist, usually at low prevalence, in susceptible fish populations remaining at the site between crops. It is also possible, but less likely, that the fungus may survive in the aquatic environment either as encysted spores or on non-fish substrate.

Accordingly, measures to eradicate the fungus should include :

- Removal of all fish (particularly all susceptible species) from ponds, reservoirs and water supply channels prior to re-stocking;
- Drying out and liming of ponds;
- Disinfection of contaminated equipment.

Exclusion

Once the fungus has been eradicated from a site, it is important to prevent re-introduction. It is likely that infection is spread by affected or carrier fish, as well as by contaminated water or equipment. Accordingly, the following measures should be considered, taking account of local conditions and likely EUS prevalence in wild stocks.

- Seed stock, broodstock etc, should be obtained, if possible, from EUS-free locations and prophylactically treated for external fungal infection (e.g. with a 1%NaCl bath treatment) prior to introduction to the site.
- All wild fish must be rigorously excluded from farms in endemic areas.
- If possible, water should be obtained only from an EUS-free source, *e.g.* from a well or bore. If water from a potentially contaminated site must be used, it should be passed through fine screens at the supply inlet (to minimise risk of entry of wild fish) and stored in a fish-free reservoir for at least 10 days (Mathews and Reynolds, 1990, showed that *A. astaci* spores remained viable for 6-9 days at 10°C and a similar length of time has been established for *A. invadans* (Campbell, unpublished)). If the risk of introduction of infected wild fish or of *A. invadans* propagules is considered high (*e.g.* there is an EUS outbreak in progress in wild fish), serious consideration should be given to treating this stored water with piscicides and a disinfectant prior to use in ponds.
- Equipment which may have been used at infected sites must be disinfected, using standard hypochlorite or iodophor treatments.

Management

Epidemiological evidence suggests EUS outbreaks in farmed fish are more severe when stocking densities are high. During high risk periods, *e.g.* when EUS prevalence is high in adjacent wild fish populations, stocking densities should be kept as low as possible and farmed populations subjected to minimal stress. In particular, fish should be monitored (see below) to ensure that bacterial and parasitic skin pathogens do not cause problems during high risk periods, as such agents are likely to provide opportunities for the fungus to establish infections. Similarly, the pond environment should be monitored to ensure that abiotic factors which may induce skin damage, *e.g.* low dissolved oxygen concentrations, are kept within acceptable limits.

A simple and effective form of prevention, which may be acceptable in some endemic areas, is to farm species which are resistant to EUS. For example, EUS has never been reported in tilapia or milkfish, and very rarely in Chinese or European carps.

Surveillance

It is important that the general health, as well as the EUS status, of susceptible fish populations is monitored regularly during the growout period. As suggested above, if a significant proportion of fish with skin damage is detected during periods when *A. invadans* is likely to be available, the cause(s) must be identified and appropriate action taken. Although sampling methods for fish in ponds have not been accurately defined, it is suggested that a representative sample of fish from each population at risk should be examined at least weekly. The best means to achieve this will vary from farm to farm and depend on the species being grown. In some cases, it may be sufficient to closely observe the fish during feeding, bearing in mind that a diseased component of the population may not feed. In general, potential stress, particularly the risk of causing skin damage, to the fish arising from collecting a representative sample must be balanced against the need to observe them.

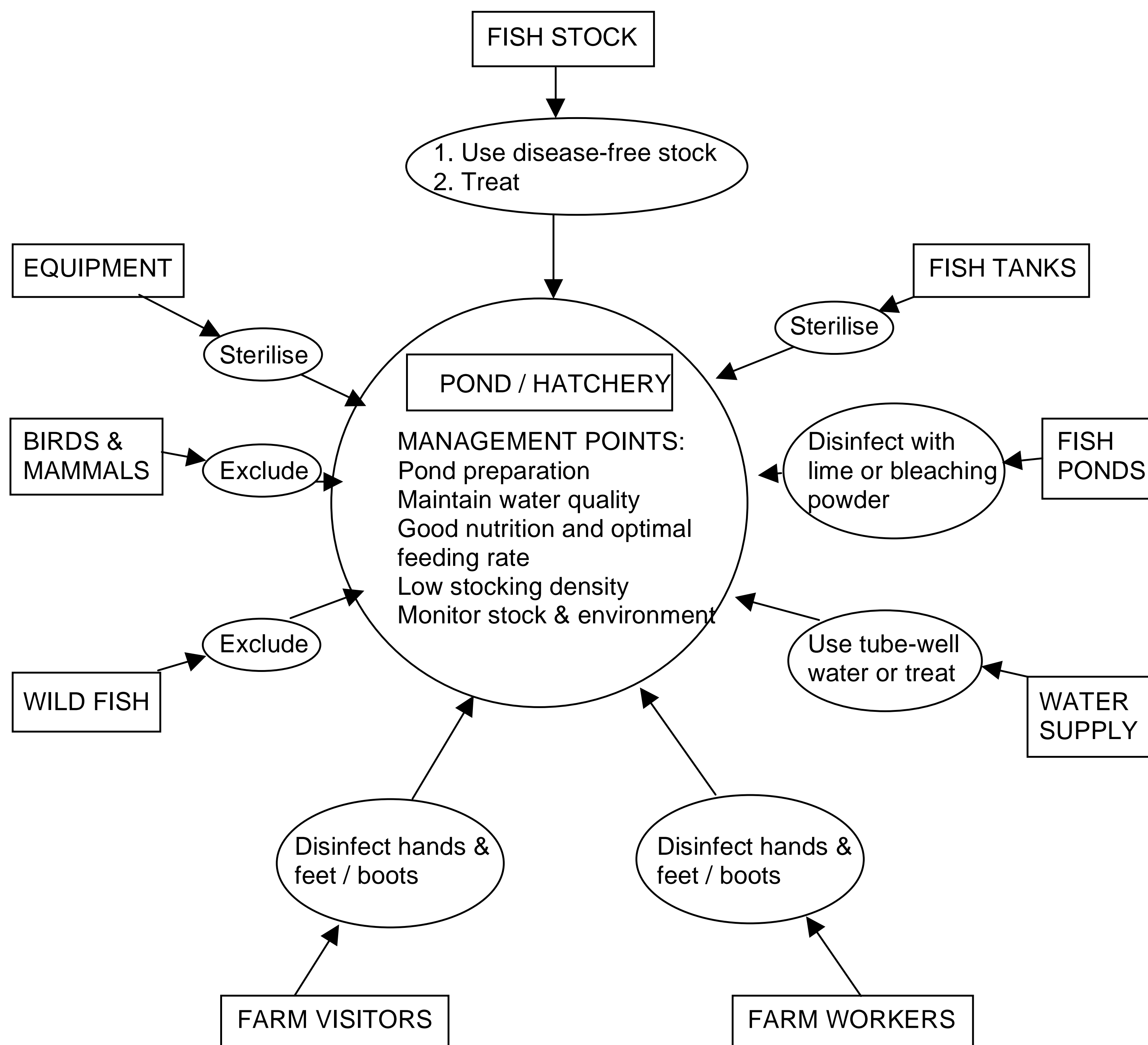
Treatment

A small number of studies has identified potentially useful treatments for preventing transmission of EUS in populations of farmed fish.

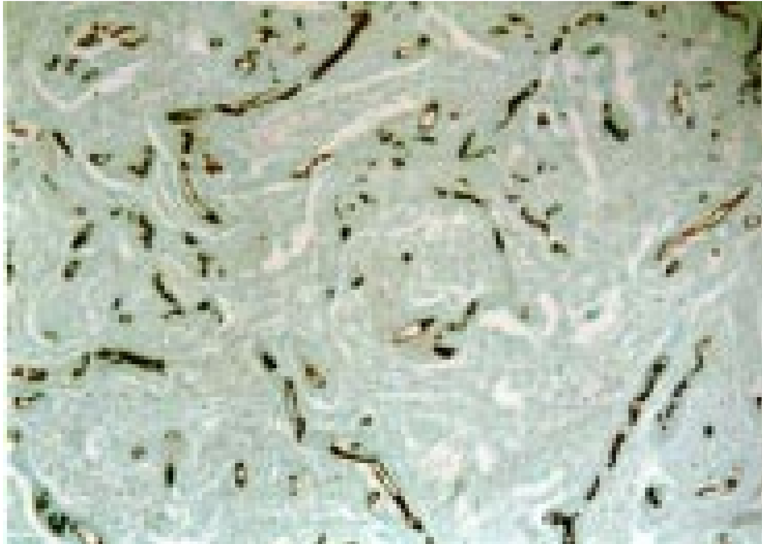
- Aquarium trials have shown that the following chemicals prevent induction of EUS lesions in abraded African catfish (*Clarias gariepinus*) fingerlings exposed to *A. invadans* propagules : 25 mg/L formalin, 5 ppt sodium chloride, 5 ppm Coptrol (a chelated copper compound) and 0.1 mg/L malachite green (Callinan, unpublished).
- Pond trials have shown that 5 ppm Coptrol prevented induction of EUS lesions in abraded African catfish fingerlings exposed to *A. invadans* propagules. Malachite green (0.1 mg/L) was only partly effective in preventing induction, while formalin (25 mg/L) was ineffective (Callinan, unpublished).
- Pond studies in Bangladesh (Ahmed and Rab, 1995) suggested that addition of agricultural lime to ponds during the culture period decreased the severity of EUS outbreaks. However, in subsequent pond trials in the Philippines, addition of lime at 2 kg/100 m^2 during the culture period failed to prevent EUS lesion induction in abraded African catfish (Callinan, unpublished).
- *In vitro* trials have suggested that malachite green, hydrogen peroxide and Proxitane 0510 (containing 5% peracetic acid in hydrogen peroxide) may have useful fungicidal activity against *A. invadans* (Lilley and Inglis, 1997).

Although no published accounts of effective curative treatments for established EUS lesions on farmed fish are available, Indian workers claim that a proprietary mixture, “CIFAX”, is curative.

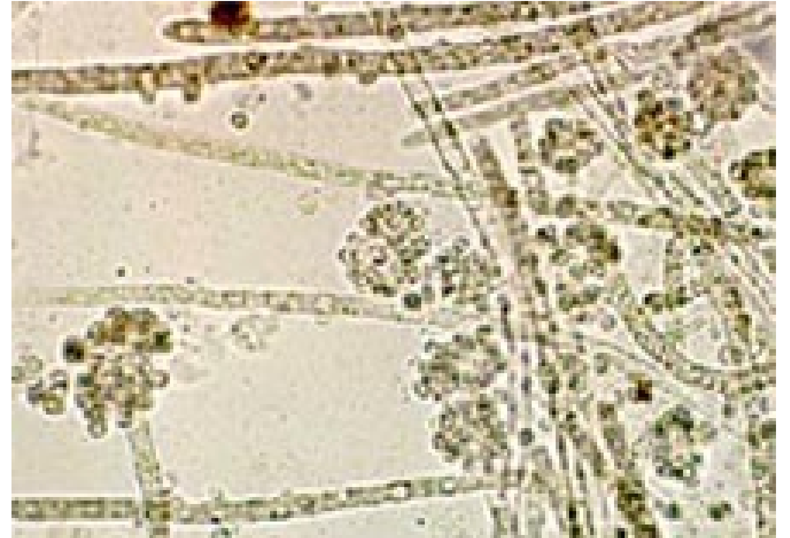
Figure 6 - Diagram summarising methods of preventing entry of infectious agents to the farm environment



Biological agents associated with EUS infection



Aphanomyces invadans **hyphae demonstrating ability to grow invasively through snakehead muscle. (Grocott's stain).**



Squash preparation of fungus showing typical *Aphanomyces* characteristics.

Moderate dermatitis in snakehead following intra-muscular injection of rhabdovirus isolate T9412. This virus is considered a probable component cause of EUS.

Typical EUS-like dermal ulceration in snakehead following intra-muscular injection of rhabdovirus T9412 followed by bath challenge with *Aphanomyces invadans* zoospores.

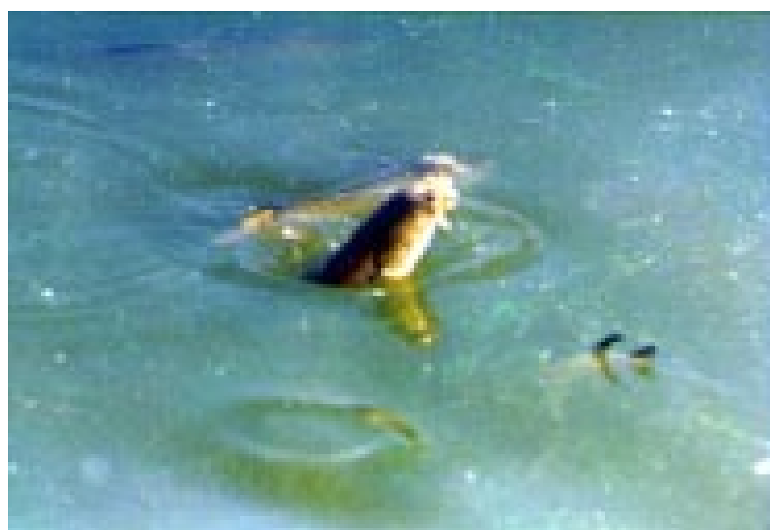
Lesions caused by *Lernaea* infestation, which may predispose fish to EUS.

Cutaneous *Flexibacter* bacterial infection which may predispose fish to EUS.

Clinical signs and gross pathology

Giant gouramy showing ulcers caused by disease unrelated to EUS.

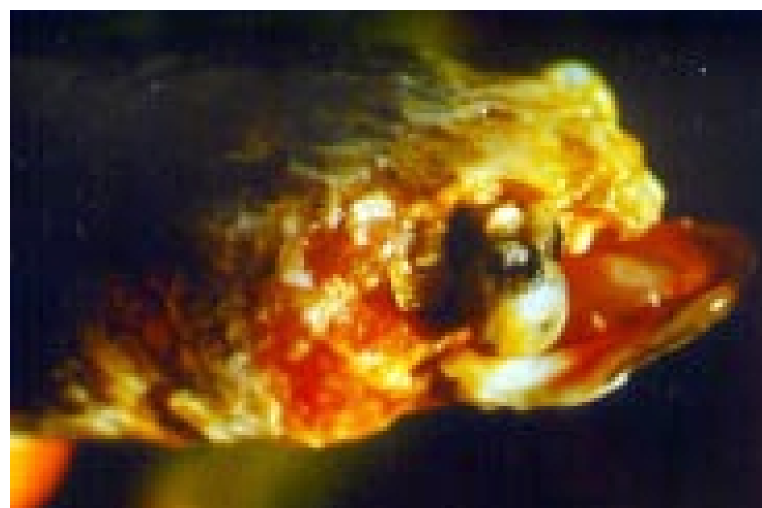
EUS snakehead fish kill in Suphanburi, Thailand.



EUS-affected snakehead, swimming with it's head out of the water.

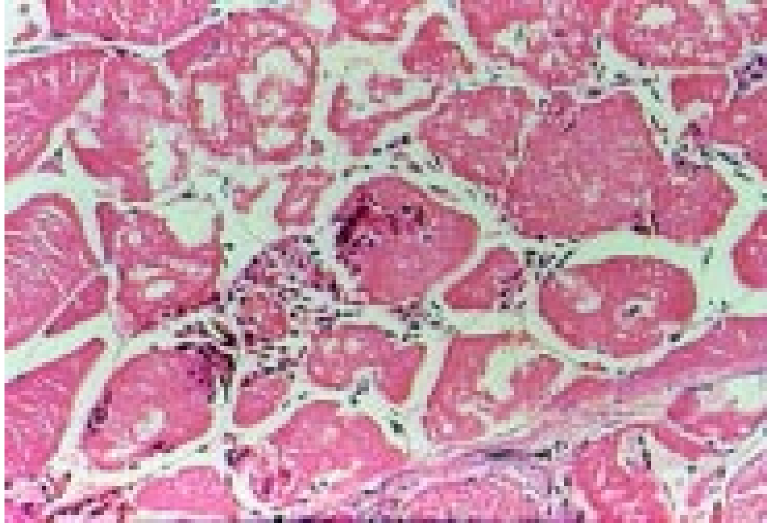
EUS-affected rohu showing moderately advanced lesions.

EUS snakehead with typical dermal ulceration.

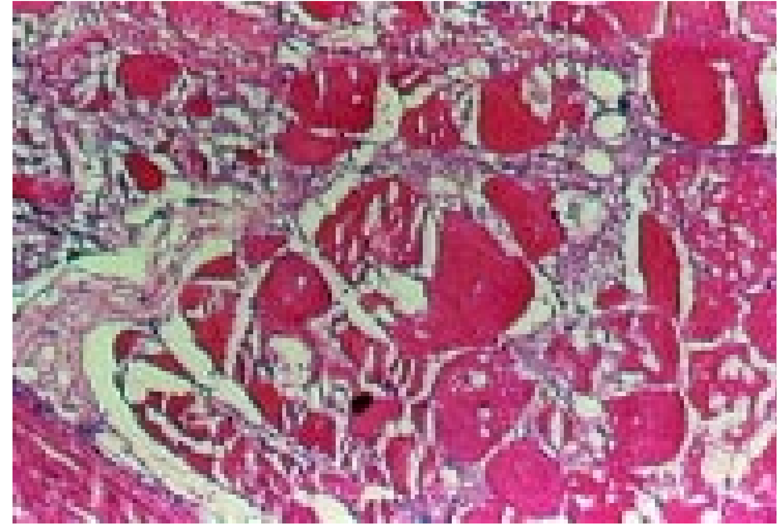


EUS snakehead with severe erosion of head tissues.

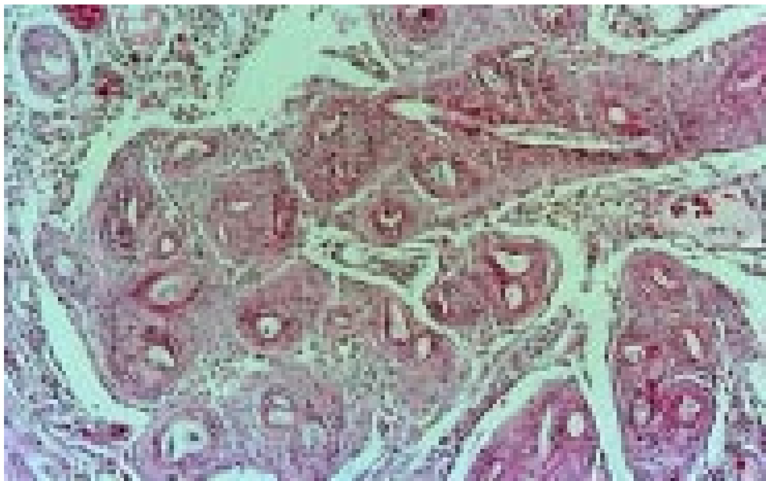
Histopathology



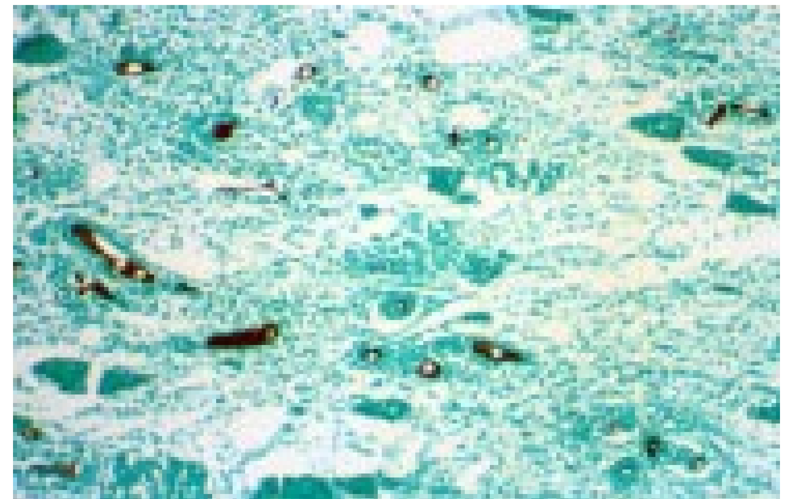
Early infection demonstrating fungal hyphae within small areas of necrosis.



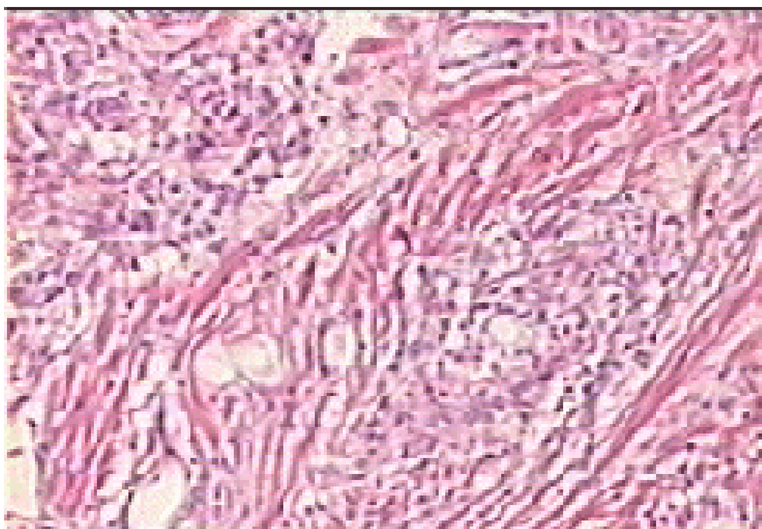
Mild sarcolysis in a moderate EUS lesion.



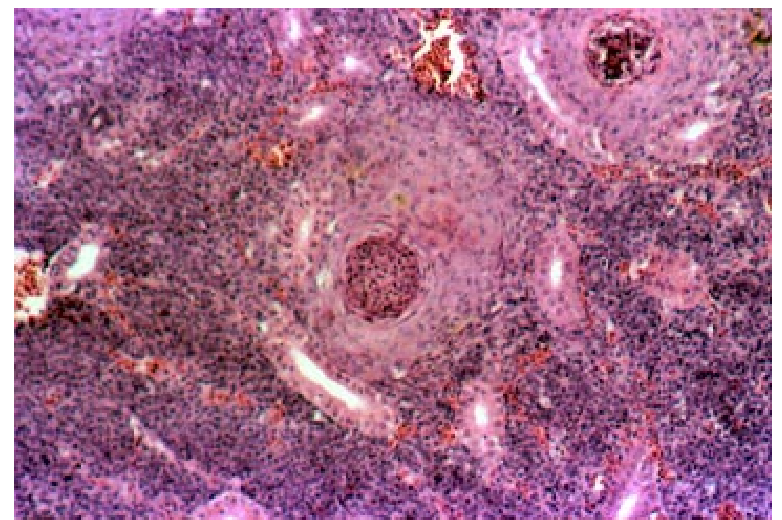
Severe mycotic granulomas in a muscle lesion from an advanced case of EUS.



Fungal hyphae (stained black) within mycotic granulomas in a muscle lesion from an advanced case of EUS. (Grocott's stain).



Fungal hyphae in the kidney of EUS infected fish.



Unrelated granulomas (left and right sides) and mycotic granulomas (centre) in the kidney of EUS infected fish.

EUS in Australia

EUS-affected sand whiting *Sillago ciliata*, caught in the Richmond River, eastern Australia (Photograph : RVL Wollongbar).

EUS-affected grey mullet *Mugil cephalus*, caught in the Richmond River, eastern Australia.

EUS-affected farmed silver perch *Bidyanus bidyanus* from eastern Australia.

Dermal ulcer on a grey mullet *Mugil cephalus*. The pale lesions on the ulcer surface are granulomas which have formed in response to invasion of skeletal muscle by *A. invadans*.

Aerial view of the junction of the main channel of the lower Richmond River, eastern Australia, and the tributary draining Tuckean Swamp, an acid sulfate soil area. The blue, acidified (pH ~ 4) tributary water can be seen mixing with the circumneutral, brown, main channel water (Photograph J Sammut).

Annex 1 - Isolation of *Aphanomyces invadans* from EUS-affected fish

Pale, raised lesions which have not yet completely ulcerated are most suitable for fungal isolation attempts. Yellow to red focal skin lesions or healing ulcers are unsuitable. Fish should be killed by decapitation and pinned, with the lesion uppermost, to a dissecting board. The scales around the periphery of the lesion should be removed and underlying skin seared with a red-hot spatula so as to sterilise the surface. If possible, the fish and board should then be removed to a laminar flow cabinet containing filtered air free of fungal elements. Using a sterile scalpel blade and sterile, fine pointed, rat tooth forceps, cut through stratum compactum underlying the seared area and, by then cutting horizontally and reflecting superficial tissues, expose underlying muscle. Ensure the instruments do not contact the contaminated external surface and otherwise contaminate the underlying muscle. Using aseptic technique, carefully excise up to 4 pieces of affected muscle, approximately 2 mm^3 and place them on a Petri dish containing the isolation medium.

Where a suitable lesion is found on the tail of a small fish (<20 cm), cut the fish in two using a sterile scalpel, by slicing a cross-section through the fish at the edge of the lesion. Flame the scalpel until red-hot and use this to sterilise the exposed surface of the muscle. Use a small-bladed sterile scalpel to cut out a circular block of muscle (2-4 mm^3) from beneath the lesion. Use sterile, fine pointed forceps to remove the block and place it on the isolation medium. In this way, it should be easy to prevent the instruments contacting the contaminated external surface of the fish.

Two different isolation media have been used successfully to obtain *A. invadans* cultures. The use of Czapek Dox agar with penicillin G (100 units/ml) and oxolinic acid (100 µg/ml) was reported by Fraser *et al.* (1992), and an adapted version of Willoughby and Roberts (1994) GP-PenOx broth is detailed below. Inoculated media are incubated at approximately 25°C and examined under a microscope (preferably an inverted microscope) within 12 hours. Emerging hyphal tips may be repeatedly transferred to fresh plates of GP-PenStrep agar until cultures are free of bacterial contamination. They may then be subcultured on GP agar at intervals of no greater than 5 days.

The fungus is subcultured by aseptically cutting a block of agar, 3-4 mm in diameter, from the periphery of a colony and placing this upside-down onto a Petri dish of fresh agar. Agar dishes should be inoculated within 24 hours of preparation and the surface should not be dried before use.

Basic GP (glucose-peptone) broth

3 g/l	glucose
1 g/l	peptone
0.128 g/l	$MgSO_4.7H_2O$
0.014 g/l	KH_2PO_4

0.029 g/l	$CaCl_2.2H_2O$
2.4 mg/l	$FeCl_3.6H_2O$
1.8 mg/l	$MnCl_2.4H_2O$
3.9 mg/l	$CuSO_4.5H_2O$
0.4 mg/l	$ZnSO_4.7H_2O$

Autoclave at 121°C for 15 minutes

GP-PenOx broth

prepare GP broth as above, autoclave, cool to 50°C and add:

100 units/ml	penicillin-K
10 µg/ml	oxolinic acid

GP agar

as GP broth with:

12 g/l	technical agar

GP-PenStrep agar

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

100 units/ml	penicillin-K
10 µg/ml	streptomycin sulphate

Annex 2 - Count method for *Aphanomyces invadans* propagules in pond water

The method involves collection of a 1 litre pooled sample (10 x 100 ml aliquots) from the pond and performance of a plate count on a 200 ml sample. Gelatin and selected metal ions are added to the subsample as nutrient supplements and antibiotics are added to reduce growth of contaminating bacteria and zygomycete fungi.

The method is a modification of aquatic fungal propagule count methods described by Anon (1992), Willoughby *et al.* (1984)and Celio and Padgett (1989). These previously described methods proved unsatisfactory for *Aphanomyces invadans* as they failed to support growth of isolates while allowing abundant growth of other fungi and bacteria. The modifications are based on the observations that *A. invadans* is one of a limited number of aquatic fungi that can utilise complex protein sources and that a large proportion of colonies attach to plastic surfaces. During the counting procedure, most of the non-*A. invadans* colonies fail to attach and are removed by washing. Bacterial contamination is minimised by the low concentrations of complex protein, absence of glucose and addition of antibiotics.

After incubation of plate count cultures, colonies consistent with *A. invadans* are identified on the basis of attachment to the plastic surface, and hyphal and sporangial morphology. Representative *A. invadans*-like colonies are subcultured for further characterisation

The technique has been trialled successfully in several artificially infected pond waters, but to date it has been possible to trial the method only once during a natural EUS outbreak. Counts of *A. invadans* propagules in pond water during that outbreak have ranged from 10 - 30 per litre.

Reagents

Gelatin
Pimaricin (2.5%)
Sodium benzyl penicillin
Oxolinic acid
Potassium dihydrogen orthophosphate (KH_2PO_4)
Calcium chloride ($CaCl_2.2H_2O$)
Magnesium sulphate ($MgSO_4.7H_2O$)
Zinc sulphate ($ZnSO_4.7H_2O$)
Ferric chloride ($FeCl_3.6H_2O$)
Distilled water

The chemicals used should be reagent (analytical) grade. To avoid contaminating the stock pimaricin suspension, aseptically dispense a 1 ml aliquot for day to day use. All antibiotics to be held at 4°C.

Glassware

All glassware used throughout this procedure must be scrupulously clean. Minimum requirement is as follows:

Soak in laboratory detergent
Rinse 3 times in tap water
Rinse 5 times in distilled water
Mark 1 litre glass bottles at 200 ml capacity.

Sample collection bottle

The sample collection bottle used is a modified 100 ml glass Schott bottle. The inlet (a small glass filter funnel) is covered with nylon mesh of 200 mm aperture size. The collection bottle is secured to a 2 m long stick so that when the stick touches the bottom sediment the inlet will be about 5 cm above the sediment. The stick is used to push the bottle to the bottom as rapidly as possible. Plastic bottles and tubing are not suitable.

Pipettes

By preference, all pipetting should be done with air displacement pipettes with disposable tips. Two micropipettesare used, one capable of 40 - 200 ml and the other 200 - 1000 ml. Where this is not possible glass capillary plunger type pipettes (Socorex) should suffice if capillaries are discarded after use. If glass pipettes have to be used they must be carefully washed. Where small volumes are required *e.g.* pimaricin, it may be necessary to prepare intermediate dilutions.

Filtered pond water (FPW)

Collect 3 litres or more of pond water from one of the untreated ponds to be tested. For each pond pooled water sample approximately 250 ml of FPW will be required. Filter through No 1 filter paper and autoclave at 121°C for 15 minutes. Store at room temperature. Before use, this water sample should be tested using the FPW positive control system described below to ensure that 100% of *A. invadans* colonies sporulate satisfactorily.

Stock metal ion solutions

Use sterile glassware to make up solutions. Keep the solutions in separate sterile disposable plastic containers at 4°C.

Dissolve the following amounts carefully in specified quantity of sterile distilled water before dispensing into storage container.

		Distilled H_2O
KH_2PO_4	0.267 g	20 ml
$CaCl_2.2H_2O$	0.588 g	20 ml
$MgSO_4.7H_2O$	2.533 g	20 ml
$ZnSO_4.7H_2O$	0.110 g	250 ml
$FeCl_3.6H_2O$	0.600 g	250 ml

Gelatin nutrient solution

The following formula is sufficient for 5 pond water pooled samples. Adjust by proportion as necessary.

Gelatin

Gelatin	0.4 g	100 ml FPW

Prepare fresh gelatin solution immediately before use. Use a clean sterile bottle and measuring cylinder to make up the solution. Weigh gelatin and transfer to sterile bottle. Add approximately 35 ml of the FPW and bring to simmering point (a microwave oven is convenient). Dissolve gelatin by vigorously shaking. When gelatin is dissolved add remaining FPW and cool to room temperature in the freezer.

Metal ions

Add 1.20 ml of each ion solution (K, Mg, Ca, Zn, Fe) to the gelatin solution.

Antibiotics

Penicillin	0.080 g
Oxolinic acid	0.080 g
Pimaricin	120 µl

Immediately before use add antibiotics as above to cooled gelatin solution (100 ml). Shake the bottle vigorously to dissolve as much of the antibiotics as possible. (The pimaricin and oxolinic acid will remain in suspension and the nutrient medium must therefore be shaken before each aliquot is removed).

Collecting a pooled water sample

Using the collection bottle collect ten aliquots of approximately 100 ml from random sites in the pond. Each aliquot should be collected as close to the bottom as possible without disturbing the sediment. The 1 litre bottle for the pooled sample must be glass and both it and the collection bottle should be thoroughly rinsed before use in the water to be tested. The pooled samples should be held at room temperature and processed as soon as possible, certainly within 1 hour of collection. The delay between collection and processing should be as constant as possible for all ponds.

Processing the pooled sample

This procedure should be carried out close to an incubator maintained at 27°C. Mix the pooled sample by gentle inversion and rolling and immediately dispense 200 ml of pooled sample into a sterile 1 litre bottle marked at 200 ml. Shake the gelatin nutrient solution and add 18 ml of solution to the subsample (use a sterile 25 or 50 ml measuring cylinder). Mix the subsample and nutrients gently by rolling and inversion, and immediately dispense the entire sample into 7 disposable plastic Petri dishes, mixing as above between dishes. By eye keep the volume in each dish as equal as possible. Transfer plates to the 27°C incubator as soon as they have been dispensed. Incubate for 48 hours in the dark. After 24 hours gently turn the plates through 360° to detach larger colonies that may be growing, otherwise do not disturb them.

Positive controls

These provide comparative material to aid recognition of *A. invadans* colonies in the test sample and ensure that reagents and glassware are satisfactory. The results of the pond water control may allow a correction factor to be calculated at the end of the experiment. Each test day set up a pond water positive control and an FPW positive control.

Approximately 14 hours before use, wash 3 x 4-day-old GPY broth mats of a vigorous *A. invadans* culture in 5 changes of FPW. Try to keep the delay between washing and use as constant as possible. Immediately before use, remove the mats with a sterile wire. Dispense 9.9 ml of FPW into a sterile glass bottle and add 100 μl of gently mixed spore suspension. Mix gently and take a 1 ml sample for a Sedgewick-Rafter count. Count motile and total spores in 30 x 1 μl squares. There should be approximately 100 spores per 100 μl and more than 50% of these should be motile.

To one 1 litre bottle add 200 ml of FPW and to another add 200 ml of the sample pond water from the same pond from which the FPW was obtained. To each control add 150 μl of the 1:100 spore suspension and proceed with addition of gelatin nutrient solution as above.

If 1:100 spore preparation contains less than 50, or more than 150 spores per 0.1 ml, adjust the volume added so that about 150 motile spores will be added to each 1 litre bottle.

Repeat the count on the 1:100 spore suspension immediately after adding the spores to the control samples. Record the number of spores (the mean of the 2 counts) added to each 1 litre bottle and the time elapsed since the mats were first washed.

Counting and Identification

Examine the positive control plates first. Gently discard the fluid and any floating fungal colonies in the plates and fill the dishes with sterile distilled water. Allow the dishes to stand for 5 minutes before discarding all but about 5 ml of wash fluid.

With a felt tip pen, close to but not obscuring the colony, mark on the plastic dish colonies resembling *A. invadans*. The colonies will be loosely adherent to the bottom and will measure 3-5 mm in diameter.

Examine the marked colonies with the 10x and 20x objectives of an inverted microscope. Mark with a circle, colonies that have the characteristic right angle branching, 10 μm hyphal diameter and the rounded tip. Hyphal diameter can be estimated with a calibrated eyepiece graticule.

At this stage hyphal tips from representative colonies in the test samples can be removed with a sharp scalpel and inoculated deep into agar plates (Czapek Dox with 100 units/ml penicillin and 100 mg/ml oxolinic acid, or GP-PenStrep: see Annex 1). Examine the plates daily with a stereo microscope for at least 5 days, and subculture fungal tips as soon as possible. Recovered fungi can be identified by sporulation features, hyphal diameter, growth rate at 22°C and failure to grow at 37°C (see section on Fungal Aetiology). Count only those colonies typical of *A. invadans*.

Spores per litre = *A. invadans* colony count (total for all 7 plates) x 5

If less than 100% of colonies sporulate satisfactorily in the FPW positive control then it may be necessary to repeat the counts after adjusting ionic strength of FPW by diluting up to 1 part FPW with 2 parts distilled water. Determine the optimum dilution by titration in the FPW positive control system.

Recording

Record the colony count for each sample and for the FPW and pond water positive control. For the latter subtract any *A. invadans* that were detected in the test sample.

Record the number of colonies subcultured, and for each subculture record the results of sporulation, hyphal diameter and 22°C and 37°C growth tests. Maintain axenic cultures of representative probable *A. invadans* colonies for possible pathogenicity studies.

Annex 3 - Maintenance of *Aphanomyces invadans* cultures

A. invadans cultures can be maintained in flasks of 200 ml GP broth (see Annex 1) at 10°C for only 6 weeks before subculturing is required. This is due to the rapid staling of the growth medium (Willoughby and Chinabut, 1996). The advantage of this technique, however, is that any bacterial contamination can be easily recognised as clouding of the medium.

Cultures can be maintained for longer periods on agar slopes in universal tubes, with sterile light paraffin oil covering the entire slope, as described by Smith and Onions (1994). Particular care should be taken to avoid contamination, as bacterial growth is not readily apparent in these cultures. GPY agar can be used for this procedure, but *A. invadans* have been sustained for longer periods (over 6 months at 20°C) using a buffered medium developed for *A. astaci* (PG-1).

GPY (glucose-peptone-yeast) agar

As GP agar (Annex 1) with:
0.5 g/l yeast extract

PG-1 (peptone-glucose-1) agar

3 g/l	glucose
6 g/l	peptone
0.17 g/l	$MgCl_2.6H_2O$
0.15 g/l	$CaCl_2.2H_2O$
0.37 g/l	KCl
0.02 g/l	$FeCl_3.6H_2O$
0.044 g/l	Na_2 EDTA
12 g/l	technical agar

Buffer with 13 mM sodium phosphate. 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 - $NaH_2PO_4.2H_2O$ - store at 4°C
71.7 g/l	solution B - $Na_2HPO_4.12H_2O$ - 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.

Annex 4 - Inducing sporulation in *Aphanomyces invadans* cultures

The induction of asexual reproductive structures is necessary in order to identify fungal cultures as members of the genus *Aphanomyces*. To induce sporulation, place an agar plug (3-4 mm in diameter) of actively growing mycelium in a Petri dish containing GPY broth and incubate for 4 days at approximately 20°C. Wash the nutrient agar out of the resulting mat by sequential transfer through 5 Petri dishes containing autoclaved pond water (APW), and leave overnight at 20°C in APW. After about 12 hours, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores should be apparent under the microscope. Features that distinguish sporulating cultures of *Aphanomyces* from *Saprolegnia* and *Achlya* are given in Annex 5.

GPY broth

as GP broth (Annex 1) with:

0.5 g/l	yeast extract

APW (autoclaved pond water)

Sample pond/lake water known to support fungal growth, and with pH 6-7. Filter through Whatman 541 filter paper. Combine one part pond water with two parts distilled water and autoclave.

Annex 5 - Identification of saprolegniacean fungal cultures

The Saprolegniaceae are aseptate, eucarpic fungi that typically demonstrate two zoospore forms. The secondary zoospores are characteristically reniform and laterally biflagellated. The two flagellae differ in type (heterokont), with one anteriorly-directed tinsel-type flagellum and one posteriorly-directed whiplash-type flagellum.

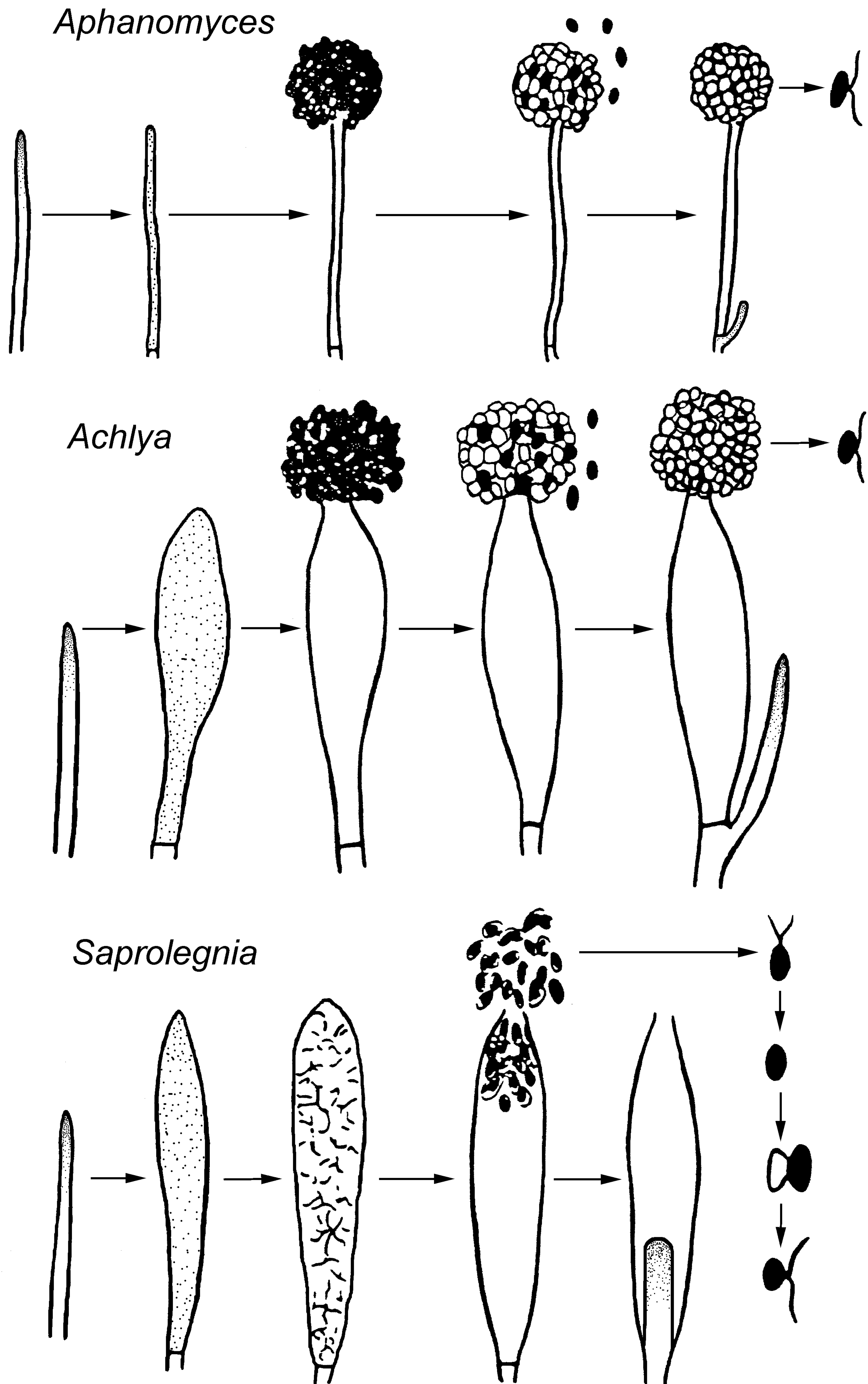
Saprolegniacean genera are distinguished primarily by asexual characters, particularly zoosporangial shape, method of zoospore release and method of zoosporangial renewal. The production of asexual characters can be induced as described in Annex 4. The variation in these characters between the three main saprolegniacean fungi associated with fish disease (*Aphanomyces, Achlya* and *Saprolegnia*) are illustrated in Figure 7. Identification of these fungi to the species level usually depends on the production of sexual structures, but these are commonly absent from fish-parasitic species, and unknown from *A. invadans*.

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, or from lateral evacuation tubes, at which time they immediately encyst and form achlyoid clusters. The primary zoospore is therefore not fully released from the sporangium. The main free-swimming stage of *Aphanomyces* spp. is the secondary zoospore which is discharged from the encysted primary zoospores. 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, although further tertiary generations of zoospores may be released from cysts (polyplanetism). Specific identification of the EUS pathogen, *A. invadans*, is discussed in the Fungal Aetiology section.

Achlya spp. zoosporangia are usually formed from terminal hyphal swellings which differentiate into the primary zoospores. These encyst, as with *Aphanomyces,* in an achlyoid manner, but only at the apical tip of the zoosporangium. Zoosporangial renewal is typically sympodial, branching from the hypha below the basal septum delimiting the spent zoosporangium.

The zoosporangia of *Saprolegnia* spp. are, as with *Achlya* spp., short terminal hyphal swellings. However in saprolegnians, the primary zoospore is fully released from the zoosporangium and remains motile for a short period before encysting and releasing secondary zoospores. Polyplanetism can be particularly pronounced among fish-parasitic *Saprolegnia* spp. Zoosporangial renewal is typically by internal proliferation, *i.e.* the secondary zoosporangium develops within the previously emptied primary zoosporangium.

Figure 7 - Zoosporangia formation and dehiscence in *Aphanomyces, Achlya* and *Saprolegnia* (reproduced with kind permission of Dr L.G. Willoughby)



Annex 6 - Isolation of viruses

The isolation of viruses from EUS-diseased specimens can be very difficult, even with access to specialised virological facilities. Two factors have been identified as critical for successful isolation. Firstly, the diseased fish need to be collected during the early period of an outbreak (*i.e.* within 1-2 weeks of the disease being noticed); and secondly, the specimens need to be alive just before collection of the tissue samples.

Tissue sampling and handling

Diseased fish with early skin lesions should be collected. Fish are sacrificed and wiped clean with tissue paper. Approximately 1g is taken of each tissue sampled. For muscle samples, tissue debris and surface fungus on the ulcerated lesions are removed using a clean razor blade. Pieces of muscle tissue are taken from beneath the lesions. For internal organ samples, the abdomen is carefully opened using clean scissors, and small pieces of tissue from kidney, spleen, intestine and pancreas are taken and pooled. If the fish are very small, the entire viscera can be taken. Tissue samples from up to 5 fish can be pooled and processed as 1 tissue extract. Tissue samples can be stored up to 48 h in HBSS (Hank's balanced salt solution) supplemented with 2% FCS (foetal calf serum), 500 IU/ml penicillin, 500 mg/ml streptomycin and 10 mg/ml amphotericin B (fungizone) at 4°C.

Samples are then homogenised using a sterile, pre-cooled pestle and mortar until a smooth paste is obtained. Sterile fine sand is added to facilitate homogenisation. Samples are diluted 1:10 by the addition of 9 ml HBSS containing 2% serum. After mixing well, the samples are transferred to sterile centrifuge tubes and spun at 1000 x g at 4°C for 15 min to separate cell debris, sand and possibly contaminating micro-organisms from the fluid extract. A further 1:5 dilution is carried out by filling 5 ml sterile disposable syringes with 4 ml HBSS (with 2% serum) and then drawing up 1 ml supernatant. These 1:50 final dilutions are mixed well and then filter-sterilised through 0.45 mm disposable filter units. The filtrates or tissue extracts are kept in 5 ml sterile bottles at 4°C which are ready to be inoculated directly onto fish cell lines or, if necessary, transported to the fish virology laboratory.

Virus isolation

Simultaneous cell culture and sample inoculation should be carried out using BF-2 and/or SSN-1 cell lines. Tests are conducted in 24-well plates. Each plate is first seeded with a single cell suspension of the indicator cell line in L-15 medium containing 2% serum and 1x antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). Each well receives 1.3-1.4 ml of cell suspension. Cell density should be sufficient to produce a 80-90% confluent

monolayer 1 day after seeding. Tissue extracts (1:50 dilution) are immediately inoculated into 2 replicate wells. The inoculum volume is 200 µl/well. An equal number of inoculated wells as negative control well should be allocated for each plate. Cells are incubated at 23-25°C and observed daily for CPE (cytopathic effect) for at least 14 days. The first blind passage of culture fluids is performed between day 7-10 by transferring 200 µl of supernatant from each well to fresh culture wells and observing the plates for a further 14 days. A second and third blind passage should also be carried out.

Samples showing CPE in which the cell monolayer changes (*e.g.* disintegrates, sloughs off the surface of the tissue culture wells, or results in cell lysis) should be passaged to provide larger quantities of the suspected virus. Two hundred microlitres of supernatant from a single well exhibiting CPE is inoculated into 25 cm^2 flasks containing a 80-90% confluent cell monolayer. The suspected virus is allowed to adsorb for 1 h. The cells are washed once with 5 ml PBS (phosphate buffered saline), then 7 ml of maintenance medium (L-15 with 2% serum) is added. Flasks are incubated at 23-25°C together with un-inoculated control flasks for comparison. When the cells show complete CPE, they are spun at 1000 x g at 4°C for 15 min. The supernatant is collected and divided into 1 ml aliquots. Some tubes are kept at 4°C for further characterisationwithin 6 months and others stored at -20°C or -70°C for long term storage.

Annex 7 - Investigation of EUS outbreaks

An outbreak can be defined as a short term epidemic or a series of disease events clustered in space and time. Such disease events are usually new cases of a disease occurring at a higher frequency than is normally expected. Throughout this section, the terms outbreak and epidemic are used more or less interchangeably.

An outbreak investigation is a systematic procedure to help identify causes and sources of epidemics, with a view to controlling the existing epidemic and preventing future ones. Usually, the primary objective of an epidemic or disease outbreak investigation is to identify ways of preventing further transmission of the disease-causing agent. The epidemiological approach to outbreak investigations is based on the premise that cases of a disease are not distributed randomly, but occur in patterns within the at-risk population. It is the role of the epidemiologist to record and analyse these patterns to help meet the primary objective.

Little is known of the means whereby EUS spreads within and between regions, although movements of subclinically affected fish are probably important in transmitting *A. invadans* infection. Until effective control and prevention measures are implemented, it is likely that the disease will continue to spread and that outbreaks will continue to recur in endemic areas. Comprehensive investigations of initial outbreaks in previously EUS-free areas as well as of recurrent outbreaks in previously endemic areas are urgently needed and will contribute important information on causal factors for EUS.

Investigation procedure

The procedure for an outbreak investigation follows 9 basic steps. Not all the steps are necessarily included in every investigation, nor do they always follow the same sequence. In practice, several steps will be undertaken simultaneously.

The 9 basic steps are :

1. Establish a diagnosis.

The initial provisional diagnosis in an EUS outbreak is usually based on species of fish affected, clinical signs, gross pathology and, perhaps, seasonality. Whenever possible, laboratory tests should be undertaken to verify the provisional diagnosis. Since some laboratory procedures (*e.g.* histopathology, fungal isolation) may take weeks, the implementation of control measures is often based on the provisional diagnosis.

2. Define a "case".

Depending on the type of investigation, an EUS case might be an individual affected fish or an aggregation of individuals such as the population in an affected pond. A useful case definition at the individual animal level might be 'a fish with necrotising granulomatous dermatitis and myositis associated with highly invasive non-septate fungal hyphae'.

3. Confirm that an outbreak is actually occurring.

This step may seem unnecessary but in many instances it is required, particularly in areas where EUS is already endemic. The disease may be expected to occur at low prevalence at certain times, but even a moderate prevalence increase, especially if ulceration is severe and/or toxigenic *A. hydrophila* is present, will lead to substantial production losses if not recognised early. Moreover, dermal ulceration caused by other agents is common in fish populations and is often macroscopically very difficult to distinguish from EUS. Laboratory confirmation of a diagnosis of EUS will usually be necessary.

4. Characterise the outbreak in terms of time, affected/ unaffected fish, and place.

From an epidemiologic viewpoint, it is important to characterise the outbreak in terms of the above 3 variables. This characterisation must be done in such a way that hypotheses can be developed regarding the source, mode of transmission and duration of the outbreak. The information is organised in an attempt to find answers to the following kinds of questions:

Time

What is the exact period of the outbreak?

Given the diagnosis, what is the probable period of exposure?

Is the outbreak most likely common source, propagated or both?

Fish

Are there any characteristics about fish for which specific attack rates vary?

Which groups have the highest and which have the lowest attack rates?

Place

What are the significant features of the geographical distribution of cases?

What are the relevant attack rates?

Time

There are 3 basic time spans used to describe disease temporal patterns: the epidemic period, which is of variable length depending on the particular epidemic; a 12 month period to describe seasonal patterns; and an indefinitely long period of years to identify long-term trends. A knowledge of seasonal

patterns and long-term trends is important when deciding whether or not an epidemic exists in the present period and in predicting future epidemics.

The temporal pattern of an outbreak is described in terms of its epidemic curve. The epidemic curve is a graph showing the onset of cases of the disease either as a bar graph or frequency polygon. The first case identified for a particular outbreak is referred to as the 'index' case. For infectious diseases such as EUS, information about the index case can be valuable in identifying the source of the outbreak.

In general, an epidemic curve has 4 and sometimes 5 segments:

i. the endemic level

ii. an ascending branch

iii. a peak or plateau

iv. a descending branch

v. a secondary peak

The slope of the ascending branch can indicate the type of exposure (propagating or common source) or the mode of transmission and incubation period. If transmission is rapid and the incubation period is short, then the ascending branch will be steeper than if transmission is slow or if the incubation period is long.

The length of the plateau and slope of the descending branch are related to the availability of susceptible animals which in turn is dependant on many factors such as stocking densities, the changing importance of different mechanisms of transmission and the proportion of resistant or immune fish in the population at risk.

The interval of time chosen for graphing the cases is important to the subsequent interpretation of the epidemic curve. The time interval should be selected on the basis of the incubation or latency period and the period over which the cases are distributed. A common error in this regard is the selection of a time interval which is too long, which may obscure subtle differences in temporal patterns. A general rule is to make the interval between one eighth and one quarter of the incubation period. Accordingly, for EUS, which has an incubation period of approximately 10 days, the incidence/ prevalence in the population should be measured every 2 days.

Outbreaks are often referred to as being either 'common source' (cases resulting from exposure to a common source, as in intoxications) or 'propagated source' (animal-to-animal transmission as in most infectious diseases). In some EUS outbreaks, it is conceivable that both types of sources could be involved, the initial cases resulting from exposure to a common source (such as contaminated water or equipment) and secondary cases resulting from fish-to-fish spread.

The duration of an outbreak is influenced by:

- the number of susceptible animals exposed to a source of infection which become infected;
- the period of time over which susceptible animals are exposed to the infection source;
- the minimum and maximum incubation periods of the disease.

Fish

Although the word 'fish' is used here, the terms 'cases' and 'non-cases' should be used to embrace the wider definitions where 'cases' might be ponds, farms, etc. For simplicity, the discussion will be restricted to individual fish only.

Species, age, sex and geographical origin are often associated with varying risk of disease. However, it should be kept in mind that fish-level patterns can be closely linked to temporal and spatial patterns of disease.

To describe patterns of disease by fish categories, it is first necessary to outline what measures of disease frequency are used in outbreak investigations. The basic measure of disease frequency in outbreaks is the 'attack rate' (AR). An attack rate is a special form of an incidence rate where the period of observation is relatively short. An attack rate is the number of cases of the disease divided by the number of animals at risk at the beginning of the outbreak. Where different risk factors for the disease are to be evaluated, attack rates specific for the particular factor must be calculated. For example, suppose there were deaths due to suspected EUS in a pond and it appeared that small fish were at greater risk of having EUS than larger fish. We might make the following calculations:

$$\text{For small fish, } AR_1 = \frac{\text{Number with EUS}}{\text{Total small fish}}$$

$$\text{For large fish, } AR_2 = \frac{\text{Number with EUS}}{\text{Total large fish}}$$

Using some hypothetical numbers, say 300 out of 1000 small fish, and 100 out of 1000 large fish, in the pond had EUS during the outbreak. The attack rates here are 30% and 10% respectively, suggesting that small fish were 3 times more likely to develop EUS than large fish. Such a finding could lend support to a hypothesis, for example, that nutritionally stressed fish are more susceptible to EUS.

Formal measures to compare attack rates among groups of fish with different characteristics are described in the next section.

With EUS, of course, the total numbers of fish in different putative risk categories would have to be estimated through representative sampling. Another problem could be the estimation of the numbers of mortalities and when they died. When investigating an outbreak of EUS, where it is postulated

that some epidermal insult (such as rhabdovirus infection) precedes infection with *A. invadans*, examination of samples of fish at different times during the outbreak should attempt to characterise the different types of lesions present and the rhabdovirus-infection status of individual fish. Thus, the 'spectrum' of disease is important here.

Place

Describing the outbreak in terms of place may lead to identification of the cause. For farmed fish, this may involve looking at the pattern in different ponds. It is often useful to consider time and place together. This can be done by drawing a plan of the ponds and recording the dates when cases occurred. Such a diagram may also give a lead to whether the outbreak is a common source or propagating. For larger scale epidemics, spot maps are useful.

5. Analysing the data.

Factor-specific attack rates for such factors as species, age, sex, pond, management system, etc are calculated and arranged in an 'attack rate table'. A theoretical example is shown below for EUS where size indicating nutritional stress is suspected:

	With factor			Without factor				
	EUS	Total	AR	EUS	Total	AR	AR Diff	RR
Small	30	100	30%	35	500	7%	23%	4.3
Medium	20	200	10%	45	400	11%	-1%	1
Large	15	300	5%	50	300	17%	-12%	0.3

In the above table, attack rates are expressed as percentages. The second last column is the difference in attack rates (sometimes called the 'Attributable Risk') and the last column is the 'Relative Risk' which is the ratio of the attack rates.

The higher the attack rate difference and the relative risk, the more important the specific factor is in increasing the risk of disease. The analysis becomes more complicated when trying to sort out the interactions and confounding among factors. Stratified and multivariate analyses are used to investigate these phenomena but such methods are beyond the scope of this handbook.

It should also be noted in the above hypothetical outbreak example that small fish were 3 times more likely to have EUS than medium sized fish and 6 times more likely than large fish. Also, medium sized fish were at twice the risk of larger fish. This dose-response phenomenon when relating size to attack rate would lend support to a hypothesis that nutritional stress as manifested in size is a 'component cause' of EUS.

6. Working hypotheses

Based on the analysis of time, place and fish data, working hypotheses are developed for further investigation. These may concern one or more of the following:

- whether the outbreak is common source or propagating;
- if a common source, whether it is point or multiple exposure;
- the mode of transmission - contact, vehicle or vector.

Any hypothesis should be compatible with all the facts.

Corrective action can be taken based on the more realistic hypotheses.

7. Intensive follow-up

This includes clinical, pathological, and microbiological examinations, together with examinations of water quality data and recent meteorological data. Epidemiologic follow-up will include detailed analyses of these data as well as the search for additional cases on other premises. Flow charts of management and movements of fish, water and equipment, for example, may be required as part of this process.

Transmission trials may be necessary where additional infectious agents, such as bacteria or ectoparasites, are suspected as component causes of the outbreak.

8. Control and prevention

Hopefully, the investigation will contribute to the termination of the outbreak and information gained will ensure that the risk of similar occurrences is reduced. Strategies to stop the epidemic must be put in place as soon as possible and will often be undertaken in the absence of conclusive findings.

Much further work is required before effective methods for treating EUS outbreaks in ponds can be developed. Detailed investigation of a number of outbreaks could provide valuable information about possibly important 'component' causes.

9. Reporting

For small outbreaks, this may take the form of a brief discussion with the farm manager outlining the important features and actions required to prevent further occurrences. However, it is wise to always produce some form of written report so that a permanent record of events exists for future use. For large outbreaks, findings should be published in scientific literature.

For substantial investigations the report should contain the following sections: background, methods, results, hypotheses, financial impact (where appropriate), recommendations and appendices containing laboratory reports etc.

Annex 8 - EUS Sampling Datasheets

To make information available for the regionwide collaborative EUS programme, please complete as fully as possible and send with formalin-fixed fish samples (see Annex 9) to: The Aquatic Animal Health Research Institute, Kasetsart University, Jatujak, Bangkok 10900, Thailand. (*Sections marked with an asterix are optional, depending on resources available).

EUS SAMPLING DATASHEET 1

SITE NAME:

Date:
Collected by:

Current EUS outbreak

Is the present outbreak:
(a) restricted to sampling site? ☐
(b) occurring throughout the local area? ☐
(c) a national problem? ☐

Date present outbreak started:
Estimated number/weight of fish lost from present outbreak:
Value of losses from present outbreak:
Conditions 3-12 days before outbreak (*e.g.* temperature, rainfall):

*Fish market price:

Species	Price/kg of unaffected fish	Price/kg of affected fish

Site description

Country:
Province:
District:
Town:
Village:

Type of water body at site:

Farm ☐ Lake ☐ Reservoir ☐
Canal ☐ River ☐ Ricefield ☐
Swamp ☐ Other:

Describe site:

Previous history of EUS at the site:

EUS SAMPLING DATASHEET 2 | **SITE NAME:**

Sampling point

How and where on the site were fish sampled:

Was selection of sampling point random? If not give reason:

Size of pond:
Depth of pond:
Fish species:
Stocking rate:
Are there wild fish in the pond?
Were fish introductions made just prior to outbreak?
What is the source of the water to this pond?
Describe attempted treatments/control strategies:

Perceived importance of the problem to local farmers/fishermen:

*Water quality data

Time	
Turbidity	
Temperature	
pH	
Alkalinity	
Hardness	
Conductivity	
NH_4	

*#Fish population data

Species	No. of fish	No. infected	% infected	Severity of infection

#when estimating EUS prevalence in the population, a random sample of at least 100 fish should be taken and examined for lesions

EUS SAMPLING DATASHEET 3

SITE NAME:

Fish sampling data

*Photo No	Code	Species	Length (cm)	Description of clinical signs	Indicate which tissue sampled			*Microscopic examination	
					Histology	*Mycology	*Bacteriology	*Fungus	*Skin parasites

Annex 9 - Procedure for sampling fish for histological examination

1. Complete EUS sampling datasheet (Annex 8) for each site, recording full details for each fish sampled.
2. Sample only live specimens of diseased fish. If fish with clinical signs of EUS are readily apparent, several samples of each species should be collected, preferably at different stages of infection.
3. Dissect large fish and take samples of skin/muscle (<$1cm^3$), spleen, kidney and liver. The muscle section should include the lesion and the surrounding tissue. Small fish (<3cm in length) can be slit along the abdomen and preserved whole.
3. Fix the tissues immediately in cold 10% formalin. The amount of formalin in the jar should be 15-20 times the volume of the tissue to be fixed.
4. Gently agitate the fixative 2-3 times over the first hour after adding the tissue.
5. The selected site should be sampled repeatedly over the outbreak period and specimens sent to a centralised diagnostic facility. If an appropriate facility is not available in-country, or to make the information available for the regionwide collaborative EUS programme, send to The Aquatic Animal Health Research Institute, Kasetsart University, Jatujak, Bangkok 10900, Thailand, along with a copy of the sampling datasheet.

Abbreviations

AAHRI -	Aquatic Animal Health Research Institute, Thailand
ACIAR -	Australian Centre for International Agriculture Research
APW -	autoclaved pond water
BF-2 -	bluegill fry cell line
CDA -	Czapek Dox agar
CPE -	cytopathic effect
DFID -	Department for International Development, United Kingdom Government, formally ODA
EUS -	epizootic ulcerative syndrome
FAO -	Food and Agriculture Organisation of the United Nations
FCS -	foetal calf serum
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-streptomycin medium
GPY -	glucose-peptone-yeast medium
GY -	glucose yeast medium (Dykstra *et al.*, 1986)
HBSS -	Hank's balanced salt solution
ICBN -	International Code of Botanical Nomenclature
ICSF -	International Collective in Support of Fishworkers
i.m. -	intra-muscular injection
IoA -	Institute of Aquaculture, Stirling University, Scotland
i.p. -	intraperitoneal injection
IPNV -	infectious pancreatic necrosis virus
MG -	mycotic granulomatosis
NACA -	Network of Aquaculture Centres in Asia and the Pacific, Bangkok
NSW -	New South Wales, Australia
ODA -	Overseas Development Administration of the United Kingdom, present name DFID
PG-1 -	peptone-glucose-1 medium
p.i. -	post-injection
PNG -	Papua New Guinea
RSD -	redspot disease
SEAADCP -	South East Asia Aquatic Disease Control Project
SGV -	sand goby virus
SHRV -	snakehead fish rhabdovirus
SHV -	snakehead fish virus
SSN-1 -	striped snakehead cell line
UDRV -	ulcerative disease rhabdovirus
UM -	ulcerative mycosis (menhaden disease)

Glossary

achlyoid:	referring to type of zoospore discharge from zoosporangium: primary zoospores encyst as they emerge from the exit pore, forming a loose, spherical cluster. Characteristic of Oomycete genera *Aphanomyces* and *Achlya*.
acinar necrosis:	irreversible degeneration of acinar tissue (exocrine pancreatic cells)
aetiology:	the science of the cause or origin of disease
alkalinity:	a measure of anions (*e.g.* HCO_3^-, $CO_3^=$, OH^-) in a solution (an alkaline solution would give a pH reaction above 7)
axenic:	cultures of microorganisms which are not contaminated by, or are completely free of, the presence of other organisms.
birnavirus:	a group of non-enveloped isometric viruses (including IPNV) with a genome of double stranded RNA in two segments
chlamydospore/gemma:	asexual spherical structure of fungi originating by differentiation of a hyphal segment(s) used primarily for perennation, not dissemination
complex zoosporangium:	zoosporangium with more than one evacuation tubes from which zoospores are released
cytopathic effect (CPE):	cell degeneration caused by viral growth, the pathological changes in cell culture are often virus-specific and can form the basis of a virological diagnosis
dermatitis:	inflamation of the skin
ectoparasiticide:	a substance capable of destroying external parasites
enterotoxigenic:	producing or containing a toxin specific for the cells of the intestinal mucosa
eosinophil:	a leucocyte with a bilobed nucleus and coarse granular cytoplasm that stains readily with acidic dyes such as eosin
epidemiological:	relating to the occurrence, transmission and control of epidemic diseases
epithelioid:	like epithelium

epizootic:	the sudden outbreak and rapid spread of a disease affecting a large number of animals over a wide area ("epidemic" is now used instead of epizootic)
erythematous:	relating to, or causing, erythema: a disease of the skin, in which a diffused inflammation forms rose-coloured patches of variable size
eucarpic:	referring to fungi that develop reproductive structures on limited portions of the thallus, such that the residual nucleate protoplasm remains capable of further mitotic growth and regeneration
exophthalmia:	abnormal protrusion of the eyeball
focal proliferation:	cell division and growth limited to a specific part of the tissue
granular occlusion:	blockage or obstruction of a duct or blood vessel by grain-like particles
granulomatous:	composed of a tumour-like mass or nodule of granulation tissue due to a chronic inflammatory response
haemoglobin:	respiratory pigment in red blood cells, a conjugated protein that can combine reversibly with oxygen
haemorrhage:	escape of blood from the vascular system
haemosiderin:	an insoluble form of storage iron, visible microscopically
hardness:	a measure of calcium, magnesium and other metals in freshwater expressed as mg/litre of calcium carbonate
heterokont:	Cell with flagellae (or other motile organelles) unequal in length or unlike in movement
hyperaemic:	a superabundance or congestion of blood, due to increased flow of blood to an area, or due to obstruction in the return of blood from an area
hyperplastic:	pertaining to hyperplasia: an abnormal increase in the number of cells
intercalary zoosporangia:	zoosporangia forming in the middle of a hyphal segment
lateral evacuation tubes:	zoosporangial tube from which zoospores are released

lymphoid: of or resembling lymph or lymphatic tissue

melanomacrophage centre (MMC): a discrete group of large phagocytic cells within haemopoetic and other soft tissues of teleost fish; yellow-brown or black in colour depending on species, age and health of the fish

mitotic figures: cells seen in histological preparations to be undergoing mitosis, sometimes an indication of neoplastic changes or cell regeneration as a result of a previous toxic insult.

monogenean: an ectoparasitic flatworm of the class Trematoda with a direct lifecycle and a single host

mycosis: disease resulting from infection with a fungus

myeloid: having the appearance of myelocytes (a precursor cell of blood granulocytes)

myofibrillar: pertaining to myofibrils: long cylindrical organelle of striated muscle, composed of regular arrays of thick and thin filaments and constituting the contractile apparatus

myopathy: disease of muscle tissue

myophagia: atrophy or wasting away of muscular tissue

myositis: inflammation of the muscles

myxosporidian: a spore-producing, parasitic protozoan

necrosis: the sum of the morphological changes indicative of cell death and caused by the progressive degradative action of enzymes; it may affect a single cell, a group of cells or part of a structure or organ

occlusion: closure or blockage of an orifice or tube

oedema: accumulation of body fluids in the tissues, generally causing swelling of a part of the body

oomycete: class of fungal-like protists, typically giving rise to biflagellate, heterokont zoospores. Hyphae are aseptate and the cell wall is believed in most species to lack chitin and contain cellulose

polyplanetism: phenomenon in successive generations of secondary zoospores are formed by repeated cycles of encystment and excystment

proliferative zoosporangial renewal: the development of a secondary zoosporangium within a previously emptied primary one. The supporting hyphae may also grow through the primary sporangium before forming the secondary sporangium. Common in the oomycete genus *Saprolegnia*

prophylactic: disease-preventing

reniform: kidney-shaped

rhabdovirus: a group of bullet-shaped viruses 130-380nm long and 60-95nm wide with a genome of single stranded RNA

rosacea: a disease of the skin characterised by red colouration and caused by chronic dilation of capillaries

septicaemia: systemic disease associated with the presence and persistence of pathogenic microorganisms or their toxins in the blood

simple zoosporangium: zoosporangium with a single evacuation pore from which zoospores are released

sporulation: sporogenesis. Formation of spores that involves division of a large cell into many small spores

sympodial zoosporangial renewal: lateral branching of a fungal hypha below the basal septum of a delimited zoosporangium, so that the lateral branch then becomes the primary axis. Common in the oomycete genus *Achlya*

syndrome: a group of symptoms or signs, which, when considered together, characterise a disease

telangiectasis: abnormal dilation of capillaries in the secondary lamellae of gills

terminal zoosporangium: zoosporangium forming at the end tip of a hyphal segment

therapeutic: relating to the treatment of disease

ulcer: an interuption of continuity of an epithelial surface, with an inflammed base

zoosporangium (plural: zoosporangia): fungal asexual reproductive structure in which zoospores are produced, and from which they are released

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Sampling for EUS affected fish

Bangladesh

Lao PDR

Pakistan