ENVIRONMENT AGENCY

CRAYFISH PHASE 1

APHANOMYCOSIS OF CRAYFISH: CRAYFISH PLAGUE

Research and Development

Technical report

W2-064

ENGLISH NATURE

ENVIRONMENT AGENCY

Figure 1 From the "Fischereibuch" of Emperor Maximillian I., 1499.

Aphanomycosis of Crayfish: Crayfish Plague

A Report Prepared for The Environment Agency and English Nature

R&D Technical Report W2-064

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1. SUMMARY

Crayfish plague is an extremely virulent fungal disease of European crayfish species, the white clawed or stone crayfish of Western Europe *Austropotamobius pallipes*, the Noble crayfish of northern Europe *Astacus astacus* and the narrow clawed crayfish of Eastern Europe, *Astacus leptodactylus*. The white claw crayfish *A. pallipes* is the indigenous native crayfish of the British Isles. Until the early 1980s there were extensive healthy populations of this crayfish in almost all suitable alkaline river and lake environments in England and Wales as far north as Northumberland. The conservation importance of this native crayfish is widely recognised. After some delay in 1986 it was protected in Britain under Schedule 5 of the Wildlife and Countryside Act 1981. It is also listed in Annexes II and V of the EU Habitat and Species Directive (1992) with a requirement that Special Areas of Conservation should be set up to protect it. It is additionally classified as globally threatened by IUCN/WCMC, is listed in Appendix III of the Bern Convention (the Red List) and now appears as a species of conservation priority on the UK Government's Biodiversity Action Plan.

Crayfish plague caused by the Oomycete fungus *A. astaci* first appeared in Europe in the third quarter of the 19th century and spread remorselessly throughout Europe. Within 100 years only Norway, the British Isles, Greece and Turkey were free of the disease. Infection reached the British Isles in about 1980 in signal crayfish, *Pacifastacus leniusculus* that had been imported as part of attempts to create a commercial crayfish farming industry. The signal crayfish is a N. American species which is resistant to crayfish plague (which is itself of N. American origin) and frequently acts as an asymptomatic carrier. So severe is the impact of crayfish plague in susceptible European crayfish that infection eliminates entire populations and in over 100 years no evidence of resistance has appeared. Since the arrival of crayfish plague in the British Isles some twenty years ago populations of native crayfish have been severely affected.

This report provides a general review of the literature of crayfish plague, including an overview of its spread through the British Isles from CEFAS records. Information on current diagnostic methods from the Office International des Epizooties (OIE) Aquatic Disease Manual is provided. Information on the taxonomy, morphology and physiology of the pathogen is reviewed, together with the pathogenicity and pathology of the disease and current means of prevention and control.

In the second part of this report the risks of the disease spreading further are considered, particularly in relation to stocking movements of fish. The Prohibition of Keeping of Live Fish (Crayfish) Order, 1996, now controls keeping of exotic crayfish so that the primary risk of transfer of infection to new areas is believed to lie with movements of contaminated equipment and transport of fish temporarily contaminated by *A. astaci*. That this is a practical risk has been demonstrated both experimentally and anecdotally in the field.

The Environment Agency has statutory powers under Section 30 SFFA in regard to introductions of fish into inland waters and responsibilities for conservation under the Environment Act. English Nature has statutory powers to prevent introductions of fish from sites containing signal crayfish into waters designated for the white clawed crayfish. The Environment Agency and English Nature have commissioned this report to provide them with an up to date review of the literature of crayfish plague and in particular to assist them exercising their statutory responsibilities by providing a good scientific background to support their decision making.

Recommendations are made on actions that may be taken to mitigate the risks of such transfers, on research needs to improve ability to diagnose infection particularly of carrier state. Molecular methods have major advantages, but need full validation before they can be implemented as diagnostic tools. The need for a suitable fungicide to use to aid in mitigation of transfer risks is emphasised and the limitations on the type of product that can be used is discussed in relation to veterinary medicines legislation is considered. Research needs to support this use are outlined.

PART I: LITERATURE OVERVIEW

2. INTRODUCTION

Crayfish plague is a highly infectious disease of all crayfish (Decapoda: Astacidae, Cambaridae) of non-North American origin. The aetiological agent is an Oomycete fungus, *Aphanomyces astaci,* which is now widespread in Europe as well as in North America. The European crayfish species, the Noble crayfish *Astacus astacus* of north-west Europe, the white claw crayfish *Austropotamobius pallipes* of south-west and west Europe, the related *Austropotamobius torrentium* (mountain streams of south-west Europe) and the slender clawed or Turkish crayfish *Astacus leptodactylus* of eastern Europe and Asia Minor are all highly susceptible. The only other crustacean known to be susceptible to infection by *A. astaci* is the Chinese mitten crab *(Eriocheir sinensis)* and this only under laboratory conditions.

The disease first occurred in Europe in the third quarter of the 19th century in the Franco-German border region. From there region a steady spread of infection occurred, principally in two directions – down the Danube into the Balkans and towards the Black Sea, and across the North German plain into Russia and from there south to the Black Sea and north-west to Finland and finally, in 1907, to Sweden. In the 1960s the first outbreaks in Spain were reported and in the 1980s further spread of infection to the British Isles, Turkey, Greece and Norway was reported (Alderman, 1996).

The reservoir for the original infections in the 19th century was never established, but the post-1960s extensions are largely linked to movements of North American crayfish introduced more recently for purposes of crayfish farming. These species *(Pacifastacus leniusculus* [the Signal crayfish] and *Procambarus clarkii* [the Louisiana swamp crayfish]) can act as largely or completely asymptomatic carriers, but can be killed by *A. astaci* under adverse conditions. Transmission is also believed to have resulted from contaminated crayfish traps and other contaminated equipment.

Clinically, infected crayfish may present a wide range of gross signs of infection or none at all. Focal whitening of local areas of musculature beneath transparent areas of thin cuticle, especially of the ventral abdomen and in the periopod (limb) joints, often accompanied by even more localised brown melanisation, is the most consistent sign. In the terminal stages of infection, animals show a limited range of behavioural signs, principally a loss of the normal aversion to bright light (they are seen in open water in daylight) later accompanied by a loss of limb co-ordination, which produces an effect that has been described as 'walking on stilts'. Eventually, moribund animals lose their balance and fall onto their backs before dying.

Diagnosis requires isolation and identification of the pathogen by microscopic morphology; no molecular, biochemical or serological methods that have been adequately validated exist. RAPD-PCR methods (Random Amplification of Polymorphic DNA – Polymerase Chain Reaction) have been developed that have provided useful information about origin and strains of *A. astaci.* Before such methods can be used to support any enforcement action the necessary formal validation of the methods is essential. The first stages of validation are now being employed, but further development will be needed before these methods can be applied to clinical cases.

Control of the spread of infection once a watershed is infected is in practical terms impossible. Prevention of all introductions of crayfish to natural waters and into enclosed waters from which they may escape to natural waters can be effective, although movement of fish can result in the movement of infected water between watersheds and can transmit infection, as can contaminated equipment such as boots and fishing gear. Sodium hypochlorite and iodophores are effective for disinfection of contaminated equipment. Thorough drying of equipment (>24 hours) is also effective (as is freezing or cooking) since the Oomycetes are not resistant to desiccation.

3. HISTORICAL OVERVIEW

Crayfish plague first reached Europe in the 19th Century and spread rapidly across the continent. There are numerous aspects of the spread of the disease in its earliest years that need to be taken into account in assessing risks and outcomes of the disease in England and Wales. A full account of the spread of the disease in Europe between the 1870's and 1945 is given in Appendix 1. The present section describes the spread of the disease in Europe since 1945 and in England and Wales since the first recorded outbreak in 1981.

3.1 Crayfish Plague in Europe post 1945

Inevitably the outbreak of World War II reduced the number of easily traceable reports of crayfish plague, although at least one further outbreak was noted in 1943 in Latvia, in L. Shvimiltis (Tzukerzis, 1964) and, post war, the series of outbreaks in the Baltic region continued e.g. Lakes Galstas and Zapsis in 1951, Lakes Ungris, Gavis, Shlavinas, Sutrinas and Shiakshtias in Latvia in 1960-63 and in Lithuania L. Spindzius and the Trakai district of Lithuania in 1967 (Mazylis and Grigelis, 1979). However in 1956, for the first time since the advance into Sweden in 1907, a completely new area of Europe was infected, the Iberian peninsula (Figure 2), where the first outbreaks appear to have occurred in the R. Duero in the Valdolidad region (Cuéllar and Coll, 1984). A further large mortality of crayfish occurred in Spain in 1965, in the R. Ucero in the Soria district. This infection may have spread from *Astacus leptodactylus* introduced from Germany, although *Aphanomyces* was never positively identified (Cuéllar and Coll, 1984) (Figure 3).

A further and perhaps inevitable extension occurred in 1971 when for the first time crayfish plague was identified in Norway in two rivers, both having their sources in Norway but flowing across the border into Sweden. In the Vrangselven infection was detected very close to the border in August (Hastein and Unestam, 1971), from where it spread upstream, despite attempts to prevent this using electric fences (Hastein and Unestam, 1971; Hastein and Gladhaug, 1974). The R. Veska was also affected.

The first positive identification of *Aphanomyces astaci* in Spain was made in 1978 from crayfish from the R. Riaza. Infection was believed to have originated from illegally imported *A. leptodactylus*. Disease then spread to the R. Guadiana in Cuidad Real and to other rivers in the region. Cuéllar and Coll (1984) record the continuing spread in Spain, in 1979 to the R. Cadagua and Bairax y Ayuga in Alava in June, in July and August the rivers Ega, Cidacos, Araquil, Leitzaran, Elorza, Salazar and Erro y Larraín in Navarre, the Guadalaviar and Jiloca y Alfambra in Teruel and the Pisuerga in Valdolidad were affected. In August and September *Aphanomyces* was identified in the Ebro and tributaries in Zaragoza and the Iregua in Logroño. Spanish crayfish mortalities continued in May 1980 the R. Júcar (Albacete prov.), Guadina (Cuidad Real) and Guadarrama (Toledo) and later in the Duero (in Zamora), Carrión (Valencia), Bernesga (Léon), Bernesga (Léon), Eresema and Esgueva (Valladolidad), Omecillo and Ayuga (Alava, Ega, Cidacos and Erro (Navarre), Iregua (Logroño), Riaza and Duratón (Segovia), Riaza and Esgueva (Burgos), Jalon (Zaragoza) and Ucero (Soria).

Crayfish mortalities still continue to occur in the "old" infected areas such as Finland where Westman and Nylund (1979) reported on the spread of infection in the Pihlajavesi waterway, which provided a good case study.

Further new extensions of crayfish plague continued with the first suspected crayfish plague mortalities in England being investigated in 1981 (Figure 4). The first outbreak of crayfish plague in Greece was detected in August 1982 (Theocharis, 1986), initially in the R. Kalamas immediately adjacent to the site at which 1000 juvenile *Pacifastacus leniusculus* imported from a

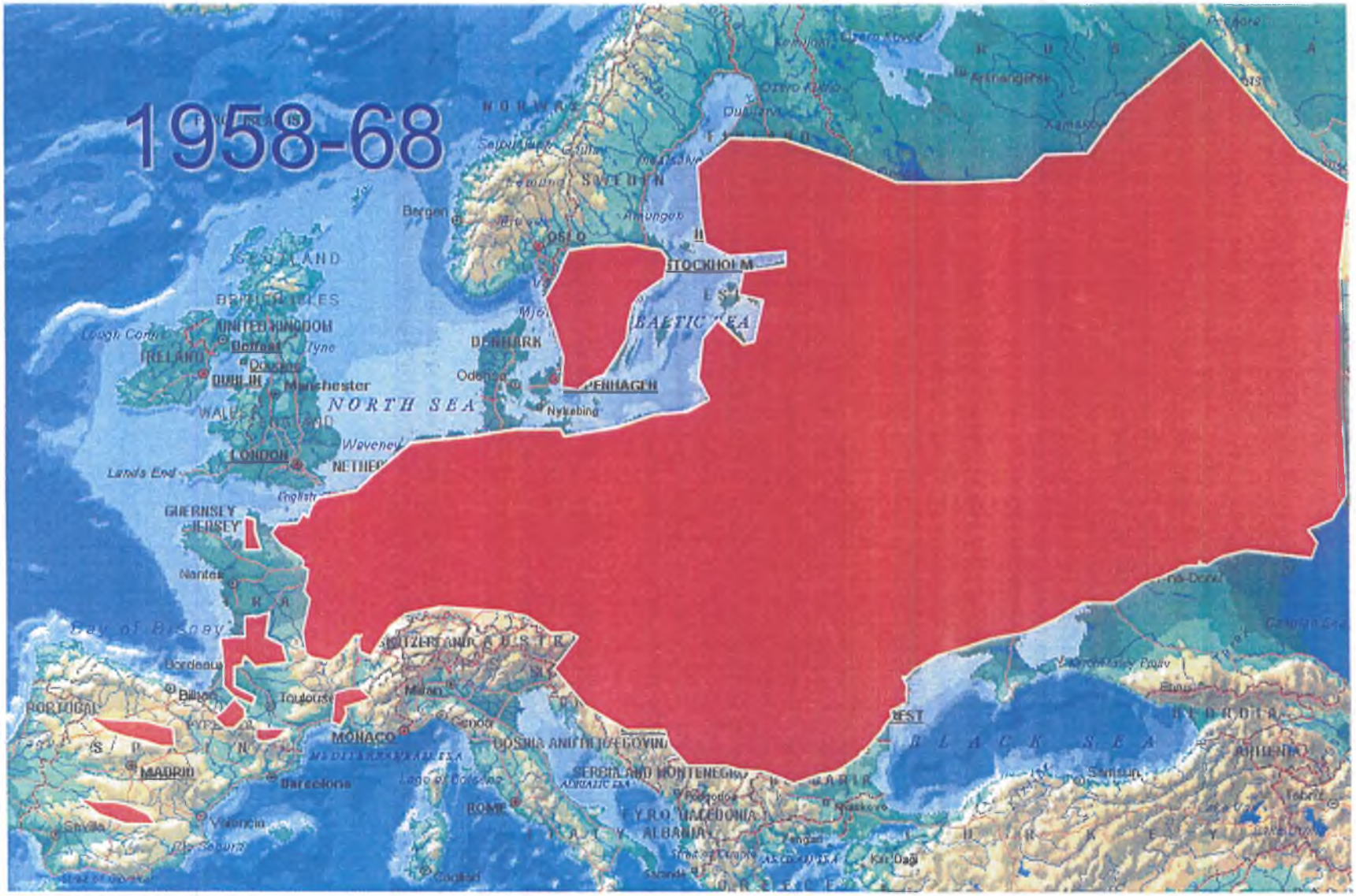

Figure 2 Post World War II extension of crayfish plague to the Iberian Peninsula

Swedish hatchery had been introduced in July. In 1983 a further outbreak was noted in the nearby R. Louros.

In late 1984 the fishermen of Lake Civiril, 200km east of Izmir in Turkey reported abnormalities on crayfish caught in the lake and in the 1985 season were only able to catch 1-5% of the usual quantity of crayfish (Rahe, 1987) (Figure 5). Fishermen from this lake were left with alternative of surrendering newly bought crayfish fishing equipment or of moving to lakes with healthy stocks. Rahe (1987) reports that they clearly did both so that by late autumn 1985 two other lakes 100km to the east were also showing disease. By the 1986 season the most important lakes Egridir, Sapanca, Manyas, Uluabat and Hirfanli were found to be affected and the failure of catches showed that 80% of the Turkish crayfish stocks were already affected. The presence of crayfish processing plants on the most important lakes, Egridir, Uluabat which together represented 50% of production was regarded as one major reason for the rapid spread of disease, together with the free movement of fishermen between the lakes and the use of wooden transport crates without any disinfectant measures. The original route by which crayfish plague arrived in Turkey remained undetermined (Rahe, 1987).

Some of the crayfish populations of the midland lakes of Ireland formed a new focus of crayfish plague (Reynolds, 1988). The route by which the infection reached Ireland could not be established, infected angling equipment was suspect. No further spread in Ireland has been reported so far.

Although no specific earlier report of crayfish plague in Switzerland has been found, since every country surrounding the country has been infected, reports in the 1980s are to be regarded as recrudescences of old infections rather than representing new infections. Similarly few reports of crayfish plague from France have been published in recent years, but plague mortalities still continue in the Seine watershed despite a feeling by some workers that the disease had disappeared from France many years previously (Machino and Dieguez-Urebeondo, 1998). Alternately the Swiss and French mortalities may represent new signal crayfish associated introductions as with the recent reported outbreaks in Germany (Oidtmann et al., 1999 and see below).

In 1990 crayfish plague returned to Norway. Although previously been found in two cross Swedish border rivers. Attempts to control spread of infection by disinfection and use of electric fences failed (Hastein and Gladhaug, 1974). However in 1990 it was the R Glomma, the most extensive river system in Norway, which was infected so that simple and drastic disinfection was not a possible option.

This final invasion of Norway completes the long history of the spread of crayfish plague in which, finally, every country in Europe west of the Urals has become infected by *A. astaci*.

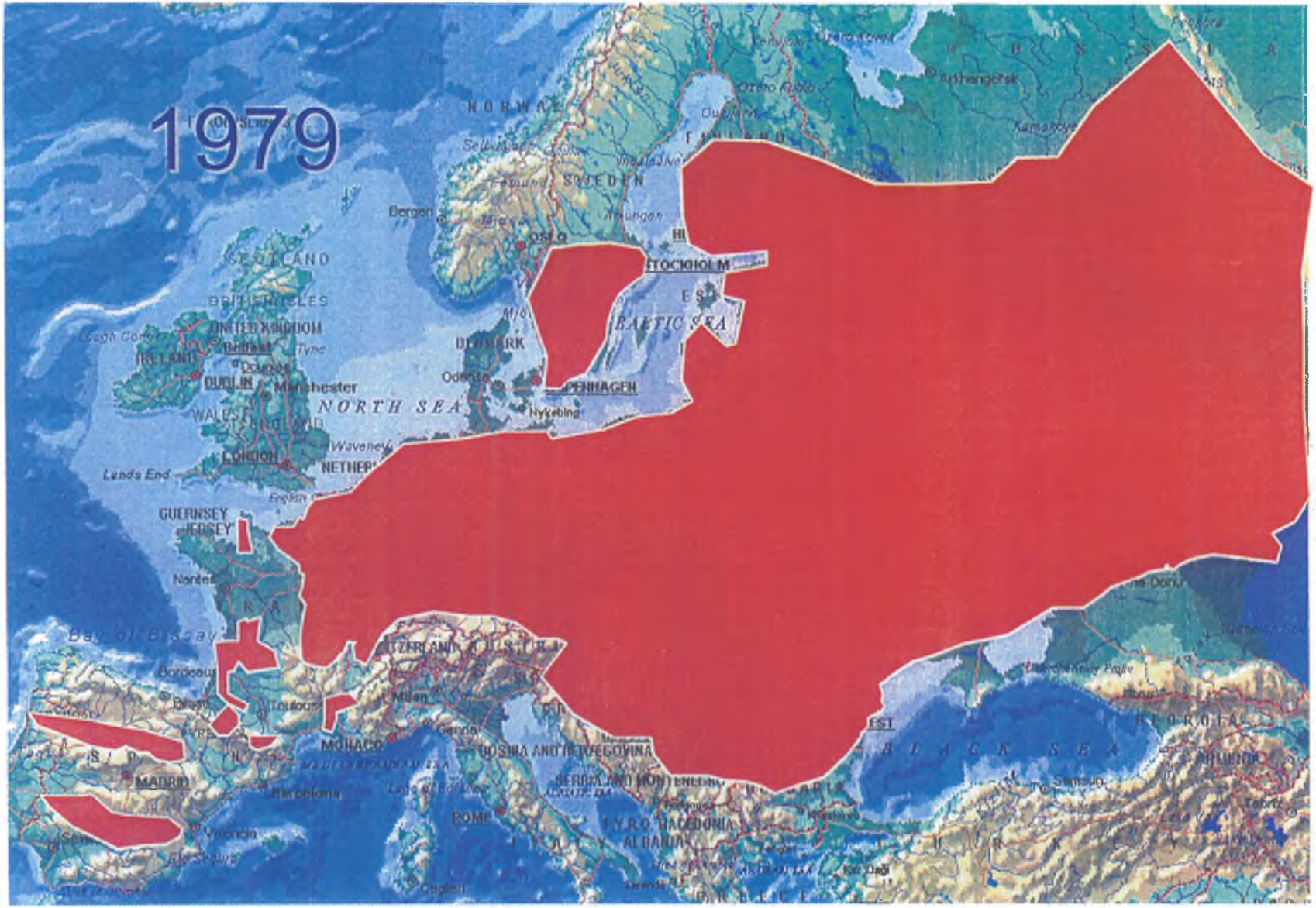

Figure 3 Continues to spread in Spain in 1970s

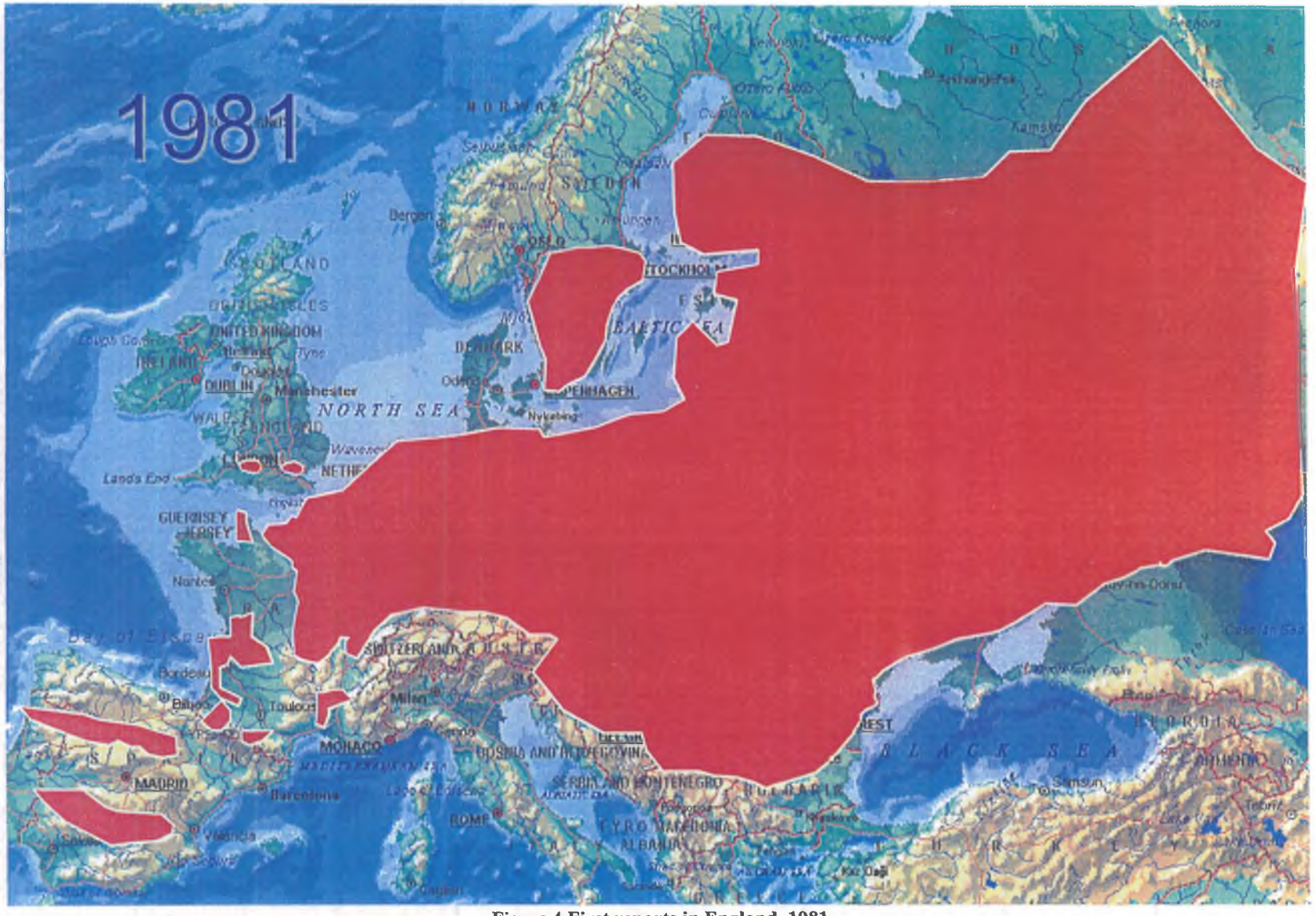

Figure 4 First reports in England, 1981

Figure 5 By 1995 infects UK, Greece and Turkey

Figure 6 Major outbreaks of crayfish plague in new areas since 1945

3.2 Crayfish Plague in England and Wales

This section of this report describes the steady spread of crayfish plague through populations of the native white claw crayfish, *Austropotamobius pallipes* Lereboullet (the sole crayfish species native to the rivers of England and Wales, see Holdich & Reeve, 1992) in the years since 1980. It does not seek to answer the question as to whether these outbreaks of crayfish plague in Britain represent first occurrence of the disease in the British Isles or a recrudescence of a disease which had arrived in Britain early in the 20th century, as part of its early spread through most of Europe. Although this question is one that can never be properly settled, the available evidence, namely the lack of any description of a plague-like mortality in native crayfish stocks in contrast to the 'torrent' of publications from continental Europe, plus the presence prior to 1980 of crayfish in every watershed in England and Wales in which there were environmental conditions favourable to their occurrence however, argues strongly against the presence of crayfish plague in the British Isles before the mid 1970's at the earliest.

The outbreaks of crayfish plague described below have largely been detected by biologists and fisheries staff of what were then Water Authority regions and then divisions of the National Rivers Authority. When crayfish mortalities were observed, material was collected and passed to the (then) MAFF Fish Diseases Laboratory in Weymouth, for diagnosis. Pathological examination together with isolation and culture of the pathogen, *Aphanomyces astaci* Schikora was carried out to confirm diagnosis of crayfish plague. Details of the methods employed have been described above.

The first outbreaks of crayfish plague appear to have started more or less simultaneously in two river systems in southern England. In the summer of 1980 crayfish mortalities were reported from the River Lee at Ware Lock and in the Sherston branch of the Bristol Avon at Easton Grey (Polglase & Alderman, 1984). Both rivers had large populations of native crayfish and the effects were dramatic with the riverbeds being strewn with dead and dying crayfish (Figure 7). In the R. Lee, signal crayfish were found having escaped from a nearby farm introduction and, although not at the first detected crayfish plague site, several populations of escapee signal crayfish have been found in the Bristol Avon. No crayfish survived below the sites of initial mortalities and in the following months mortalities spread relatively slowly upstream in tributaries such as the Tetbury branch of the Bristol Avon and the Rib and Beane in the Lea system. *A. astaci* was isolated from crayfish in these cases (Alderman, Polglase, Frayling & Hogger, 1984).

In the summer of 1983 further crayfish mortalities occurred in the Thames basin. Such mortalities are essentially ephemeral since even exoskeletal remains will survive for only a few weeks even at winter water temperatures. Delayed recognition of the disappearance of crayfish from the R. Blackwater in Surrey in the summer of 1982 (Figure 8) left no remains which could be investigated, but the mortality in the nearby R. Wey in September 1983 was reported at a very early stage, allowing dead and dying animals to be obtained and isolation of *A. astaci* to be achieved, confirming that crayfish plague was responsible (Figure 9). This mortality commenced in the upper reaches of the river and within 2 weeks, no live crayfish were to be found from Alton downstream to the R. Thames. The Blackwater and Wey catchments are not directly linked, but local riparian owners on the Wey reported that the only known introduction was a fish stocking from the Blackwater. This led to the investigation of the Blackwater, where on the previous survey, native crayfish had been common. In the Blackwater, although no surviving native crayfish could be found, escapee signal crayfish were widespread. It was inferred that, had the fish stocking taken place at the time of a plague outbreak on the Blackwater, this could have been responsible for a transfer of infection to the R. Wey.

In March 1984, a mortality was reported from the upper Hampshire Avon, between Amesbury and Salisbury, and was confirmed to be crayfish plague (Figure 10). Within three weeks no

Figure 7 Bristol Avon and Rib (red)

crayfish were left alive in 60km of river downstream of that site and more crayfish were dying as a zone of mortality began to move up the major tributaries from their confluence with the main river.

A point of interest in this mortality is that although large numbers of dying crayfish were littering the bed of the river (admittedly in highly coloured winter river) through the centre of the city of Salisbury, they were not observed or reported by the public. A small crayfish cultivation unit using signal crayfish was identified in the headwaters of the Hampshire Avon in Pewsey. The disappearance of crayfish from the R Kennet recognised later that summer was completely unobserved and although the only probable cause was plague, proof is lacking (Hogger, pers. comm.). Similarly, native crayfish had also apparently disappeared from the Dorset Frome and Stour. In the case of the Kennet, escapee signal crayfish were found later and the crayfish site at Pewsey was only a short distance from the Kennet and Avon Canal (Figure 11). The R. Stour headwaters were the site of the major crayfish importer to the British Isles where signal crayfish were held in small ponds above and only a few metres from the river with no form of protection against escape. Signal crayfish have been found in the Stour.

Plague was next confirmed in tributaries of the R. Colne, to the west of London including the Missbourne in 1985 and then there was a gap in further reported mortalities until a further case was confirmed in the south-east in the R. Darenth in 1988 (Lowery, pers comm.). A previously unconfirmed mortality in the Sevenoaks region reported to Weymouth in 1986 may have been an earlier case in gravel pits associated with this river system. Although not confirmed, signal crayfish may have been introduced into these gravel pits.

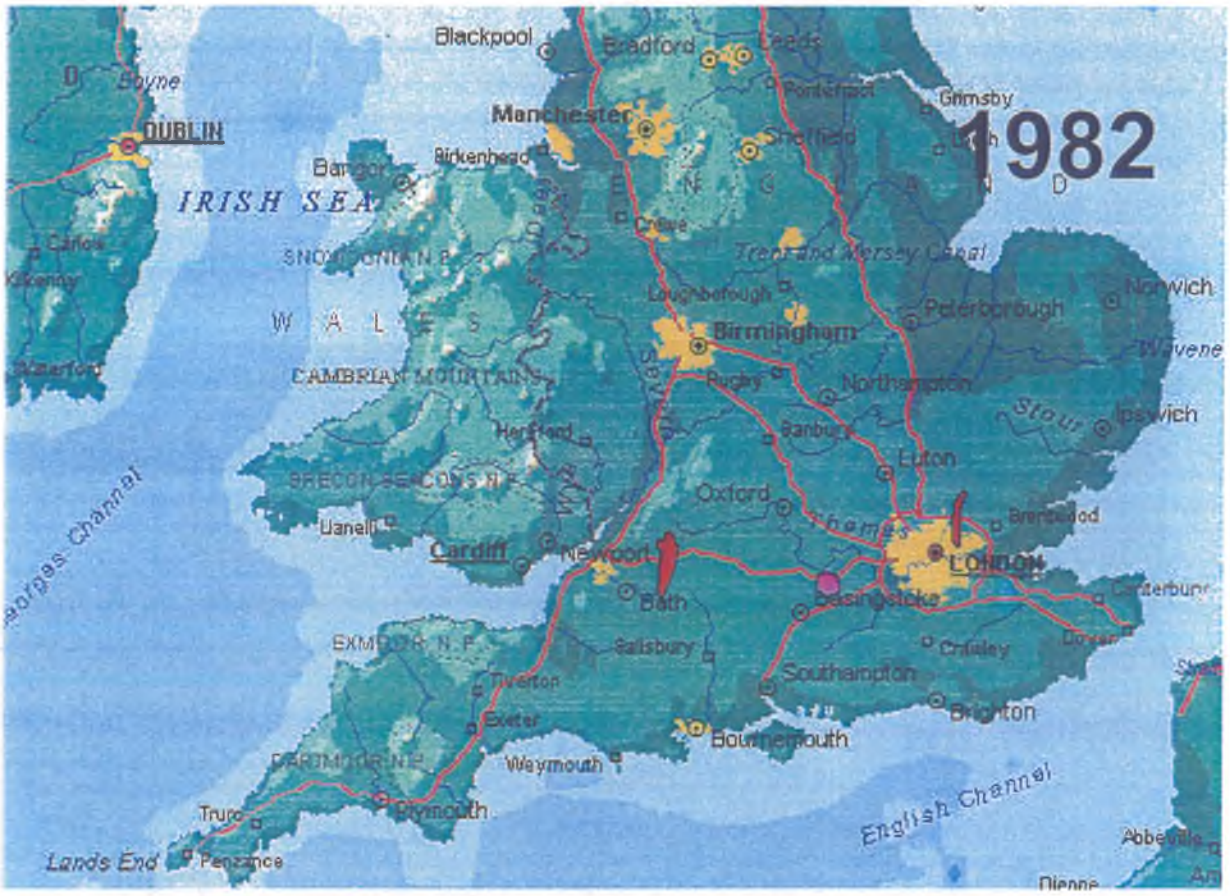

Figure 8 Confirmed and extended in Bristol Avon and Lee, R. Blackwater probable (purple)

These outbreaks of crayfish plague and disappearances of crayfish had all been in the Thames and Wessex areas, but in the autumn of 1988, a small stream (the Dowles Brook) in the Forest of Wyre, well away from the infected waters of southern England was found to be infected. Signal crayfish were found in a pond within the area, which was within a nature reserve. This effectively exposed any crayfish in the lower Severn watershed to crayfish plague (Figure 12).

No new cases were reported in 1989, but 1990 saw several major extensions of infection. In the spring, several tributaries of the Bristol Avon in which native crayfish had survived despite the infection of the main river since 1981 were affected. These included the By Brook to the north and the Somerset Frome and its tributary, the Mells River to the south of the Avon. Field investigations showed that the mortality in the R. Mells was clearly one which had started in the headwaters of the river rather than extending upstream from the main river, since the animals in all parts of the river died over a very short timescale. A few side streams still contain surviving populations (Frayling, pers. comm., 1992, 2001).

In the East Midlands mortalities from the R. Ise, a tributary of the R. Nene, were confirmed to be plague in July and in August, the R. Camlad a tributary of the upper Severn was found to be infected. In July reports of crayfish deaths in side streams of the R. Wye (Herefordshire) could not be confirmed, but samples collected from the R. Arrow, a tributary in the Wye catchment, in September were found to be crayfish plague (Figure 13). The mortality in the R. Arrow appeared to have commenced in the vicinity of a fin fish farm where signal crayfish were reported to have been introduced (NiChallanian, pers. comm.), but no examination of these animals has been possible. Rumours of signal crayfish being introduced into the vicinity of the R. Camlad could not be confirmed.

The effect of these extensions of 1990 meant that all major crayfish bearing river systems south of the Trent were infected by crayfish plague. A further major northwards extension is believed

to have occurred in March 1991 with crayfish mortalities in the R. Wye (Derbyshire) below Buxton, with further mortalities being reported in the main R. Derwent above the Wye confluence in the following August (Figure 14).. Unfortunately no samples were collected so that the cause of these mortalities remained unconfirmed as crayfish plague (NRA, Severn-Trent Division, pers. comm.) until samples from a small tributary (the R. Bradford) were found to be positive in the following year.

On the Welsh borders a further crayfish mortality in the R. Clun, a tributary of the R Teme, which joins the R. Severn below Worcester, was confirmed as crayfish plague in the autumn. This completed the spread of infection to all of the major tributaries draining into the Severn from the west.

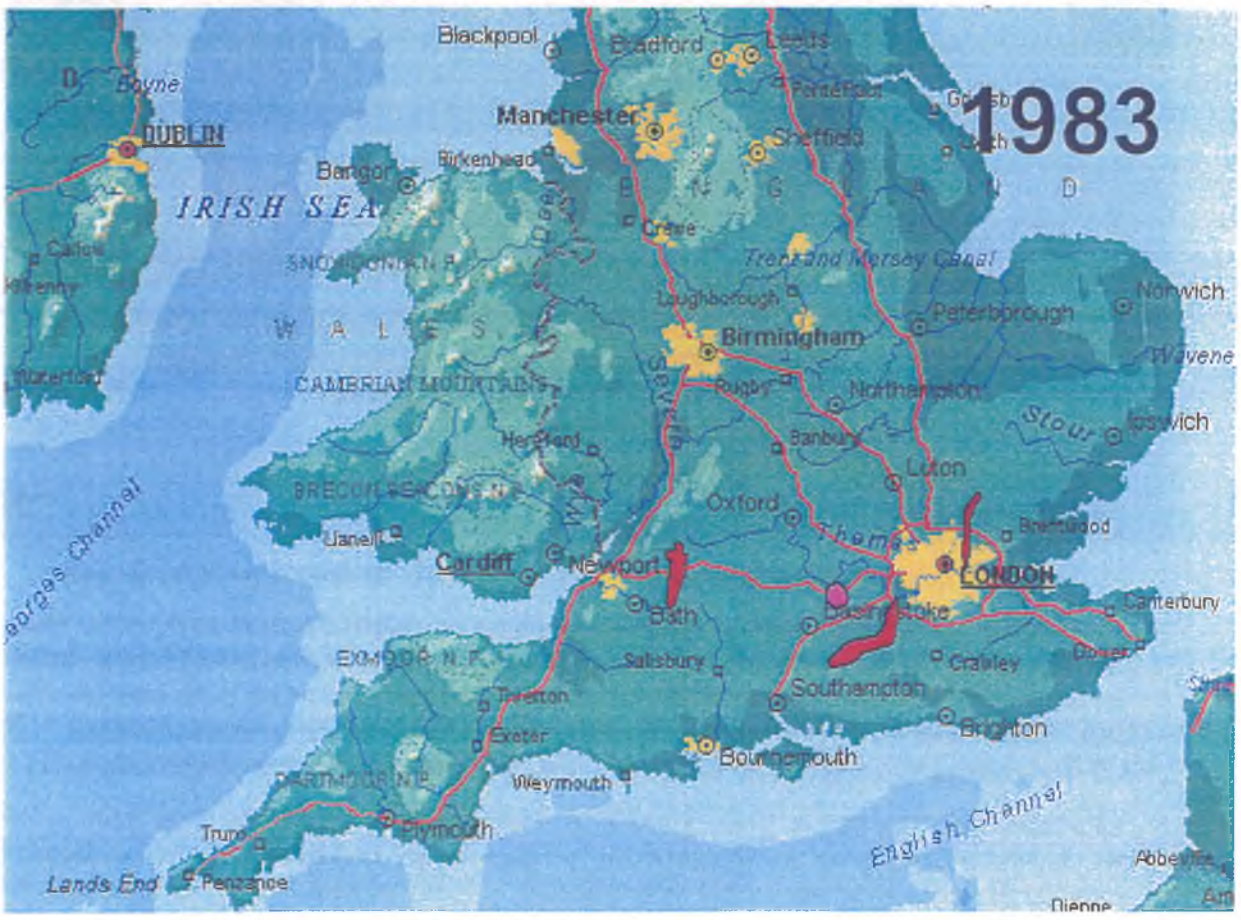

Figure 9 R Wey infected

Certainly in the early 1990's, some smaller southern river catchments and the upper Thames still retained crayfish populations, but continuing erosion of these surviving populations, as occurred in northern Europe during the 1930s and 1940s (Schäperclaus, 1935, 1954), is to be expected . Significant populations of escapee signal crayfish are now widely established in the catchment. An example of this occurred in January 1992 when the small Thames tributary catchment of the High Wycombe Wye and Wycombe Dyke was infected.

By the early 1990's the present author commented that only crayfish populations in Northumbria, the Pennines, the Yorkshire Dales together with the Vale of Eden and the rivers of eastern Lakeland apparently uninvaded. It should be noted however that CEFAS Weymouth has received reports of a number of additional crayfish mortalities where data had been insufficient and no suitable samples had been available to confirm cause. Sites involved included the Blythe in Northumberland, the Ure and the Weare in N. Yorkshire and the Eden in Cumbria.

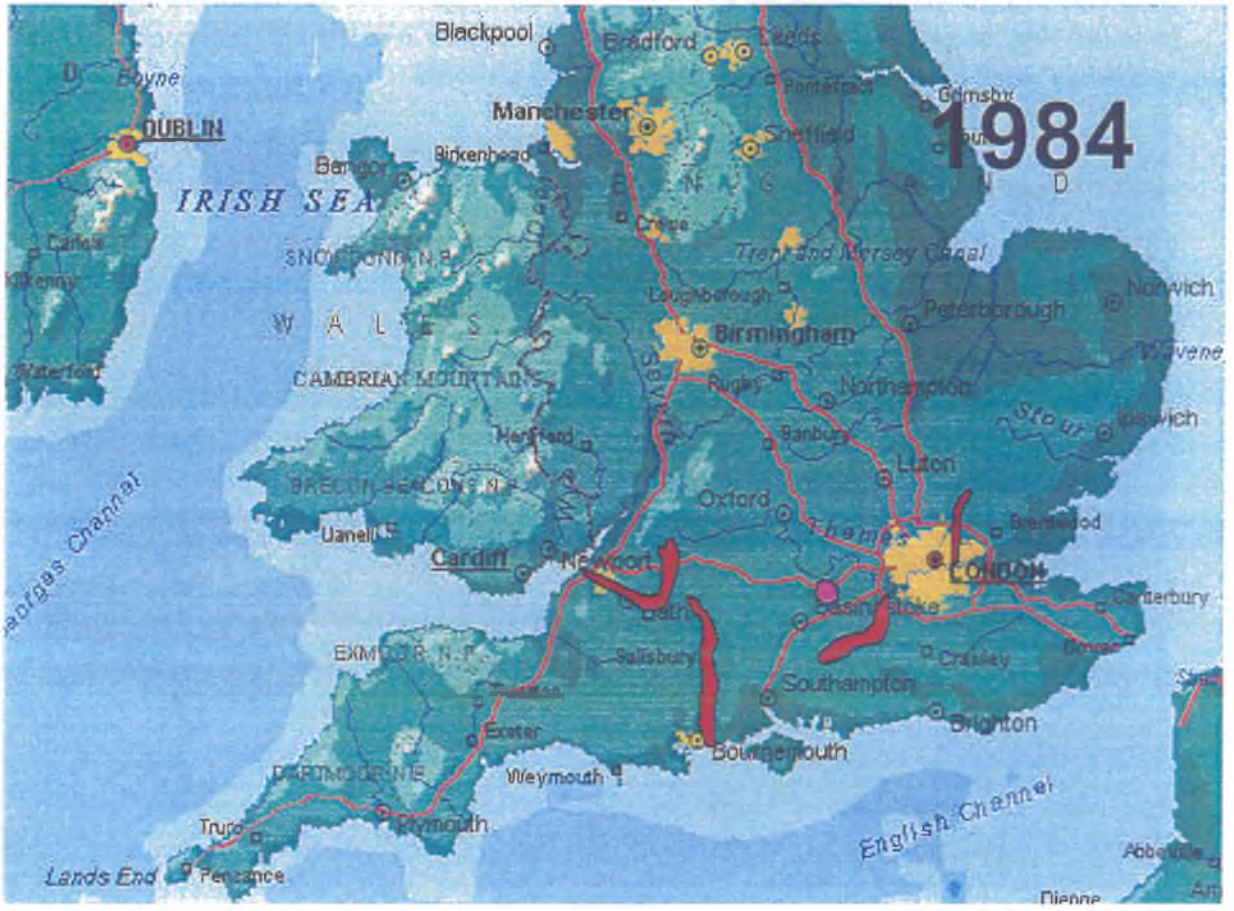

Figure 10 Hampshire Avon

From 1992 no new reports of crayfish mortalities were received for several years. Possibly the introduction of strict controls on crayfish movement and introductions introduced at that time may have had almost immediate effect. Equally, lack of observations and interest may have led to some cases of extension of crayfish plague to have remained unreported. Unreported to MAFF / CEFAS a number of mortalities in the Great Ouse system may have occurred during the 1990s and a report of crayfish plague apparently confirmed by PCR (Aldridge, 2000) exists for the Rivers Rhee and Shep in Cambridgeshire. Confirmed extension to the R. Ribble with isolation of *A. astaci* in 2000 was viewed seriously and EA took action to try to save representatives of the local crayfish gene pool by transferring animals to an isolated site in the hope that restocking would eventually be possible (Figure 16). Reports suggested that the crayfish plague infection might have been associated with a fish stocking transfer from Yorkshire. In the following year, 2001, reports were received of mortalities on the Ure but, although the description of the mortality agrees with crayfish plague, confirmation has not so far been possible, the reader is reminded that previous reports of crayfish mortalities in that river had also remained unconfirmed.

It will be recalled that the R. Darenth in Kent had been affected by crayfish plague in 1988, so that the author was somewhat surprised to receive a report of a crayfish mortality in the main river in summer 2001. Samples were obtained by local EA staff and confirmed to be crayfish plague in *A. pallipes*. After some discussion with EA staff, the case seems to be typical of that reported from plague areas in Europe. The outbreak in the main river appears to be associated with stock from a small side tributary. Some distance above its confluence with the main river this tributary passes through a culvert some 100m long that could act as a barrier to crayfish movement. It is believed that crayfish above this culvert were isolated and not affected by the first outbreak in 1988 and eventually repopulated the stream and then the main river at its confluence. Clearly crayfish plague is still present in the watershed and eventually the

repopulated stock became infected and a mortality sufficient to be observed took place. It is to be hoped that the culvert will again act to isolate and protect remnant crayfish populations, but a continuing cycle of repopulation and disease may be expected.

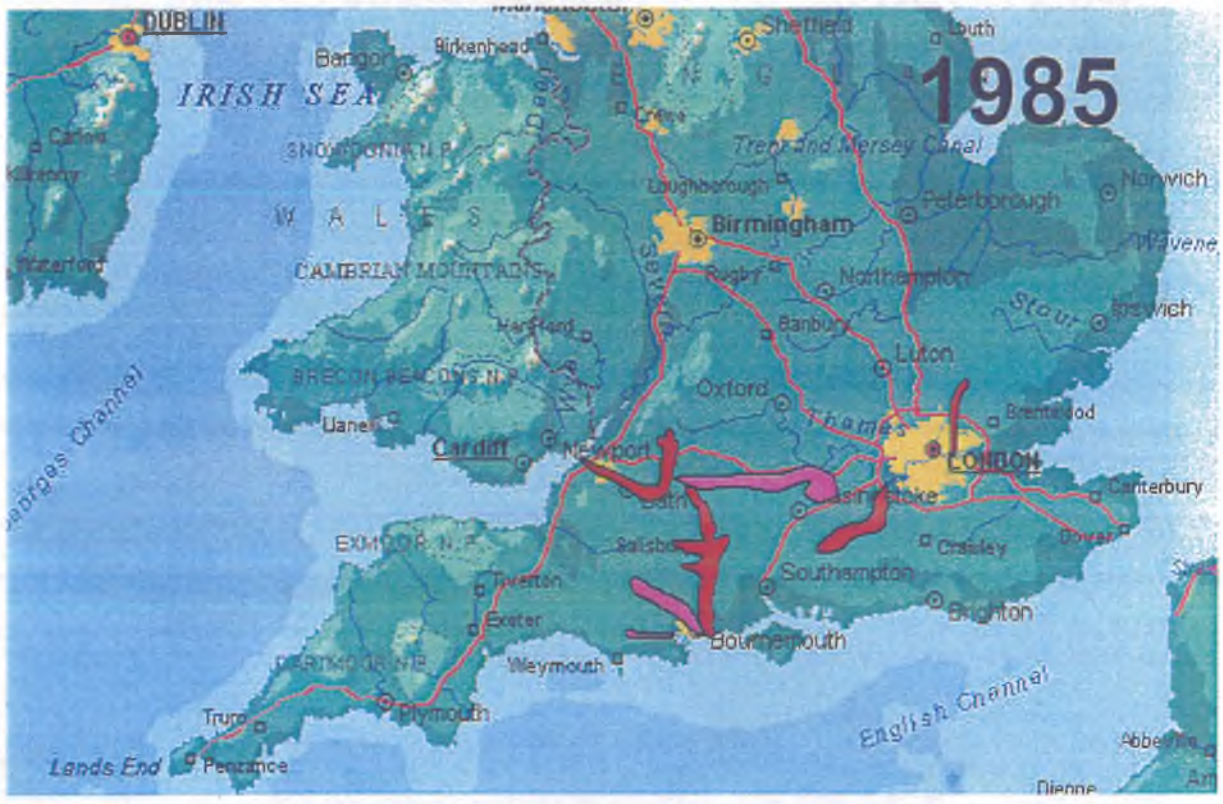

Figure 11 Kennet, Dorset Stour and Dorset Frome probable

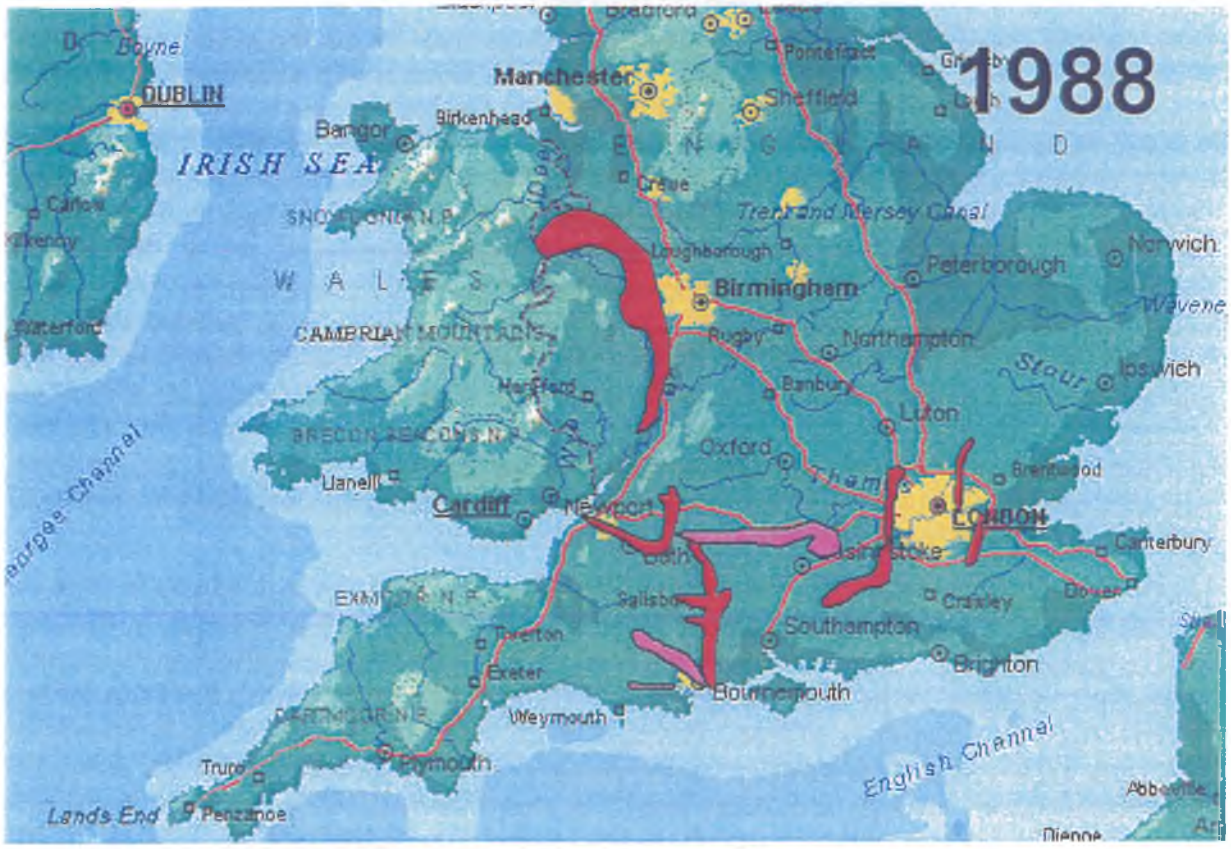

Figure 12 Forest of Wyre and Upper Severn, R. Darenth, Kent

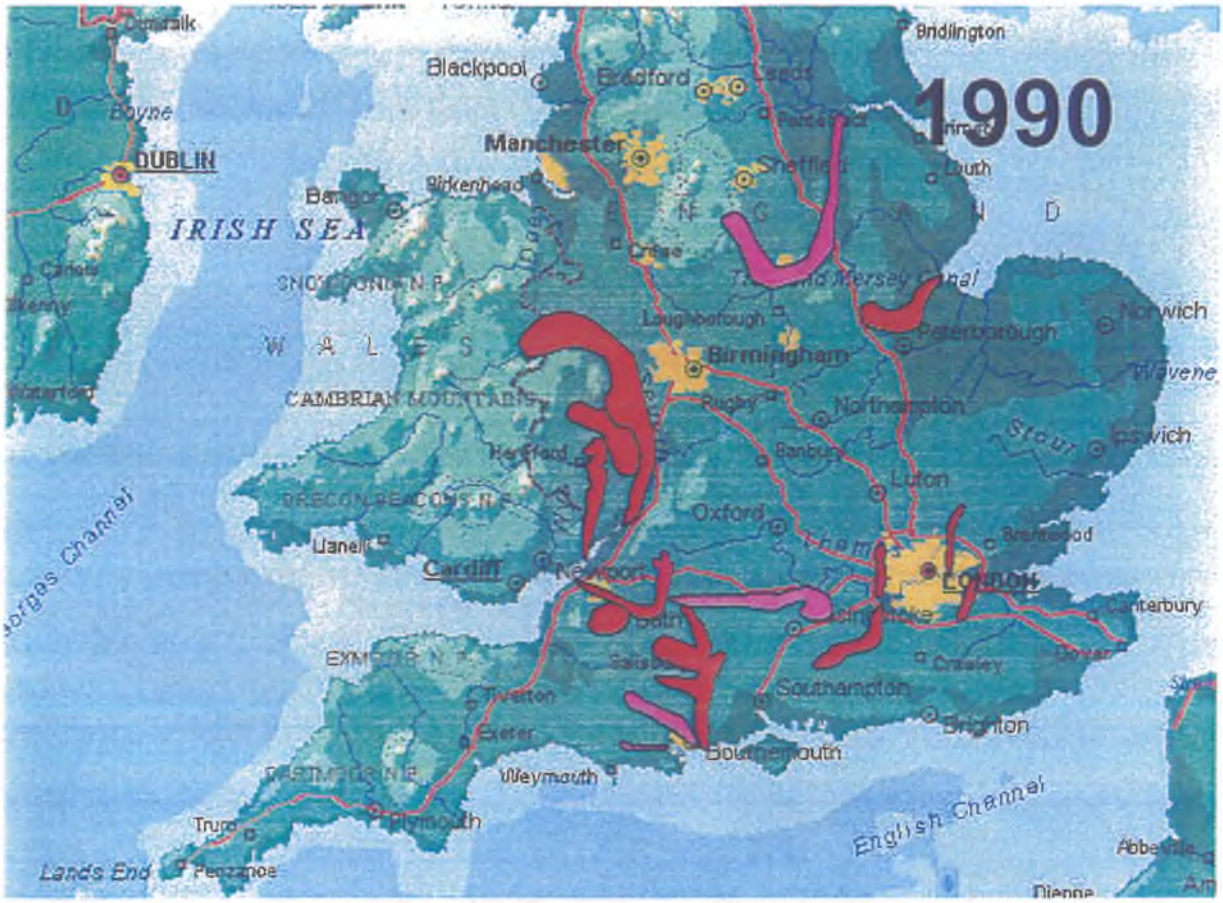

Figure 13 R. Arrow. R. Ise and R. Clun confirmed

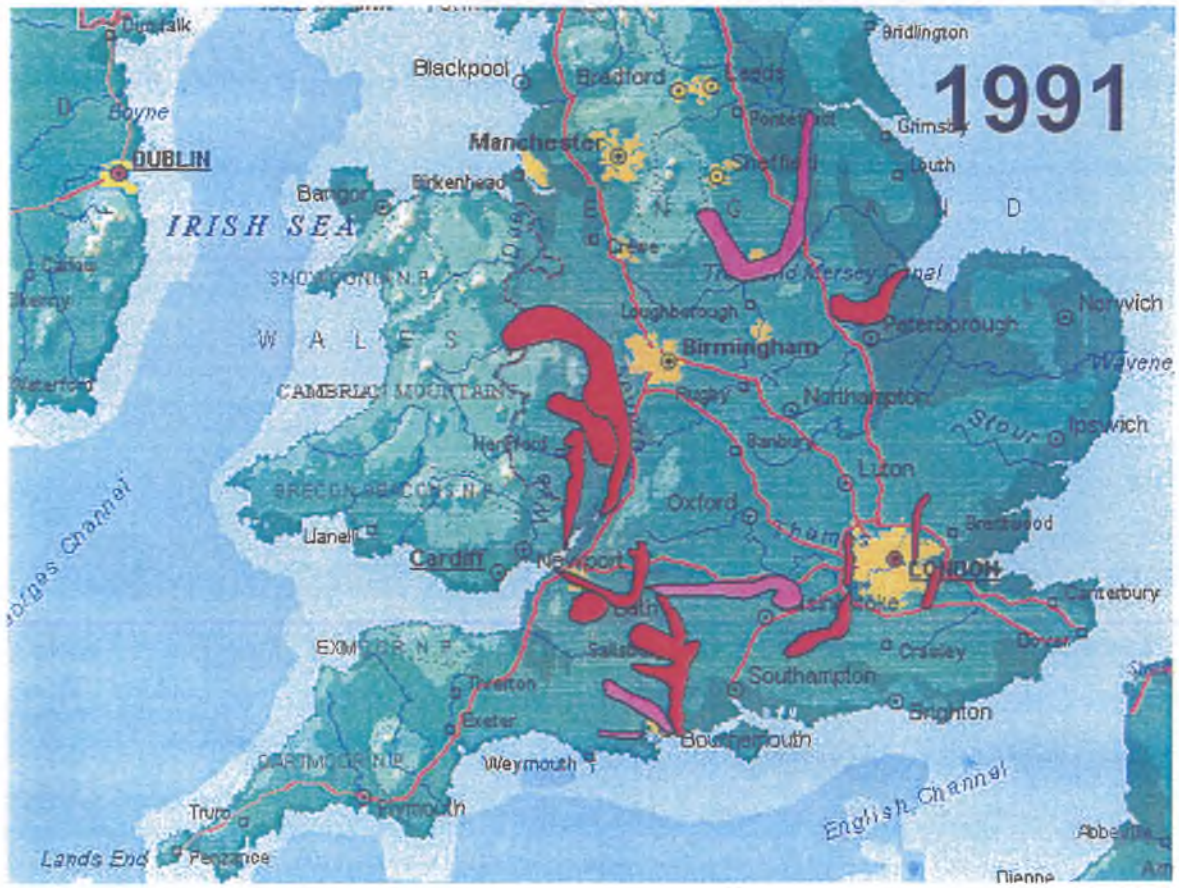

Figure 14 Probable plague mortality in Derwent, new mortalities in Bristol Frome

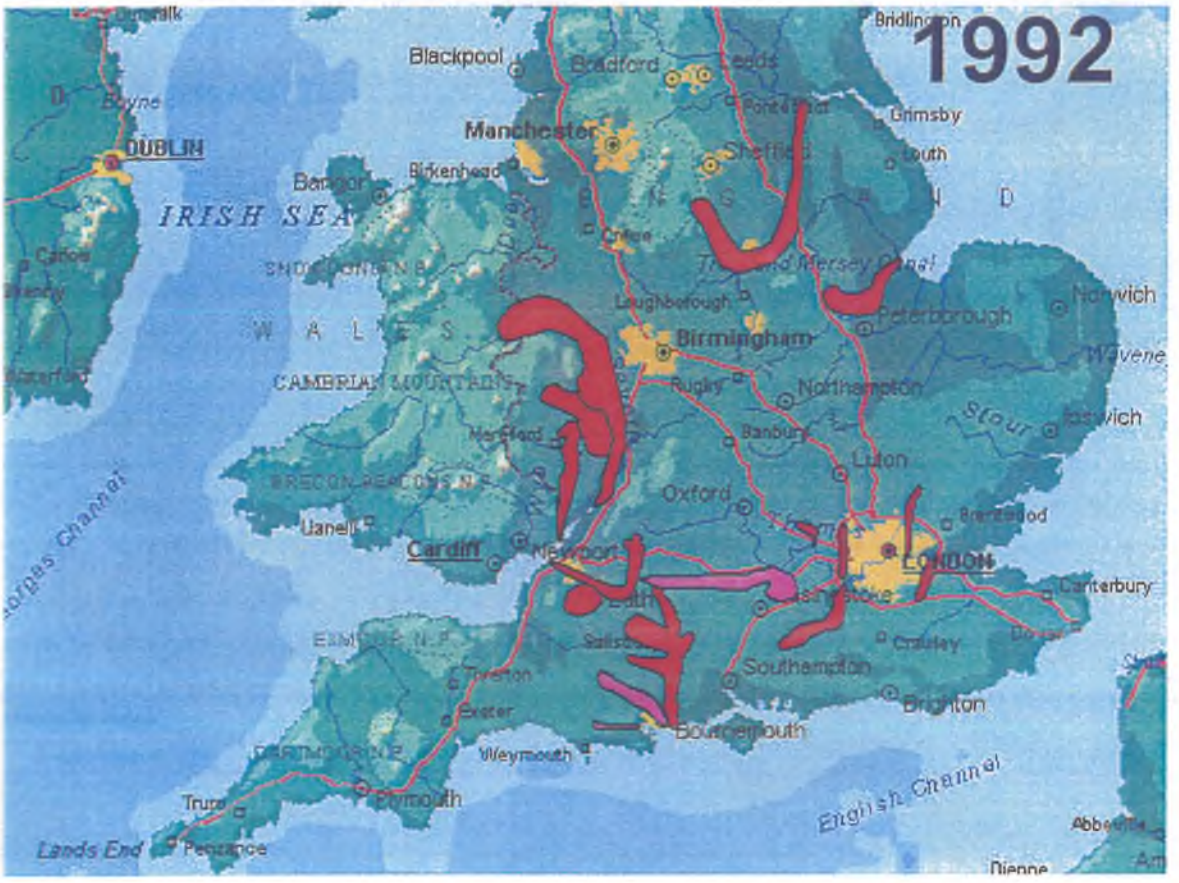

Figure 15 Derwent area confirmed

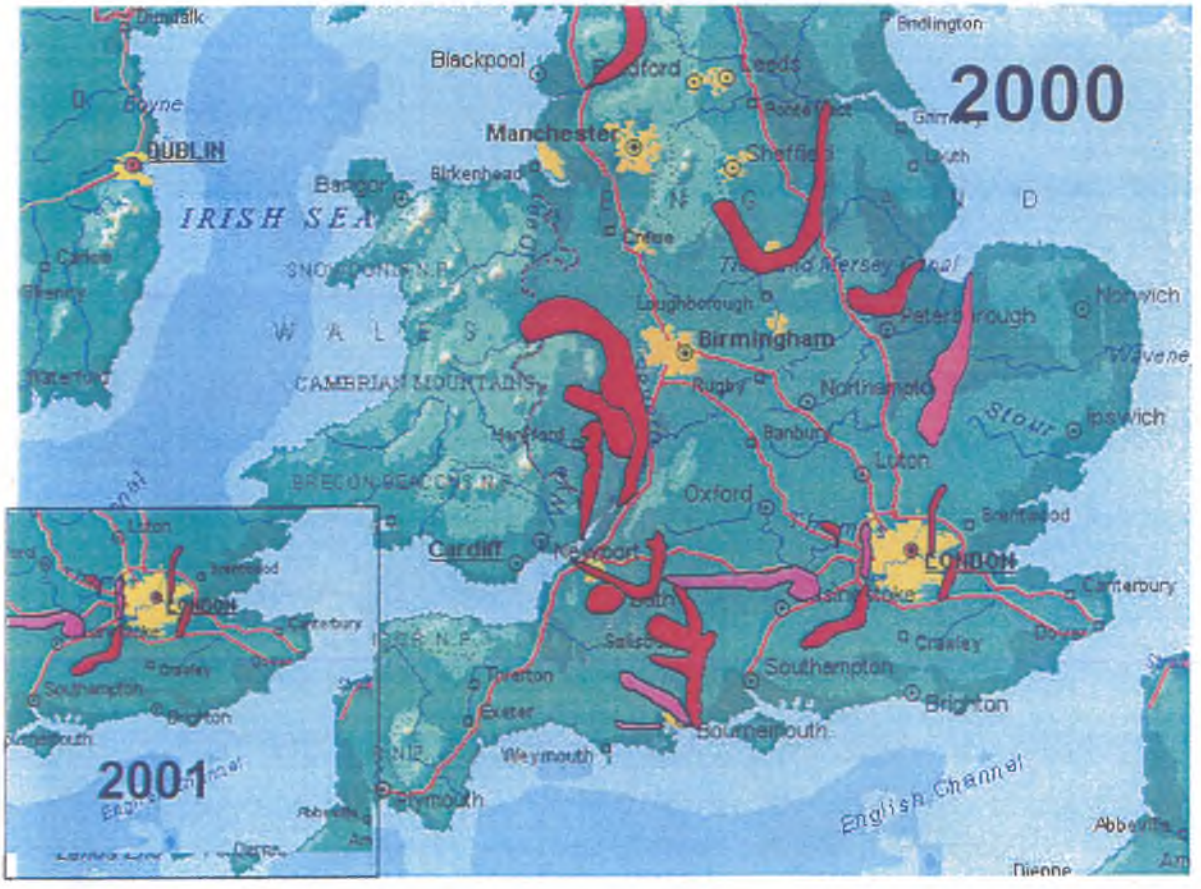

Figure 16 R. Rhee and Shep, Cambs, R. Ribble, new outbreak in 2001 in R. Darenth

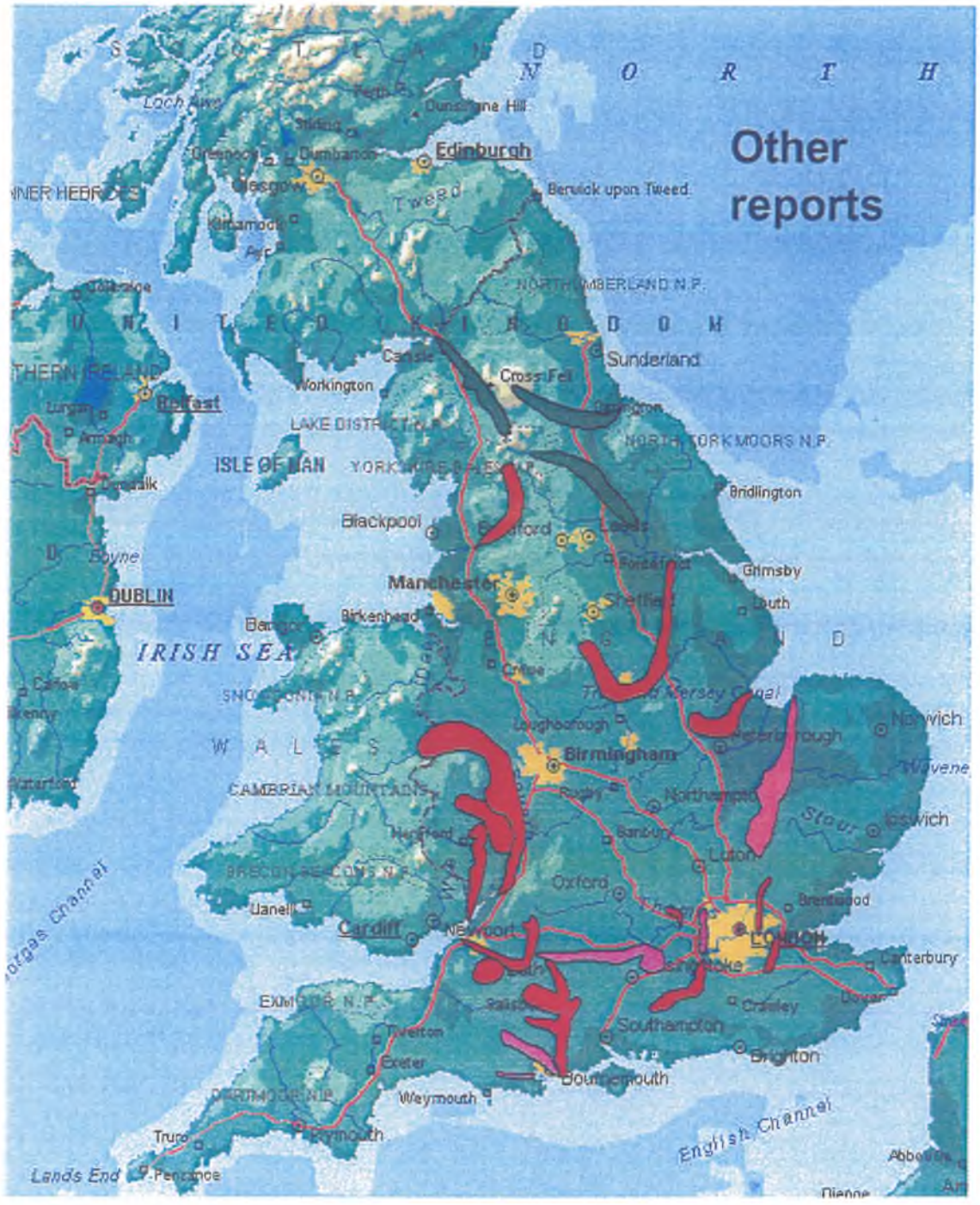

Figure 17 Summary of other unconfirmed crayfish mortalities 1981-2001 (green)

4. ROUTES OF TRANSMISSION

4.1 Crayfish as carriers

As Unestam (1969, 1970, 1975a) suggested, the marked resistance of N. American crayfish species to *A. astaci* and the susceptibility of non N. American species, strongly suggests that *A. astaci* is a N. American organism in origin. *A. astaci* and N. American crayfish appear to have a reasonably balanced host-pathogen relationship in which drastic epizootics are rare. This contrasts strongly with the unbalanced destruction wrought by *A. astaci* in Europe. This would suggest that *A. astaci* was introduced into Europe in the mid nineteenth century, presumably on infected, carrier N. American crayfish, although evidence is lacking. An alternative suggestion of import in ballast water could also be possible, but equally lacks proof.

Whether the crayfish mortality reported from the Po basin in the 1860's was crayfish plague or not, the main focus from which crayfish plague spread to the rest of Europe was clearly the French / German border area in Alsace Lorraine and no record exists that might indicate how *A. astaci* arrived in an area that drains into France, Belgium and Germany. The arrival of contaminated ballast water in such an area is of course unlikely.

Schikora (1922) discussed the role of commercial movements of crayfish in some detail in his review entitled "50 years of crayfish plague" and was in no doubt that it was predominantly the wholesale crayfish trade which brought crayfish plague to Berlin in the north and to Munich in the south of Germany from the original Alsace Lorraine focus and then continued to import infected crayfish both for stocking and for table purposes.

Some German crayfish wholesalers had developed sufficient expertise to recognise infected stocks and to play a role in attempting to restrict the spread of infection (Schikora, 1926), others were much less careful. The problems of lack of sufficient disease free stocks to satisfy the German market was evident, without imports the wholesalers were unable to continue in business, yet continued imports brought the certainty that some of the imports would be infected and would continue to "top up" infections in German waters. The economic returns for indiscriminate imports of crayfish were clearly attractive. Cases of illegal imports of crayfish into Germany were reported in which indiscriminate purchases were brought in in rucksacks or as hand luggage and then "sent on inland as postal packets as stocking crayfish" (Schikora, 1926). Parallels with modern illegal movements of cold water ornamental fish can only too easily be drawn.

The most popular American crayfish species for culture in Europe in modern times is the signal crayfish *Pacifastacus leniusculus* which Unestam and Weiss (1970) have shown to be infected in its native environment and which Persson and Soderhäll (1984) have demonstrated to be a resistant carrier of crayfish plague. *P. leniusculus*, however, was not introduced into Europe until the mid twentieth century in a bid to replace populations of *Astacus astacus* destroyed by crayfish plague. Thus, although it has been shown to be a vector in recent crayfish plague epizootics in Europe (Alderman *et al.*, 1990 and in cases described above) the signal crayfish was clearly not associated with the original introduction of crayfish plague well over 120 years ago. Another N. American crayfish, *Orconectes limosus*, the spiny cheeked crayfish, had certainly been introduced into Europe by 1890 and is now long naturalised in French and German waters (Vivier, 1951), but this first recorded introduction is well after the first indisputable outbreaks of crayfish plague. The rainbow trout, *Oncorhynchus mykiss* (Walb.), another N. American species introduced for aquaculture, may be a potential carrier of crayfish plague (Alderman and Polglase, 1984; Hall and Unestam, 1981) was not introduced into Europe before 1879, a few years before the first cases of crayfish plague in Europe. Some of the more recent outbreaks in Spain may relate to the introduction of another N. American crayfish, the

Louisiana swamp crayfish *Procambarus clarkii* which is now extensively naturalised in Spain (Figure 18).

Because the presence of crayfish plague in the British Isles prior to the 1980's was not established scientifically, its absence, a negative, equally cannot be established beyond doubt, particularly to the satisfaction of those who felt accused of introducing the infection in the mid 1970's. Nevertheless, it is clear that prior, to 1980 the British Isles had good stocks of native crayfish and from 1981 onwards a series of confirmed plague outbreaks has occurred. Equally, it is clear that the first significant introduction of exotic crayfish into the British Isles was of the signal crayfish, *Pacifastacus leniusculus* (Dana), in the period from 1976 onward, unlike much of the rest of Europe where *Orconectes limosus* (Rafinesque) has been established since before 1900. In Sweden and Finland, the signal crayfish is recognised as a carrier of crayfish plague and is associated with plague mortalities in susceptible native crayfish.

Similarly in most cases of crayfish plague in England and Wales since 1981, the presence of signal crayfish, *Pacifastacus leniusculus*, in close proximity to the outbreak is notable. The evidence for the presence of the signal crayfish has not always been firmly established, but only in one or two cases is there no discernible link between the disease and the presence of signal crayfish.

It also must be made clear that, apart from two cases, it has not been possible to obtain samples of suspect signal crayfish to determine presence or absence of the carrier status in those populations. In one, signal crayfish obtained from commercial sources held in tanks at the University of Nottingham transmitted infection to native crayfish held there. Examination proved these signal crayfish to be carriers of *A. astaci* (Alderman, Holdich & Reeve, 1990) by isolation of the pathogen into culture. The Nottingham crayfish had been obtained from the British Crayfish Marketing Association (BCMA), a now defunct organisation created by importers of signal crayfish to market the crayfish produced by members. The BCMA operated a central sales system where crayfish from all producers were mixed together, so that the source of the plague positive Nottingham signal crayfish could not be traced. Clearly however, at least one of the BCMA producers was distributing infected signal crayfish capable of infecting *A. pallipes.*

In the second case, signal crayfish from gravel pits near Ringwood, Dorset have also been found to be carriers of crayfish plague (Alderman, unpublished), these latter were reportedly imported and introduced directly from N. America and not from BCMA or any European source.

In some cases naturalised populations of signal crayfish are clearly not carriers of plague, since they have coexisted in the same waters as native crayfish for more than five years without any evidence of plague in the susceptible stock (e.g. the By Brook and St. Catharines Brooks in Avon (Frayling, pers. comm.).

Although the majority of stocks imported into Britain came from Swedish crayfish hatchery sources where precautions against disease transmission were practised, some at least are known to have come directly from N. America. Lacking any check on health status of crayfish imports into the Britain, the possibility that farmed signal crayfish were responsible for the introduction of plague into the British Isles could not be proven at the time of the original outbreaks, although the temporal relationship between the introduction of signal crayfish farming in England and the commencement of plague outbreaks was clear and as indicated above, some at least of those commercial stocks are infected and infectious.

In the last few years work by the Uppsala group to develop molecular methods have yielded valuable information about the origin of different outbreaks of crayfish plague. Huang *et al.* (1994) applied RAPD PCR to a range of isolates of *A. astaci* from a range of sources, principally northern Europe but also Turkey and N. America. RAPD fingerprints were obtained which clearly divided into two groups that clearly related to the source host species and geographical origin. One group was from noble crayfish populations and were interpreted as representative of

the "old" crayfish plague established in Europe for many years and some had been isolated as early as 1962. The second group came from both native Swedish noble crayfish and from signal crayfish. Signal crayfish were first introduced into Sweden for large scale stocking in 1969 and Huang *et al.* (1994) interpreted their results as indicating that signal crayfish were vectors of a new strain of *A. astaci* which transferred into susceptible native crayfish. The isolate from Turkey fell into the same RAPD group as did the "old established" crayfish plague strains from Sweden. This suggested that the 1980's outbreaks in Turkey did not derive from importation of signal crayfish, but instead represented a new spread of the original disease strain. The authors postulate that until the introduction of signal crayfish in the late 1960's only a single genotype of *A. astaci* had been present in Europe.

A further study by the Uppsala group included a strain from the U.K. (Lilley et al., 1997). This isolate had been supplied from the Weymouth *A. astaci* collection to the first author for comparison with *A. invadens* from Epizootic Ulcerative Disease (see below) and was from the outbreak on the R. Arrow in Herefordshire. The authors found it to fall into the "recent" signal crayfish associated RAPD PCR group of *A. astaci*, supporting the view that recent UK outbreaks related to import of signal crayfish for farming. In fact the R. Arrow outbreak was one in which a signal crayfish introduction onto a nearby fish farm was known so that the link was tentatively already established.

Further work by the same group (Dieguez-Urebeondo et al, 1995; Oidtmann et al., 1999) led to the recognition of two more *A. astaci* RAPD PCR groups, each represented by a single isolate, one from signal crayfish in Canada and the other from *Procambarus clarkii* in Spain. Strains of *A. astaci* are now referred to as belonging to Groups A ("old established"), B ("recent signal crayfish associated", C (Canadian) and D (Spanish). German isolates from recent plague outbreaks were studied and found to belong to Group B.

The German plague outbreaks were reported (Oidtmann et al., 1999) as being at two sites 80km apart with no known or traceable introductions of crayfish for at least 16 years in one case. *Procambarus clarkii* have however been introduced in the area.

The twenty-one years from 1980 to 2001 have seen the destruction of the majority of crayfish stocks in England and Wales south of the Pennines with several undetermined mortalities to the east of the Pennines and one very recent outbreak confirmed to the west in the Ribble. The presence of signal crayfish in the vicinity of many of these disease outbreaks suggests that these exotic crayfish may well have been carriers of the disease and therefore have been responsible for the rapid spread of crayfish plague. Indeed, as described above, PCR data links at least one UK plague outbreak to Group B *A. astaci* which is believed to have been introduced into Europe through Sweden with signal crayfish imports since 1969.

It is also clear that there have been many introductions of signal crayfish into fish farms and would be 'crayfish farm' sites throughout England and Wales and from most of these signal crayfish have escaped to the wild resulting in the establishment of naturalised populations (Holdich & Reeve, 1992). Prior to 1983 deliberate release of such exotic crayfish to the wild was not prevented by legislation since controls that had been established on transfer of crustaceans were established under the Sea Fisheries Act and thus did not apply. Some of the naturalised populations result from early direct releases to the wild, which, whilst not illegal at the time, cannot be regarded as responsible actions.

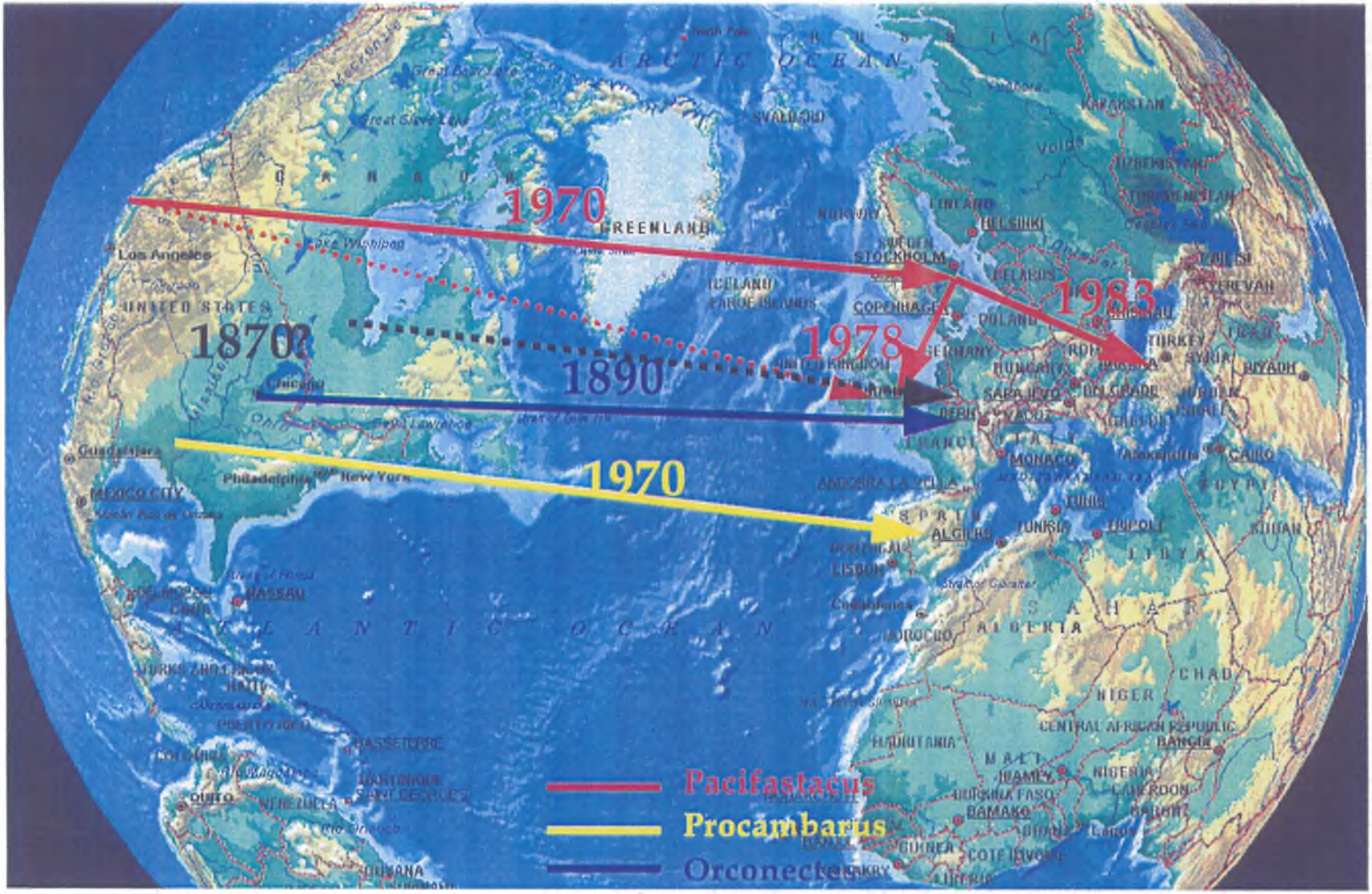

Figure 18 Probable routes of transfer of crayfish plague from N. America. Solid lines indicate recorded introductions, dotted lines indicate unrecorded possible introductions

The evidence from the R. Darenth in 2001 confirms that as seen over many years in Europe, that once *A. astaci* reaches a watershed, plague epizootics occur rapidly, but remnant susceptible crayfish populations may re-establish (even quite widely) to the point at which further epizootics occur. Major crayfish mortalities have not always been observed in England when they occurred, so that low level mortalities in remnant populations can easily be overlooked. With low crayfish populations, transfer of infection will be slower, fewer spores will result in a longer time from infection to death.

In theory, once crayfish have been eliminated from a body of by *A. astaci*, the pathogen will not persist for long (3 months has been estimated by Söderhäll). This may occur in simple bodies of water, but in any natural river system some animals at least may be expected to be protected by distance - either by weirs or culverts etc or by low crayfish population densities. This will slow down the spread of infection, but if sufficient animals survive a chronic mortality situation will arise. This may mean a series of crayfish population recoveries followed by acute population crashes, or alternatively that population densities never recover. Where signal (or other N. American species) are present, then if these animals are infected, a continuing reservoir of *A. astaci* will remain in the river system for the foreseeable future.

4.2 Non Crayfish Routes of Transmission

4.2.1 Equipment: angling gear, nets, rods and boots

It has been clearly demonstrated that infected crayfish, whether of susceptible or resistant species will transmit crayfish plague if moved into new watersheds. However there is considerable evidence to show that this is not the only possible route of transmission.

As mentioned in the description of the history of crayfish plague in Appendix I of this report, much of the information on the spread of crayfish plague, particularly in the period 1880 to 1920 is accompanied by comments about the role played by commercial crayfish trappers and wholesalers in the spread of the disease. Schikora (1922) was in no doubt that it was predominantly the wholesale crayfish trade that brought crayfish plague to Germany from the original Plateau de Langres. The spread of plague eastwards from Germany has been attributed to introduction of infected crayfishing gear from Germany and the activities of mobile crayfish catchers (Arnold, 1900; Tzukerzis, 1964) moving from river to lake to river in Russia. Certainly an examination of the developing railway system of eastern Europe makes it clear that by the 1890's reasonable transport systems then existed that could transfer an essentially highly perishable product such as live crayfish to German and other western European markets from the great rivers of Russia.

More recently, the spread of crayfish plague to Ireland has also been attributed to infected (non-crayfish) fishing gear, rods, boots etc. (Reynolds, 1988) and rumours of illegal introductions of crayfish are dismissed. Taugbøl et al., (1993) published a detailed consideration of how *A. astaci* may have reached and infected rivers in Norway. Despite the close proximity of long infected waters in Sweden and cross border rivers, Norway had remained plague free for more than 60 years. The first infections in the cross border Veska / Vrangselva river systems occurred in 1971-4. These rivers rise in Norway and drain into Sweden and infection entered the country form downstream. Attempts were made to control upstream spread of infection using electric fences were unsuccessful and crayfish appeared to have gone from the system below a concrete weir by 1974. Some repopulation, both natural and fisherman aided appears to have occurred since. The much more important Glomma river system (the longest in Norway) was found to be infected in 1987, but many side streams have remained uninfected, perhaps as a results of protection from weirs and crayfish free sections of river etc. Lake Store Le and Halden watersheds further south on the Swedish border were first recorded with crayfish plague in 1986-9. Norwegian authorities could find no proof of the origin of infection, but speculated that

known crayfish plague epizootics in Sweden only a few hours by road from the Glomma could have allowed transfer by tourists and their equipment. There is no evidence of illegal movement of signal crayfish into Norway. The Store Le system is another cross border river system so that again plague could have entered from Sweden by natural movements of infected crayfish. At one point the Store Le and Halden are only a short distance apart, movements of boats between the two were prohibited once infection was known to be in the Halden, but Taugbøl et al (1993) acknowledge that the enforcement was difficult and may not have been effective.

Recommendations for the disinfection of equipment have been made.

4.2.2 Transmission on other animals.

In the UK, there is (strong) circumstantial evidence that fish movements were associated with transfer of infection from the R. Blackwater to the R. Wey and the recent Ribble outbreak has been suggested to have similar origin. In the case of the Wey, the fish movement took place at a time of (presumed) acute crayfish mortality on the Blackwater where considerable numbers of escapee signal crayfish were present. The risks of such transmission are discussed more fully in Part II of this report where means to prevent such fish movement transmission are considered.

Transmission of crayfish plague by movements of animals other than crayfish and fish is a risk that is often mentioned during discussion of the disease, but there is no evidence, circumstantial or otherwise that would show that this has ever occurred. Possible mechanisms are on the feet and feathers of birds and on the feet and fur of wild mammals. Although both would feed on dead and moribund crayfish, gut transmission can be ruled out. Mammalian and avian body temperatures are too high for *A. astaci* to survive passage.

When the first plague mortality was observed on the Sherston branch of the Bristol Avon the numbers of dead crayfish were sufficient to attract carrion feeders and crayfish had been pulled out onto the bank. Birds were observed to be attracted (Frayling pers. comm.) and mammals such as foxes, mink, etc would also obviously be attracted in the right circumstances. Whilst birds could contaminate feet and feathers with *A. astaci* and fly sufficiently rapidly between watersheds to transfer viable *A. astaci* spores to a new river site, mammals would appear to represent an unlikely route of transmission between watersheds. Except in unusual circumstances feet and fur would not remain infectious over the time taken for mammals to move between two watersheds. However a role in upstream spread above weirs and other physical obstructions cannot be ruled out.

4.2.3 Effects of weirs on transmission of *A. astaci* .

Weirs have been noted to act as a significant and permanent barrier to upstream migration of crayfish plague. One example is the weirs on the Sherston branch of the Bristol Avon at Easton Grey, another (discussed elsewhere) a culvert on a tributary to the R. Darenth in Kent. Even when a major plague mortality is underway, the main route of upstream migration of infection appears to be by the movement of crayfish rather than of spores or on fish. Indeed the effect of plague on crayfish behaviour may enhance movement of crayfish.

Weirs of sufficient height may not be passable by infected crayfish and significant stretches of stream unsuitable for crayfish (culvert or water type) may thus act as a barrier. No weir should however be regarded as offering any guarantee of breaking upstream migration of infection. Although Easton Grey weirs protected the upper Sherston branch of the Bristol Avon, an equally substantial weir on the Tetbury branch of the same river did not.

5. DISINFECTION AND CONTROL

Control of crayfish plague has two aspects - attempts to prevent and control human actions that will bring about transfer and extension and attempts to prevent and control natural spread of the disease.

Human actions include movement of equipment, including protective clothing and fishing gear and secondly transfers of aquatic animals, whether crayfish for "farming" or fish for stocking purposes. Alderman et al. (1987) demonstrated that (admittedly heavily) contaminated fish nets could transmit crayfish plague (Figure 19 and see below) and similar hazards will be associated with crayfish traps, waterproof clothing and boots. Indeed it was strongly believed that such equipment was responsible for spread of infection in Russia at the turn of the 19th Century. The practicability of adequate disinfection of boots and gear was demonstrated by Alderman & Polglase (1985a,b), who demonstrated that standard fisheries disinfectants are effective. They showed that sodium hypochlorite at 100ppm available chlorine would kill *A. astaci* in less than 30 seconds and that iodophores (such as Wescodyne) at 100ppm available iodine was also effective, but that the latter had less penetrating power so that it should only be used on clean surfaces and not where mud might protect the pathogen from its action. Although not specifically investigated, proper drying of equipment and nets may also be expected to be effective in disinfecting equipment from *A. astaci*. The same workers, mindful that Hall and Unestam (1980) had demonstrated that *A. astaci* can grow on detached fish scales also examined the efficacy of the traditional fisheries fungicide malachite green against *A. astaci*.

A further potential route of transmission that also must be considered for completeness, is the risk of transfer by cross watershed movement of aquatic diseases such as crayfish plague by pumped water and also by canal (e.g. the Kennet and Avon Canal)

To investigate the ways in which crayfish plague might be transmitted by commercial fish movements and contaminated equipment, Alderman et al. (1987) infected a tank of crayfish and then introduced rainbow trout introduced into the tank for 24 h. Twenty of the trout were transferred directly (after a 20 s draining period to ensure the transfer of minimal amounts of water) to a 300 l tank containing 20 healthy crayfish. Two further groups of 20 trout, each in 100 l of water from the mortality tank, were transferred to two fish transport tanks on a road vehicle which was then driven on a circular 60 min 'journey' to simulate commercial transfer of fish from one fish farming site to another. During the last 30 min of the journey, one of the tanks of fish was treated with 1 ppm malachite green oxalate. Both fish and water from transport tanks were then transferred into two further 300 litre tanks, each with 20 clean crayfish. Finally, one 26x26x30-cm fine mesh net was placed in the tank of dying crayfish for 1 h then removed, allowed to drain for 2 h in a dry, empty bucket at ambient temperature (15°C) before being placed in another tank of 20 clean crayfish.

Transmission of infection was most rapidly and efficiently transmitted by combined movement of water and trout, which produced results as effective as those from introduction of zoospores at moderate-challenge doses (Figure 19).. Movement of fish alone and the use of the contaminated fish nets also transmitted the infection, but with a prolonged time to first mortality of 58 and 61 days, respectively. Interestingly, despite the prolonged incubation time before the first death, the ensuing mortality rates were rapid, the mortality curves for both the net and fish only challenges being very steep (Figure 19). The first animals to die showed some marked signs of crayfish plague, in contrast to the latter mortalities from these challenges which showed the minimal gross signs of the disease.

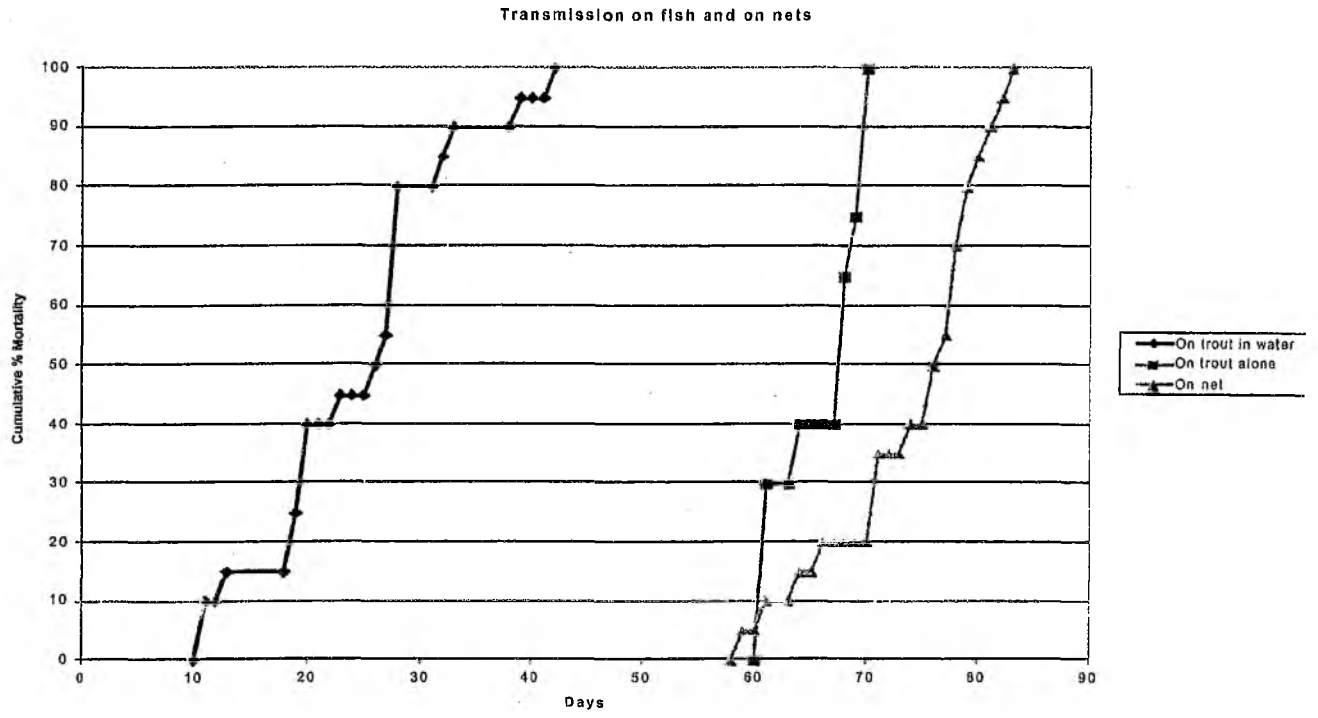

Figure 19 Transmission of crayfish plague on fish and on nets

No mortalities occurred in any of the control tanks of crayfish, which were provided for all experiments, nor were there any mortalities in the crayfish exposed to trout and transport water treated with malachite green.

Alderman et al. (1987) interpreted the results of their transmission experiment, to indicate that zoosporulation occurred on the first animals to die (both in the net and in the fish only transmission experiments) produced secondary infections which resulted in a much-accelerated mortality pattern for the late survivors. In those laboratory experiments, in conformity with welfare needs, dead animals were removed from the experimental tanks immediately on death. This will have tended to reduce the number of zoospores, which could produce secondary infections. Under natural conditions, such zoospores would be available to produce further infection. These results demonstrate that the concept of an LD_{50} is not practicable with an organism such as *A. astaci.* The 100% mortality of all experimentally-exposed animals indicates the pathogenic ability of *A. astaci* and also shows why resistance to crayfish plague has not developed in European crayfish populations. The transmission experiments showed how easily crayfish plague may be transferred from one river system to another by fish movement or by contaminated boots or gear.

These studies also showed that the use of a suitable fungicide such as malachite green could prevent such transmission on fish. Malachite green had the advantage that it bioaccumulates but is no longer acceptable as a fungicide in fisheries use. Since the purpose would be to disinfect fish being transferred from a potentially plague infected water to a non infected water, it could be argued that this use of malachite green would be as a disinfectant and not as a veterinary medicine. This argument would however not be acceptable in terms of residues of malachite green present in the stocked fish. Directive 96/23/EC requires that European Member States operate a monitoring programme for illegal veterinary medicines and malachite green is specifically listed as an illegal substance to be monitored for in this programme which has now been in place for a number of years. Malachite green as a residue is now known to accumulate rapidly in fish tissues, but to deplete slowly, several thousand degree days may be required for residues to be completely eliminated after a single treatment. Since malachite green is

considered to be a potential carcinogen and since stocking fish could in theory at least be caught for food within a very short period after stocking, the effective withdrawal period would be zero time. Effective though it is therefore malachite green cannot be used.

The question therefore arises "is there any other compound that could be used legally to prevent transmission of crayfish plague on fish?". The most likely possible compounds are formalin and the new fungicide Pyceze being developed by Novartis Animal Vaccines, but specific data is lacking. These possibilities, together with consideration of the scale of the risk of transfer of crayfish plague with movements of fish will be considered further in the second, forward look part of this report.

The transfer of crayfish plague on crayfish themselves, particularly the N American species, *P. leniusculus*, the signal crayfish and *P. clarkii*, the Louisiana swamp crayfish is a clear hazard. As mentioned above, MAFF had imposed controls on introductions of fish, molluscs and crustaceans for some considerable time. When the first attempts to introduce signal crayfish for crayfish farming were made in the late 1970's, the available legislation was examined. Legal advice at the time was that the Disease of Fish Act 1937, could not apply since crayfish were not therein regarded as fish and that the Crustacean controls could not be employed in fresh water because they had been introduced under the Sea Fisheries Act. There was therefore no means available to control these introductions. It was recognised that crayfish plague could well be introduced by these imports and that any initial damage would be well advanced before any new legislation could be introduced.

Some protection was afforded by The Wildlife and Countryside Act, 1981, which made it an offence to release or allow to escape into the wild any animal of a kind not ordinarily resident in Great Britain in a wild state (unless a licence has been issued by MAFF under the Act). Persons farming crayfish needed to make sure that they had taken reasonable steps to ensure that stock could not escape into the wild and, in particular, into ponds or adjoining river systems. The native crayfish was subsequently given protection under schedule 5 of the Act, which then prohibited the taking of any native crayfish for any purpose, except as licensed by the appropriate national nature conservation body. Signal crayfish were placed on schedule 9 of the Act prohibiting further release even though populations existed that were "normally resident".

The Diseases of Fish Act 1983 which replaced the earlier 1937 Act also included shellfish and introduced registration of fish farming enterprises which required that fish and shellfish farms should register their business (at no charge to the producer) with the appropriate Departments in England, Wales and Scotland. A full listing of crayfish farms was thus created for Departmental use in relation to the Act, but the data protection requirements of the Act meant that the information was not publicly available.

It should be noted that in 1991 European legislation had been introduced (Directive 91/67/EEC) which established a general framework for aquatic animal health and disease control within the Community. The original Directive 91/67/EEC was accompanied by legislation (Commission Decision 92/532/EEC) which laid down the rules and methods which member states had to follow in developing plans for carrying out sampling and diagnoses for detection and confirmation of fish and shellfish diseases listed in the Annex to the Directive. Action to be taken on identification of listed diseases was then defined in Council Directive 93/53/EEC.

The general principles of Directive 91/67 were summarised in the preamble to the Directive as follows. "To contribute to the completion of the internal market, avoiding the spread of contagious diseases, taking into account that the animal health situation for aquaculture animals is not the same throughout the territory of the Community, to introduce the concept of approved zones which are free for one or more diseases. To define the concept of approved farms situated outside free zones and enjoying a special animal health status. To define the trade patterns between approved zones, approved farms and non approved zones."

Directive 91/67 (and amendments) covers the following:

aquaculture animals

- fish
- crustaceans
- molluscs

aquaculture products

- for reproduction: eggs, gametes
- for human consumption

The Directive groups aquatic animal diseases into three lists defined as follows:

List I diseases

- diseases which are exotic to the Community
- which are expected to result in serious economic impact
- and for which eradication measures must be implemented (Directive 93/53/EEC)

List II diseases

- which are expected to result in serious economic impact
- which are present in the Community
- for which approved (free) zones and farms (in non approved zones) can be identified
- for which control measures are defined (Directive 93/53/EEC)

List III diseases

- diseases from which a Member State is free or has an accepted control programme against that disease in force

Within this framework - movement patterns for live fish, molluscs, crustaceans, eggs and gametes were defined.

Crayfish plague is a List III disease. Because of the difficulty of implementing a control programme for a disease already widely spread, when consideration was given to control of the problems created by the introduction and farming of signal crayfish, the approach employed was designed towards the control of further spread and introductions of exotic (and therefore potential disease carrying) crayfish, rather than on crayfish plague *per se*.

Thus after the "new" crayfish farming industry had been in existence for 10 years and had only achieved farmed productions of less than 10 tonnes per year (data collected under the Diseases of Fish Act 1983 confirming this), whilst crayfish plague had simultaneously appeared and spread and many signal crayfish had escaped, it was agreed that enough evidence had now accumulated that new legislation to attempt further spread of the problem could be justified. The Prohibition of Keeping of Live Fish (Crayfish) Order, 1996 (England and Wales) came into force in February 1996. Its object was to control the holding of exotic crayfish in those areas of Great Britain where there were still good stocks of native crayfish or, as in Scotland where there were no crayfish present. It defines a number of areas based on watersheds particularly in southern and central England in which it would continue to be permitted to keep (in any water from gravel pit sized pond to aquarium) any crayfish other than our native *Austropotamobius pallipes* without a licence. In all other areas of England and Wales the keeping of such crayfish are prohibited without a licence issued under this order and there is a general presumption that such licences will not be issued. The only exceptions to this were:-

- Existing Crayfish Farms Registered under the Diseases of Fish Act 1983 within permitted areas.
- Existing crayfish farms outside the permitted areas registered under the above Act which were issued with a licence of right to maintain their existing operations.
- Restaurants and direct suppliers of restaurants
- Restaurants and wholesalers supplying restaurants with crayfish for direct consumption.

A Code of Practice for such bodies was prepared and distributed widely. A similar The Prohibition of Keeping of Live Fish (Crayfish) Order, 1996 (Scotland) came into force in February 1996 and completely prohibited the keeping of exotic crayfish in any waters in Scotland, again with the exception of restaurants and wholesalers supplying restaurants for direct consumption.

Permitted and Prohibited Areas were defined as the areas in which the keeping of exotic crayfish are permitted and those in which it is prohibited. The areas concerned were defined in terms of Postcode Areas and Postcode Districts, since it was felt that this provided the least ambiguous definitions and since it was felt that would be crayfish farmers could not claim ignorance of their postcode. Since the introduction of the Order, the view of what sites are permitted as farms has been considerably tightened. The Crayfish Order now gives reasonable protection from deliberate crayfish introductions and movements.

Finally, although the present situation in the UK is such that little further natural spread is unlikely, since there are few unaffected natural populations left, some consideration needs to be given into the way and the rate at which crayfish plague can spread once introduced. Evidence from both UK and the rest of Europe (e.g. Finland (Westman and Nylund, 1979)) suggests that crayfish plague spreads downstream from point of introduction at the speed of the river itself. Upstream spread has been seen at rates of 2 to 4km per year. This is believed to be a combined result of migratory and erratic movements of infected crayfish between infection and death and perhaps includes a small element from the motility of zoospores that exhibit a positive chemotaxis to crayfish.

Attempts to stop crayfish migration were attempted unsuccessfully in Norway (Taugbøl et al., 1993) using electrified fences or barriers. Natural or man made barriers such as waterfalls or weirs do have effect (e.g. in Norway and in UK on the Sherston branch of the Bristol Avon), so that creation of an artificial barrier could be effective.

Rantamäki et al. (1992), building on previous understanding of requirements for zoosporulation of *A. astaci* demonstrated that addition of $MgCl_2$ at concentrations above 20mM prevents sporulation and concentrations above 200mM prevent all growth. The 20mM inhibition of zoosporulation is temporary and when replaced with normal water zoosporulation resumes. In a laboratory challenge it was found that a more than>90% reduction in infection rate could be achieved when crayfish were held in 100m,M $MgCl_2$ and crayfish survived for longer. Since the effect is fungistatic rather than fungicidal, this information has little practical application.

The practical effect of introduction of *A. astaci* into any watershed with susceptible crayfish populations is that infection will spread downstream very rapidly and upstream at up to 4km a year. Only crayfish proof barriers, artificial or natural will stop this up river spread.

6. ATTEMPTS TO RESTOCK PLAGUE AFFECTED WATERS

In the early years of the spread of crayfish plague Europe restocking attempts were tried a number of times, sometimes with short-term success. Crayfish released into the Main were thriving in 1892 (Scherpf, 1892). In contrast infection was still present in 1893 in the Altmühl (Anon, 1893), with introduced animals dying rapidly and in 1894 in Lake Boethin where crayfish had begun to repopulate naturally, a further mortality occurred (Schäperclaus, 1979). Similarly in the Nurnberg area, the R. Selbe a re-established population was again destroyed.

Schikora (1926) reported that Italy had made "energetic" attempts to restock the rivers of Lombardy (destroyed by plague in the 1860's) which were halted by World War I and "subsequent economic weakness". The same author reported that a French "Commission for the repopulation of the rivers in the ravaged areas" was established in the Rhineland had approached a Berlin wholesaler for 100,000 restocking crayfish, a request which was refused, apparently partly from "patriotic" (the Saarland was occupied) and partly from commercial reasons. Whether disease free crayfish could have been obtained in such numbers for such a purpose from Berlin is uncertain.

With the development of programmes to farm and (again) to restock waters with crayfish in the mid 20th century a new phase of commercial transfers of crayfish began with the spread of the signal, *Pacifastacus leniusculus* and the swamp crayfish *Procambarus clarkii*. Sweden undertook extensive investigations to find a suitable plague resistant crayfish that could be introduced to replace plague destroyed populations of noble crayfish (Ackefors and Lindqvist, 1994). Good populations of this introduced species now exist in southern Sweden, presumably filling the ecological niche of the plague destroyed native species. However in European terms this introduction has been little short of a disaster. The introduction of the signal crayfish has been clearly linked with several of the most recent extensions of infection including Greece the UK and Spain, plus what are clearly new disease introductions in Germany.

During the main series of outbreaks of crayfish plague in continental Europe in the last quarter of the 19th Century, crayfish containing waters were "bypassed" by infection and survived, some until they were later infected, others until the present day. Similar "chance survivals" may be expected to occur in Britain, particularly with enclosed waters, such as isolated reservoirs, gravel pits and other similar sites. Also during the main series of European plague outbreaks, some populations of crayfish appeared to recover, largely as a result of expansion of isolated populations which had escaped the initial outbreak into areas from which crayfish had disappeared.

The R. Darenth in Kent is a specific case of repopulation from isolated surviving populations. Discussed more fully elsewhere, this is the case of a population of white clawed crayfish apparently isolated from the original plague outbreak in the main river by a 100 metre culvert. The isolated population eventually expanded downstream and repopulated the main river until a surviving source of infection from elsewhere on the catchment resulted in a new mortality.

Deliberate repopulation of affected waters is also possible and the practicality of this has already been demonstrated in Britain in the upper reaches of the Tetbury branch of the Bristol Avon where reintroductions have resulted in the establishment of a population which is now well established (Frayling, pers. comm. 1992). This, together with the proposal to establish 'reserve' areas for *A. pallipes*, will mean that the total disappearance of the British native crayfish is unlikely (Holdich & Reeve, 1992). The large number of naturalised signal crayfish populations, some of which at least, are infected with *A. astaci*, will however mean that crayfish plague will continue to prevent the possibility that *A. pallipes* will permanently repopulate many of the areas that have been affected. Repeated outbreaks of plague occurring if surviving susceptible crayfish populations reach thresholds sufficient for acute outbreaks is the most likely prognosis.

7. DIAGNOSIS

7.1 Diagnostic Procedures

The text of this Section of this report is based on a draft prepared for the Chapter on crayfish plague diagnosis of the 2002 edition of the Office International des Epizooties (OIE) Diagnostic Manual of Aquatic Diseases, replacing the current edition published in 2000 (OIE, 2000). As such it describes the diagnostic methods approved by the OIE which is the world veterinary body with authority on matters of animal disease.

Diagnosis of crayfish plague strictly requires the isolation and characterisation of the pathogen, *A. astaci,* using simple mycological media fortified with antibiotics to control bacterial contamination (Alderman and Polglase, 1986). Isolation is only likely to be successful before or within 12 hours of the death of infected crayfish. However, there is no other disease or pollution effect that can cause such total mortality of crayfish while leaving all other animals in the same water unharmed, so that isolation of the pathogen is desirable but not essential, particularly in regions where further spread of infection is known to be a potential hazard. Clinical signs of crayfish plague include behavioural changes and a range of visible external lesions. The range of these lesions is so large that, except for the experienced eye, such clinical signs are of limited diagnostic value.

7.1.1 Standard screening methods for Crayfish Plague: Isolation of *A. astaci*

Isolation methods are as described by Alderman & Polglase (1986). An agar medium (isolation medium) is used that contains yeast extract and glucose in river water with antimicrobial agents (penicillin G and oxolinic acid) to prevent the growth of most bacteria and enable easy and rapid isolation of the pathogen.

Isolation medium (IM): 12.0 g agar; 1.0 g yeast extract; 5.0 g glucose; 10 mg oxolinic acid; 1000 ml river water; and 1.0 g penicillin G (sterile) added after autoclaving and cooling to 40°C. River water = any natural river or lake water as opposed to demineralised water.

Simple aseptic excision of infected tissues, which are then placed as small pieces (1–2mm^2) on the surface of isolation medium plates, will normally result in successful isolation of *A. astaci* from moribund or recently dead (<24 hours) animals. Depending on a range of factors, foci of infection in crayfish may be easily seen by the naked eye or may not be discernible despite careful examination. Such foci can best be seen under a low power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases a brown coloration of cuticle and muscle may occur and in others, hyphae are visible in infected cuticle in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and tail, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.

Provided that care is taken in excising infected tissues for isolation, contaminants need not present significant problems. Small pieces of cuticle and muscle may be transferred to a petri dish of sterile distilled water and there further cut into small pieces with sterile instruments for transfer to IM isolation medium. Suitable instruments for such work are cataract knives and fine electron microscope or instrument grade forceps and scissors.

Identification of *A. astaci*

On IM agar, growth of new isolates of *A. astaci* is almost entirely within the agar except at temperatures below 7°C, when some superficial growth occurs. Colonies are colourless. Dimensions and appearance of hyphae are much the same in crayfish tissue and in agar culture.

Vegetative hyphae are aseptate and (5)7–9(10) µm in width (i.e. normal range 7–9 µm, but observations have ranged between 5 and 10 µm). Young, actively growing hyphae are densely packed with coarsely granular cytoplasm with numerous highly refractile globules. Older hyphae are largely vacuolate with the cytoplasm largely restricted to the periphery leaving only thin strands of protoplasm bridging the large central vacuole. The oldest hyphae are apparently devoid of contents. Hyphae branch profusely, with vegetative branches often tending to be somewhat narrower than the main hyphae for the first 20–30 µm of growth.

When actively growing thalli or portions of thalli from broth or agar culture are transferred to distilled water, sporangia form readily in 20–30 hours at 16°C and 12–15 hours at 20°C. Thalli transferred from broth culture may be washed with sterile distilled water in a sterile stainless steel sieve, before transfer into fresh sterile distilled water for induction of sporulation. Thalli in agar should be transferred by cutting out a thin surface sliver of agar containing the fungus so that a minimum amount of nutrient containing agar is transferred. Always use a large volume of sterile distilled water relative to the amount of fungus being transferred (100:1). Sporangia are myceloid, terminal or intercalary, developing from undifferentiated vegetative hyphae. Sporangial form is variable: terminal sporangia are simple, developing from new extramatrical hyphae, while intercalary sporangia can be quite complex in form. Intercalary sporangia develop by the growth of a new lateral extramatrical branch, which forms the discharge tube of the sporangium. The cytoplasm of such developing discharge tubes is noticeably dense, and these branches are slightly wider (10–12 µm) than ordinary vegetative hyphae. Sporangia are delimited by a single basal septum in the case of terminal sporangia and by septa at either end of the sporangial segment in intercalary sporangia. Such septa are markedly thicker than the hyphal wall and have a high refractive index. Successive sections of vegetative hypha may develop into sporangia, and most of the vegetative thallus is capable of developing into sporangia.

Within developing sporangia the cytoplasm cleaves into a series of elongate units (10–25 x 8 µm) that are initially linked by strands of protoplasm. Although the ends of these cytoplasmic units become rounded, they remain elongate until and during discharge. Spore discharge is achlyoid, that is, the first spore stage is an aplanospore which encysts at the sporangial orifice and probably represents the suppressed saprolegniaceous primary zoospore. No evidence has been observed for the existence of a flagellated primary spore, thus, in this description, the terms 'sporangium' not 'zoosporangium' and 'primary spore' not 'primary zoospore' have been used. Discharge is fairly rapid (<5 minutes) and the individual primary spores (=cytoplasmic units) pass through the tip of the sporangium and accumulate around the sporangial orifice. The speed of cytoplasmic cleavage and discharge is temperature dependent. At release, each primary spore retains its elongate irregularly amoeboid shape briefly before encystment occurs.

Encystment is marked by a gradual rounding up followed by the development of a cyst wall, which is evidenced by a change in the refractive index of the cell. The duration from release to encystment is 2–5 minutes. Some spores may drift away from the spore mass at the sporangial tip and encyst separately. Formation of the primary cyst wall is rapid, and once encystment has taken place the spores remain together as a coherent group and adhere well to the sporangial tip so that marked physical disturbance is required to break up the spore mass.

Encysted primary spores are spherical, (8)9–11(15) µm in diameter, and are relatively few in number, (8)15–30(40) µm per sporangium in comparison with other *Aphanomyces* spp. Spores remain encysted for 8–12 hours. Optimum temperatures for sporangial formation and discharge are between 16 and 24°C, but the discharge of secondary zoospores from the primary cysts peaks at 20°C and does not occur at 24°C. In new isolates of *A. astaci*, it is normal for the majority of primary spore cysts to discharge as secondary zoospores, although this varies with staling in long-term laboratory culture. Sporangial formation and discharge occurs down to 4°C. *Aphanomyces astaci* does not survive at –5°C and below for more than 24h.

In many cases, some of the primary spores are not discharged from the sporangium and many sporangia do not discharge at all. Instead, the primary spores appear to encyst *in situ* within the sporangium, often develop a spherical rather than elongate form and certainly undergo the same changes in refractive index that mark the encystment of spores outside the sporangium. This within-sporangial encystment has been observed on crayfish. Spores encysted in this situation appear to be capable of germinating to produce further hyphal growth.

Release of secondary zoospores is papillate, the papilla developing shortly before discharge. The spore cytoplasm emerges slowly in an amoeboid fashion through a narrow pore at the tip of a papilla, rounds up and begins a gentle rocking motion as a flagellar extrusion begins and spore shape changes gradually from spherical to reniform. Flagellar attachment is lateral; zoospores are typical saprolegniaceous secondary zoospores measuring 8x12 µm. Active motility takes some 5–20 minutes to develop (dependent on temperature) and, at first, zoospores are slow and uncoordinated. At temperatures between 16 and 20°C, zoospores may continue to swim for at least 48hours.

7.1.2 Presumptive Diagnostic Methods for Crayfish Plague

The first sign of a crayfish plague mortality may the presence of numbers of crayfish at large during daylight (crayfish are normally nocturnal), some of which may show evident loss of co-ordination in their movements, and easily fall over on their backs and are unable to right themselves. Often, however, unless waters are carefully observed, the first recognition that there is a problem will be the presence of large numbers of dead crayfish in a river or lake.

In susceptible species where sufficient numbers of crayfish are present to allow infection to spread rapidly, particularly at summer water temperatures, infection will spread quickly and stretches of over 50km may loose all their crayfish in under 21 days from first observed mortality. Crayfish plague has unparalleled severity of effect, infected susceptible crayfish do not survive – 100% mortality is the norm. Resistant North American species survive infection in many cases and then act as largely asymptomatic carriers, although under adverse conditions (stress, concurrent infections), mortality may occur.

It must be emphasised, however, that presence of large numbers of dead crayfish, even in crayfish plague affected watersheds is not on its own sufficient. The general condition of other aquatic fauna must be assessed. Mortality or disappearance of other aquatic crustaceans as well as crayfish, even though fish survive, may indicate pollution (e.g. insecticides).

7.1.3 Confirmatory Diagnostic Methods for Crayfish Plague

Strictly, the identification of Oomycetes to genus depends on sporangial morphology and to species on the morphology of the sexual reproductive stages (oogonia and antheridia). Such sexual stages are absent in *A. astaci* so that identification is based on general morphology of isolates from crayfish involved in an outbreak of crayfish plague. Since no other crayfish disease produces such swift and drastic mortalities this normally presents no practical diagnostic problem.

Exposing susceptible crayfish (e.g. *A. leptodactylus* or *A. pallipes*) to zoospores produced by suspect isolates (see above) will result in characteristic rapid mortality and with subsequent re-isolation of the fungus, give firm confirmation of crayfish plague. However, susceptible crayfish species should only be used for confirmation of diagnosis in exceptional circumstances since some are endangered species (Berne Convention) and populations may be protected under

7.1.4 Molecular biological diagnostic methods for Crayfish Plague: status

As discussed in the next Section, *A. astaci* is an Oomycete, a member of the Oomycotina and not directly related to the true fungi, the Eumycota. Oomycetes are widely distributed in fresh waters and *Aphanomyces* spp. are common with more than 30 recognised species. There are a

number of reports of isolation of fungi other than *A. astaci* from crayfish. Some (e.g. Alderman and Polglase 1984) such as *Fusarium* are members of the Eumycota, whilst Dieguez-Uribeondo *et al.* (1995) reported isolation of *Aphanomyces* spp. other than *A. astaci* from crayfish, presumably as opportunist invaders of damaged crayfish tissues or simply as isolates growing from water contamination. *Saprolegnia* spp. rapidly establish in lesions on plague infected and moribund crayfish and Söderhäll *et al.* (1981) report the recovery of *S. parasitica* from crayfish during a crayfish plague mortality in *A. leptodactylus* in Turkey. In the author's experience no problem has been found in distinguishing between *A. astaci* and perthotrophs such as *Saprolegnia* spp.

Conventional isolation and morphology does differentiate well between *A. astaci* and other fungi, including other *Aphanomyces* spp., preferably backed up by confirmation of virulence in a challenge trial with susceptible crayfish, but does require significant experience and technical skill.

Immunological and molecular biological diagnostic techniques, require their own specific skills, but these are skills that are less specialist and may be applied to a wide range of organisms, they are not organism specific. In the absence of specific mycological and pathological skills and experience therefore, these more general methods offer major advantages for diagnosis of *A. astaci.*

Although valuable developments in use of molecular techniques to identify *A. astaci* had been made over a number of years, first by Söderhäll's group in Uppsala (Huang *et al.*, 1994) and more recently by Oidtmann in Munich (1999, 2002), none of the methods so methods had been adequately validated for cross reaction with other species of *Aphanomyces*. The problem of lack of validation of these approaches is now being addressed. Oidtmann *et al* (2002) have recently published the results of a study in which they attempted to characterise the DNA of *A. astaci* using primers developed to amplify a 1050 bp segment of the 28 S rDNA region. This was repeated with a number of other fungi, most of which are either known parasites of freshwater crayfish cuticle or can be found in their natural environment. Several species of Aphanomyces were included in the study. Then the amplicons obtained were digested with the restriction enzymes AluI, HindIII and AvaI. With this method *A. astaci* DNA was distinguishable from the DNA of other fungal species tested. Unfortunately as indicated above, there are well over 30 recognised species of *Aphanomyces* in the scientific literature and only about six of these are available from culture collections. This therefore does create some problems for validation and in particular the absence of *A. invadens* from Oidtmann et al. (2000)'s validation set in regrettable. *A. invadens*, is the causative agent of Epizootic Ulcerative Necrosis, a disease that has devastated production of farmed tropical fresh water fish in the Asia Pacific region. The pathogen is also associated with serious mortalities of estuarine species in the warmer waters of the E coast of the USA and has been shown to be capable of infecting Rainbow trout. Any diagnostic method must be capable of distinguishing between *A. astaci* and *A. invadens* and between these and opportunist perthotrophs including other *Aphanomyces* spp. The addition of *A. invadens* to the validation set would not be difficult and, if *A. astaci* and *A. invadens* prove to be distinguishable by the method (or a further development thereof), the limited range of *Aphanomyces* spp. tested should not present a problem in the use of the method.

To summarise the current status of molecular methods for diagnosis of *A. astaci* infections:

1. There are four key papers on the use of molecular techniques to detect and discriminate between the causative agent of crayfish plague, *A. astaci* and the wide range of other fungi that have been reported from crayfish. Huang *et al.* (1994), Dieguez-Uribeondo *et al.* (1995), Oidtmann *et al.* (1999) and Oidtmann *et al.* (2002).
2. The papers of 1994, 1995 and 1999 describe the use a randomly amplified polymorphic DNA (RAPD) to distinguish between a large number of isolates from diseased crayfish and the assignment of four *A. astaci* genogroups (named after the host from which they are isolated

or associated). *Astacus* strain (group A), *Pacifastacus* strain I (group B), *Pacifastacus* strain II (group C) and *Procambarus* strain (group D).

3. Most recently, Oidtmann *et al.* (2002) have exploited the sequence differences between the *A. astaci* isolates, other *Aphanomyces* and non-related fungi at the 28s rRNA gene level to develop a PCR-based to detect the crayfish plague fungus.
   The PCR primers are based on *A. astaci* sequence generated by Oidtmann *et al.* and were shown to be specific for the Oomycetes, generating the appropriately sized amplification products for both the *Aphanomyces* species and *Saprolegnia* species tested, with a sensitivity of 1fg (184 genomic equivalents). Products derived from *A. astaci* were differentiated on the basis of the absence of an AluI endonuclease restriction site.
   The work of Oidtmann is preliminary and as the author admits in the discussion further validation is required before this technique can be usefully applied to clinical samples..
   The areas that need attention are 1) The specificity and sensitivity of the primer set in the presence of crayfish tissue 2) and the ability to differentiate *A. astaci* in the presence of other Oomycetes. Presence of an undigested PCR product together with the expected digestion products of *A. laevis* or *A. helicoides* could indicate either a partially digested of the *A. laevis/A. helicoides* amplicon or the presence of *A. astaci* in a mixed infection.
   From the paper it is also not clear if the primers (P1 and P2) have been evaluated against representatives from each of the four *A. astaci* genogroups identified by Huang *et al.* (1994),and Dieguez-Uribeondo *et al.* (1995).
4. Nonetheless, the putative 28S rRNA gene sequence data has been submitted to Genbank and it should be possible with limited resources to produce a modified protocol suitable for routine screening for low level infection of *A. astaci* in crayfish. Initially, the sensitivity of the assay could be significantly improved by increasing the number amplification cycles from 30 to 40, and the specificity could be enhanced by increasing the annealing temperature slightly. Using the sequence data it should also be possible to design a second primer set for use in a second round or nested PCR assay. The sensitivity of the assay can also be increased by targeting the *A. astaci* RNA that is more abundant rather than the genomic DNA.

These results therefore represent a major development towards a practical molecular biological diagnostic method for *A. astaci,* but have not yet reached the stage at which they can be applied to clinical samples.

8. TAXONOMY, MORPHOLOGY AND PHYSIOLOGY OF *A. ASTACI*

8.1 Saprolegniaceae

Taxonomy has long been a problem in the Saprolegniaceae. This family, prominent in the Oomycotina and consisting of some 12- 15 genera and 125-150 species, was first studied seriously in the 19th century. Today the Oomycotina and thus the Saprolegniaceae, are considered better placed in either the Kingdom Protoctista or the Kingdom Chromista and, regardless of which, are not considered to be closely related to the "true fungi", the Eumycota. They are perhaps more correctly to be regarded as pseudo fungi. This has considerable relevance to any work carried out with potential fungicides. Agricultural fungicides are designed to have effects on biochemical pathways of relevant Eumycota. The taxonomic gulf between Eumycota and Oomycotina is such that few shared pathways exist.

The Saprolegniaceae (water moulds) are a family of filamentous, coenocytic organisms living in fresh water habitats or in wet soils. Nutritionally they live as saprobes, perthotrophs, or parasites or (in some cases) as all three, depending on circumstances. A number of species that one might nominally consider as saprobes can live as perthotrophs given suitable dead cells or tissues on the bodies of living, prospective hosts. There is little question that under the right conditions some of these perthotrophs invade the living tissues of the host and thus become true parasites. Some are important and effective pathogens (e.g. *Aphanomyces astaci* and *Aphanomyces invadens*) of aquatic animals and others are plant pathogens. The taxonomically important features of the developmental cycle are described below and illustrated in Figure 20. An understanding of this much of the morphology and life cycle of the Saprolegniaceae is essential to understanding the limitations of methods of identification of members of the Saprolegniaceae and therefore of the constraints under which identifications including those in this report are made.

Asexual reproduction in the Saprolegniales is accomplished by means of unicellular, biflagellate zoospores (planonts) produced in (usually) terminal sporangia separated from the hyphal filaments by basal septa. Zoospores are usually both diplanetic and dimorphic, i.e., there are two different swimming stages involving two different kinds of zoospores: pyriform primary zoospores and reniform secondary zoospores. Each type of zoospore not only has a different shape but also a different point of flagellar insertion. Furthermore, either or both of the two swimming stages may be suppressed to varying degrees in some genera in the family. Both zoospore types encyst, in some cases immediately, in others after swimming for varying periods of time. Primary zoospore cysts germinate to release secondary zoospores. Secondary zoospore cysts may bear hooked hairs on their surfaces. However few genera in the family have been examined by transmission electron microscopy to ascertain the nature of the surface of their secondary zoospore cysts.

Sexual reproduction is by means of gametangial contact which leads to fusion of haploid oospheres (eggs) produced in lateral or terminal female gametangia (oogonia) with sperm nuclei carried to the oogonia by antheridial branches and to the oospheres by fertilisation tubes. Both gametangial types (oogonia and antheridia) are separated from hyphal filaments by septa. The zygotes may undergo a resting period but in time they usually germinate to produce hyphal filaments that soon form terminal zoosporangia and zoospores.

8.1.1 Identification to genus

Taxonomically, genera in the Saprolegniaceae are delimited (and therefore identified) by the morphological and developmental features of the zoosporangia and the manner of zoospore

formation and escape (or failure to escape) from the zoosporangia. Despite its inadequacies this persists as a primary character for separating genera.

Thus in *Saprolegnia* spp., zoospore discharge results in the release of motile primary zoospores from a zoosporangium which is wider than vegetative hyphae. In *Achlya* spp the primary zoospore is suppressed and discharge aplanonts encyst at the mouth of the sporangium, subsequently swimming as secondary zoospores. In *Aphanomyces*, discharge is Achlyoid, but the sporangia are no wider than the vegetative hyphae.

8.1.2 Identification at species level

Traditional morphology also gives the criteria for delimiting species within the group. These are based largely on the morphology and development of the oogonia and their contents. Such features as oogonial size, presence and types of ornamentations on oogonial walls, presence of pits (thin spots) in oogonial walls, number and size of oospores in oogonia, or visual differences in stored lipid-like globules are all important in this regard. The origins of antheridia are also of some importance here; whether they arise from the same hypha as the oogonia or from distant hyphae is an important character. Various of these taxonomically important features and some of their relationships to one another are illustrated in Figure 20 which shows the general zoosporangial morphology and stages in zoospore release for six characteristic genera in the Saprolegniaceae. The six genera shown also serve to illustrate the complete range of zoospore release patterns found in the family (see legend, Figure 20). Included in the group are the four genera most commonly found on fishes: *Saprolegnia*, *Achlya*, *Aphanomyces*, and *Dictyuchus*.

Therefore, to identify a member of the Saprolegniaceae to the generic level, asexual reproductive structures are required, followed, for speciation, the sexual stages. Zoosporangia are formed readily in nature and may easily be induced. In contrast sexual stages require several weeks to form and are often suppressed, particularly in those members of the Family, which are animal parasites such as *A. astaci*, *A. invadens* and *S. parasitica*.

8.2 *Aphanomyces astaci* morphology

Much of the detail that is required for the identification of *A. astaci* has already been presented in the preceding section on diagnosis of crayfish plague. It remains only to emphasise that apart from the earliest description by Rennerfelt (1936) and Schäperclaus (1935), no-one has reported a sexual stage for *A. astaci.* The latter reported from crayfish tissue that was unlikely to be unifungal. It is the author's opinion that Rennerfelt's description was also erroneous and that *A. astaci* is entirely asexual.

The other aquatic animal pathogen in the genus *Aphanomyces, A. invadens* is the aetiologic agent of Epizootic Ulcerative Necrosis (EUS) a highly invasive pathogen of freshwater fish in Australasia and SE Asia. No sexual stages are known for this pathogen either.

Further support for the absence of sexual stages in the *A. astaci* life cycle comes from the results of Huang et al. (1994) who investigating a wide range of strains of *A. astaci* by RAPD PCR. The fact that the Turkish isolate of *A. astaci* was clearly closely related to old Swedish strains led to the view that this represented a very old introduction of *A. astaci*, probably direct descendants of the original introduction. They noted that the degree of genetic variation was lower than in other fungi and suggested that the virtually unchanged genotype over considerable temporal and geographical distance supported the absence of a sexual cycle.

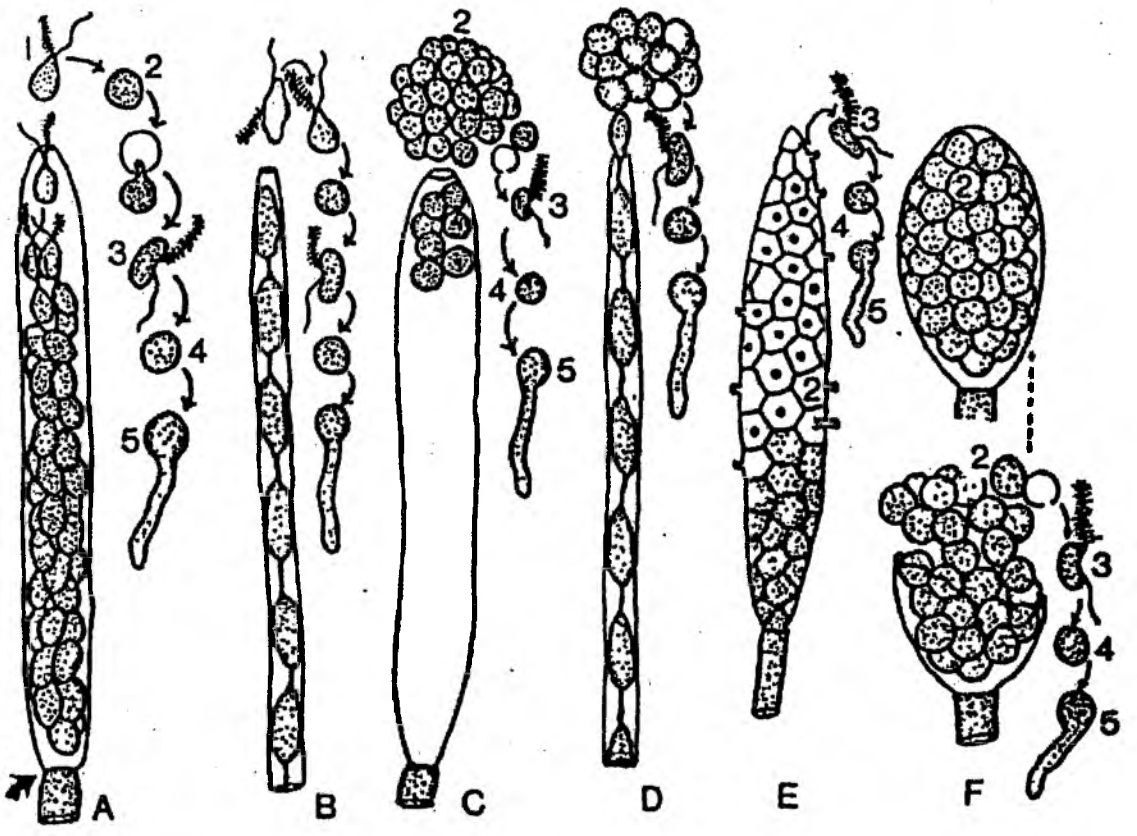

Figure 20 Sporangial types and zoospore escape patterns in six genera of the Saprolegniaceae.

A) *Saprolegnia*, characterised by clavate zoosporangia and diplanetic zoospores; primary zoospores (A1) swim away from the sporangium on release, B) *Leptolegnia* has narrow, filamentous zoosporangia but a *Saprolegnia*-like diplanetism, C) *Achlya*, zoosporangial shape resembles *Saprolegnia* but primary zoospores encyst (C2) immediately on exiting the sporangial tip, D) *Aphanomyces* has narrow filamentous, sporangia (as in Leptolegnia) but a zoospore escape pattern like Achlya, E) Dictyuchus possesses clavate sporangia, as in Saprolegnia, but primary. zoospores encyst within the zoosporangium. and do not escape, instead forming a net of cells within the sporangium and germinating to release secondary zoospores (E3) singly through papillae in the sporangial walls, F) *Thraustotheca*, zoosporangia disintegrate to release encysted primary zoospores (F2) which then germinate to release typical reniform secondary zoospores. A1) primary zoospore, A2) primary zoospore cyst, A3) secondary zoospore, A4) secondary zoospore cyst, AS) germinating secondary cyst. A, C, D, & E, are the genera most commonly implicated in saprolegniasis of fish (After Hughes 1994).

8.3 *A. astaci* physiology

Comments here relate mainly to the temperature range at which *A. astaci* will grow, sporulate and survive. These factors relate strongly to the pathogenicity of *A. astaci* and to the pattern of clinical disease that results (see Pathogenicity, below). The temperature / growth curve for mycelial growth of a typical British isolate of *A. astaci* is shown in Figure 21. Sporulation temperatures are more restricted, in this isolate peaking at 22°C and ceasing at 25°C. Dieguez-Urebeondo, J. et al., (1995). reported that isolates from *Procambarus clarkii* in Spain showed a higher thermotolerance than N. European isolates. This may represent selection for Spanish fresh water temperatures or could mean that some Spanish *A. astaci* isolates derive from introduced Louisiana crayfish and represent a warmer adapted N. American *A. astaci* strain.

Ability of stages of the life cycle of *A. astaci* to survive freezing and cooking temperatures are of obvious interest to those countries that remain free of crayfish plague such as Australia and a brief description is presented here of some studies carried out on behalf of AQIS (now Biosecurity Australia) to determine whether *A. astaci* mycelium or spores or *A. astaci* in infected crayfish would remain viable after cooking or freezing.

The effects of temperature on viability were tested using a modification of the methods employed for testing candidate fungicides (Alderman 1982). The method used for testing of fungicidal compounds is shown diagrammatically in Figure 22, and was modified from Alderman (1982) in that, instead of exposure to different fungicidal compounds, the mycelial discs of *A. astaci* isolate FDL 457 and supporting membrane filters were exposed to different temperatures for varying time periods (from 5 minutes to 14 days exposure to test temperatures).

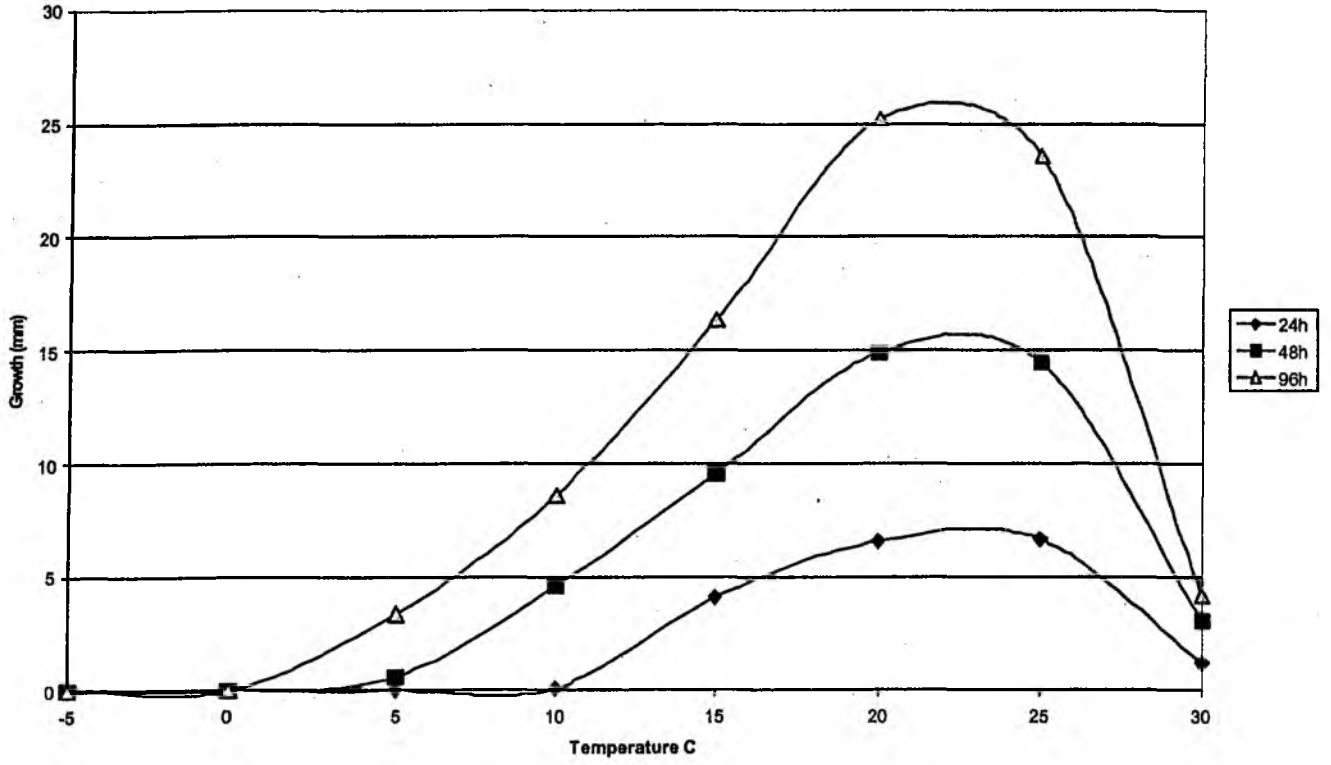

Figure 21 Temperature / growth curve for typical British isolate of *A. astaci*

Standard 90mm plastic petri dishes containing RGY agar were inoculated by placing inverted 4mm diameter plugs of actively growing culture of *A. astaci* at their centres. These plates were incubated at 15°C until the new colony had a diameter of between 40 and 60mm. At this time, using a sterile punch the whole growing surface of the culture plate was "converted" to fresh plugs of inoculum.

Polycarbonate membrane filters, Nuclepore and Whatman were employed. These polycarbonate membrane filters are strong and have considerable wet strength with extremely low retention, unlike paper, precipitated or woven filter media. They autoclave well and, being packed with interleaving papers, could easily be picked up with fine forceps. Batches of 50 to 100 (as appropriate) were placed in glass petri dishes and packed in sterilisation bags and autoclaved at 121°C for 15 minutes.

When needed, packs of sterile membrane filters were opened and, using aseptic techniques, the filters were transferred to the surface of new plates of RGY agar. Up to seven 25mm filters could be accommodated on the surface of a standard 90mm petri dish. Newly cut *A. astaci* agar

plugs were inverted (colony surface down) at the centre of each membrane filter. Plates were then incubated until the *A. astaci* colonises the surface of the membranes. Some manipulation of incubation temperature was used to ensure that the time taken to cover the filter surface was appropriate to the time need to start the temperature exposure.

When the membrane supported colonies were 25mm in diameter, a pair of hot flamed curve tipped forceps were used to excise the original inoculum plug from the centre of the colony to leave a largely naked fungal colony on the membrane filter surface.

Petri dishes containing 10ml of sterile demineralised water were prepared in advance and placed in lab freezer room / freezer / refrigerator / cooled incubator / incubator / oven as appropriate to the test temperature concerned. These were given time to adapt to the test temperature (time predetermined by tests with a logging thermometer system).

When ready the membrane filter supported *A. astaci* colonies were transferred aseptically to the petri dishes and left for the appropriate length of time.

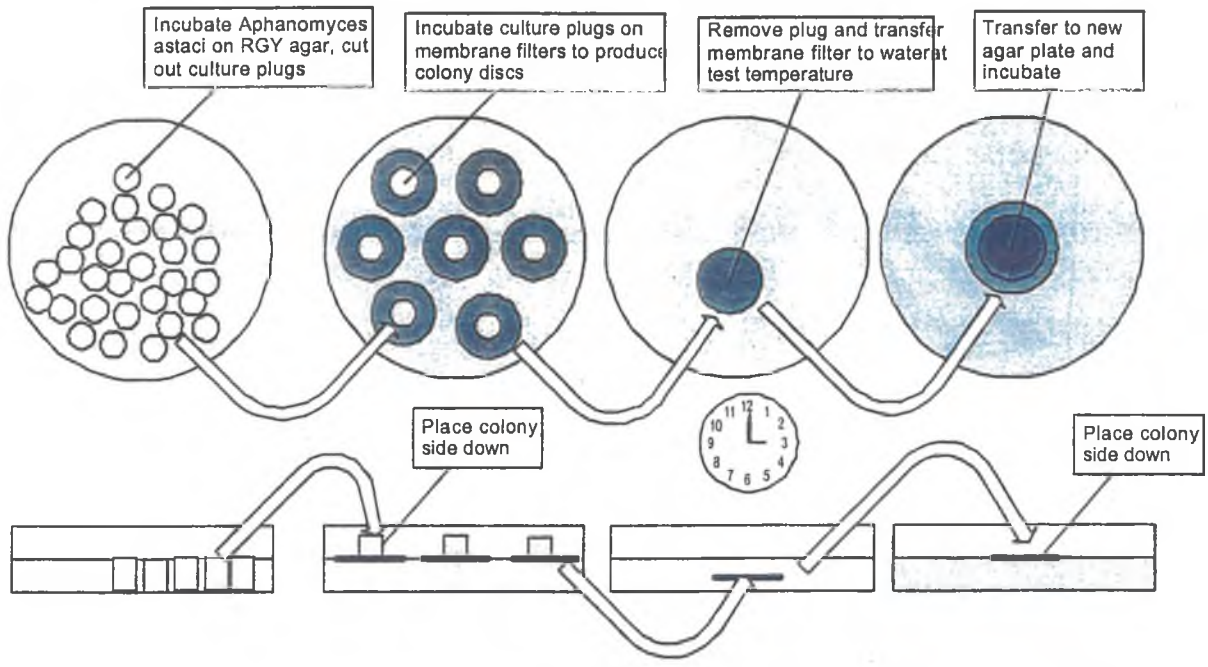

Figure 22 Diagrammatic representation of filter disc procedure

At the predetermined intervals, dishes were removed from the test temperature and the membrane filter supported colonies were transferred to new RGY plates by inverting them colony down on the agar surface.

These plates were be incubated at 15°C for 24 h. Any new growth from the edge of the colony were measured as increase in colony diameter at 2 points at 90 intervals, using electronic digital calipers to the nearest 0.5mm. After a further 24h incubation, growth was measured again. The intention was to determine both any inhibition of growth and any delay in recommencement of growth.

At least 5 x 4 colonies were tested (diameters at 90°) for each time / temperature combination, giving 160 growth data points for each time temperature combination. Similar colonies passed through the same physical movements, but incubated (exposed to) at a temperature of 15°C were provided for each time / temperature combination.

A. astaci survived well at those intermediate temperatures which are at or near its normal growth range (0 to 10°C). At 24 hours after the return of the fungal colony to its normal culture temperature of 15°C, no new growth had occurred from colonies exposed to temperatures of -

20°C and -15°C for more than 10 minutes. At -10°C, exposure for more than 20 minutes prevented further growth at 24 hours as did exposure to -5°C for than 24 hours. At the opposite end of the exposure temperature scale, no new growth had developed in 24 hours from colonies exposed for 5 minutes or more to +60°C or +70°C.

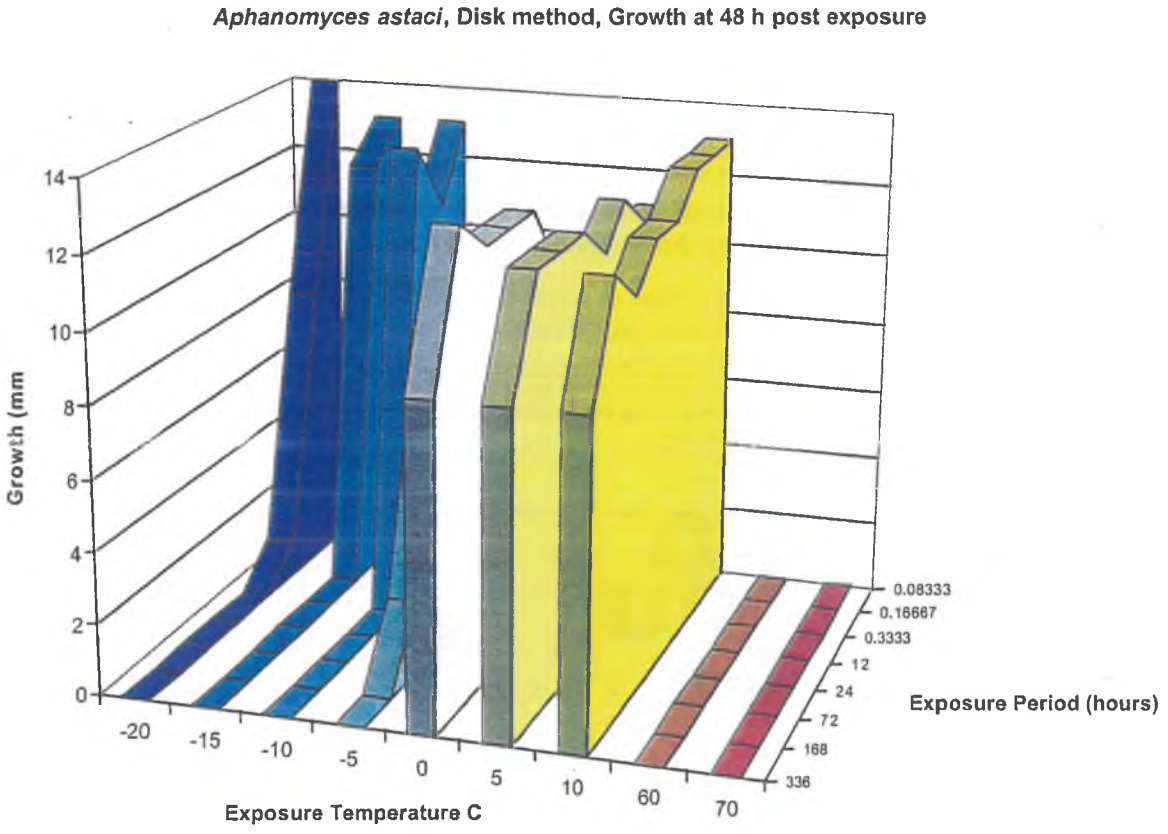

Figure 23 *A. astaci* Growth after exposure to high or low temperatures

That *A. astaci* is able to recover from limited exposure to "abnormal temperatures" was demonstrated in colonies at 48 hours after return to normal 15°C culture temperature. Some limited survival was seen at 20 minutes exposure to –20°C and at –10°C, whilst at –5°C, growth was possible after 3 days exposure.

The culture plates from the –20, -15 and –10°C 12 hour exposures were maintained for a further 4 days at 15°C (a total of 6 days post exposure to those temperatures) and no new growth of *A. astaci* was recorded. At +60°C and +70°C no new growth occurred from colonies exposed to those temperatures for as little as 5 minutes.

When the effects of exposure of zoospores to different temperatures was investigated, the results obtained were superficially fairly straightforward. When numbers of colonies were counted after 5-8 days incubation at 15°C (Figure 24), the numbers of colonies increased with length of exposure time to temperatures between 0°C and 15°C. A marked increase in numbers of new colonies occurred with temperature exposure times in excess of 24 (15°C) to 72 hrs (0°C). Below 0°C, exposures of up to 12h at –5°C and –10°C did not result in any reduction in the number of colonies produced on return to incubation at 15°C, but very few propagules survived 24h exposure to these temperatures. None survived to germinate when exposed for 72h. At –15°C and –20°C a few spores survived to germinate when exposed for 5 or 10 minutes, none survived 20 minutes at these temperatures.

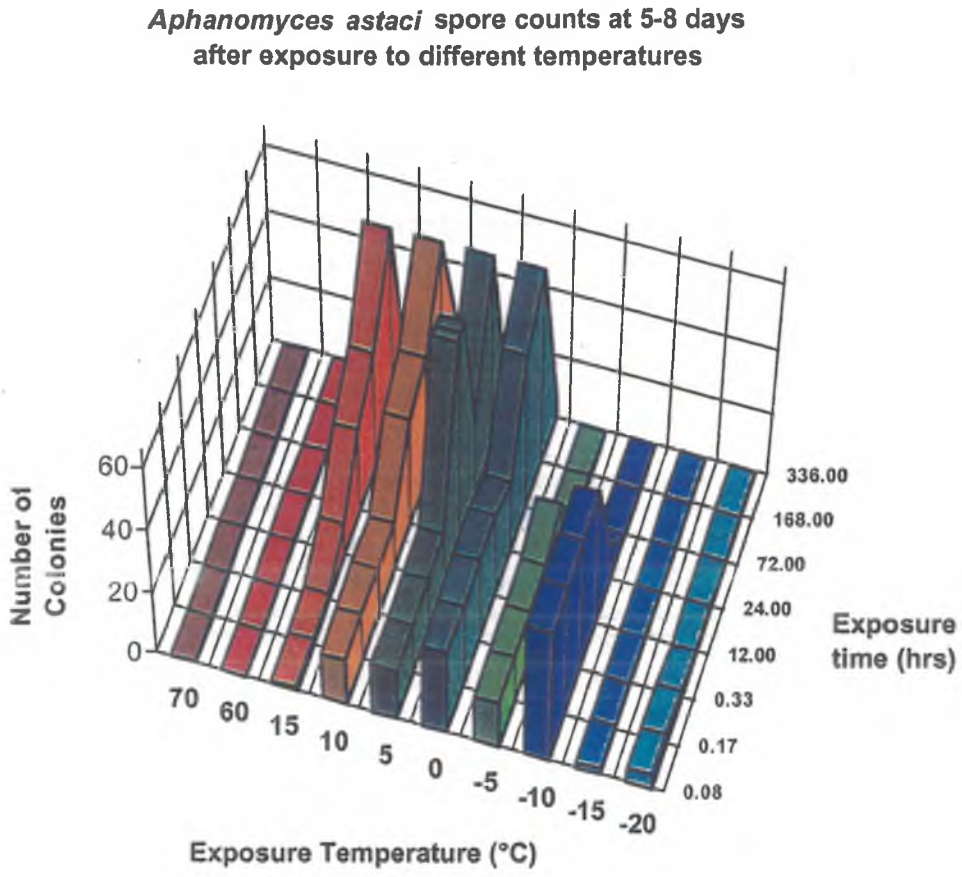

Figure 24 *A. astaci* effect of temperature on spore viability

These results indicate that propagules of *A. astaci* have only limited ability to survive for periods of exposure to temperatures below 0°C for more than 24h and below –10°C for less than 20 minutes. At and above 60°C, no propagules survive even for 5 minutes. Therefore normal freezing or cooking procedures will ensure that no viable propagules will be present.

This simple interpretation of the results of this study in relation to commercial transfer hazards disguises a number of other scientifically interesting and difficult to interpret aspects of the results.

Oomycete zoospores react to fairly small physical shocks by encysting. At 15°C the spores will have received two such shocks in quick succession (5 to 20 minutes interval) when they were pipetted into the 6 well dishes and then out and onto the incubation plates. At a temperature at which the spores are active, the resulting poor survival should not be regarded as unexpected. When the transfer shocks were separated by 12h or more survival improved.

Although some suggestions can be made, the very interesting increase in numbers of surviving propagules which occurs after exposures of between 12 h and 72h at temperatures between 15°C and 0°C is much more difficult to explain. The result would seem to be linked to metabolic or physiological factors since the effect is delayed as exposure temperature is lower.

The short (5 to 10 minutes) period of survival at –15°C and –20°C reflects the difficulties of cooling the spore suspension down rapidly and it is unlikely that these results represent true temperature exposures for the full time. The survival of spores for up to 24h at –5°C and –10°C

is however a little unexpected. Oomycetes are known to contain high molecular weight sugars in vacuoles in the cytoplasm. These have been suggested to offer possible limited cryoprotection to the cells for at least short periods. Certainly, *A. astaci* must normally be able to survive low water temperatures for periods in crayfish in Norway and in Sweden, but these temperatures are lower than would be expected in nature beneath ice.

Most interestingly the length of exposure at which low temperatures (–5°C and –10°C) result in a sudden fall in survival of propagules is very much the same at that at which there is an increase in survival at 0°C and above. This gives added confidence in the results obtained both above and

The results suggest that some delayed germination or growth effect is occurring, the length of the lag period for which is affected by the temperature of exposure, increasing with reduction in temperature to 0°C. Below 0°C instead of increased numbers of colonies forming, all propagules are killed.

Published and unpublished observations on *A. astaci* indicate that under normal temperature conditions, germination will take place fairly rapidly (24h) and visible germlings can be observed on the agar surface in this time at 15°C. These observations refer to spores deposited directly onto RGY agar and incubated immediately at 15°C. In the present study, spores were held in distilled water at the test temperature. In both cases the spores were produced from mycelial culture that had been washed several times in sterile distilled water before incubating in distilled water to induce zoosporulation. Availability of nutrients in the spore containing water was therefore low.

Encysted spores of Oomycetes can germinate under low nutrient conditions to produce germlings or they may excyst directly to produce another motile zoospore. Such germlings are short lengths of narrow vegetative mycelium which, as reported, can then produce a small spherical terminal cyst structure capable of releasing a single zoospore. This has been termed "repeated emergence" by Willoughby. Whilst there is no specific report of repeated emergence occurring in *A. astaci,* germlings are produced in distilled water and these may become septate. The possibility exists that such septate germlings may be fragmented during transfer and thus act as more than one propagule giving a possible explanation for the increase in colonies noted after 24 to 72h exposure at 15°C down to 0°C.

This interpretation leads to the suggestion that the colonies appearing after short exposures represent largely zoospores, which have not encysted and have remained motile and able to germinate rapidly. Those growing after the longer exposures could thus represent encysted spores and germlings. Few zoospores (or encysted spores or germlings) were able to survive exposures to –5°C or –10°C for 12h and none for exposures of 24h

9. PATHOGENICITY

Until the studies of Alderman *et al.* (1987) information on the severity of the effects of crayfish plague on susceptible crayfish populations relied almost exclusively on information collected in the field or in commercial crayfish stock-holding facilities, for example, Schikora (1906) and Schäperclaus (1928) and such field studies were hampered by the very varied nature of gross signs of the disease. It was established early (Schikora 1922) that *Aphanomyces astaci,* is highly pathogenic to native European crayfish species, whilst American species are resistant. Schikora (1906), using a small group of infected animals as an initial challenge, successfully transmitted the infection from one crayfish to another 15 times over a 4-month period. He was, however, unable to control his experimental conditions. Having cultured *A. astaci,* Nybelin (1936) was able to use zoospores to initiate infection but limited his experiments to two or three animals at a time. Unestam, in his investigations into crayfish plague, carried out a number of laboratory infection experiments with different crayfish species (Unestam 1969, 1972; Unestam & Weiss 1970). In particular, Unestam & Weiss (1970) compared the susceptibility of *Astacus astacus* and *Pacifastacus leniusculus* to different concentrations of zoospores and estimated the LD_{50} for *A. astacus* to be 3 zoospores/ml, slightly higher than the lowest concentration of 2.5 zoospores/ml tested. In that experiment, three of seven crayfish died with a mean time to death of 14 days at 16.5°C but, in a footnote, the authors indicated that the final mortality in the experiment was 100%, with one animal dying at 28 days and three at 36 days post-challenge, all showing slight signs of fungal infection. These authors therefore did not establish an LD_{50}, the lowest dose that they employed was higher than the LD_{100}.

The only investigators to develop a laboratory challenge model for crayfish plague and then use it to investigate the disease under controlled conditions were Alderman et al. (1987) who studied a range of factors affecting the pathogenicity of A. *astaci* under controlled laboratory conditions and compared their results with results obtained from natural field challenges. Their studies provided experimental explanation for many of the observed patterns in natural outbreaks crayfish plague which had caused confusion in investigators e.g. "Krankheitstbild mit so unglaublich variablem Symptomenkomplex zu Stande kommt, wie es keiner andern bekannten Epizootie der Fall ist" (Schikora, 1922) (a clinical picture with such an unbelievably variable symptom complex unlike anything produced for any other known epizootic disease).

The authors' field challenges were carried out at a time when a major natural epizootic was taking place in the River Avon (August to November 1983). Healthy stocks of *Austropotamobius pallipes* obtained from a then plague free river were placed in a plastic-coated metal-mesh cage (0 75 x 0.75 x 0.75 m) moored on the river bottom. In the laboratory infection trials, experimental animals included both *A. pallipes* and *Astacus leptodactylus.* The latter were then readily available being imported from Turkey for restaurant sale via the Rungis in Paris to Billingsgate fish market in London.

Zoospores for experimental challenge were produced from mycelium transferred from broth cultures to sterile river water which was then used as an experimental challenge to crayfish, either at full concentration or at selected dilutions. Numbers of zoospores were determined by counting germlings from plated aliquots.

Zoospores were added to tanks of crayfish, temperature controlled water was re supplied 12 - 14h after the introduction of the zoospore challenge. Dead and dying animals were removed for detailed examination and randomly selected animals used to re-isolate *A. astaci* to confirm its presence in the tissues of the dying animals. All experiments were accompanied by appropriate controls.

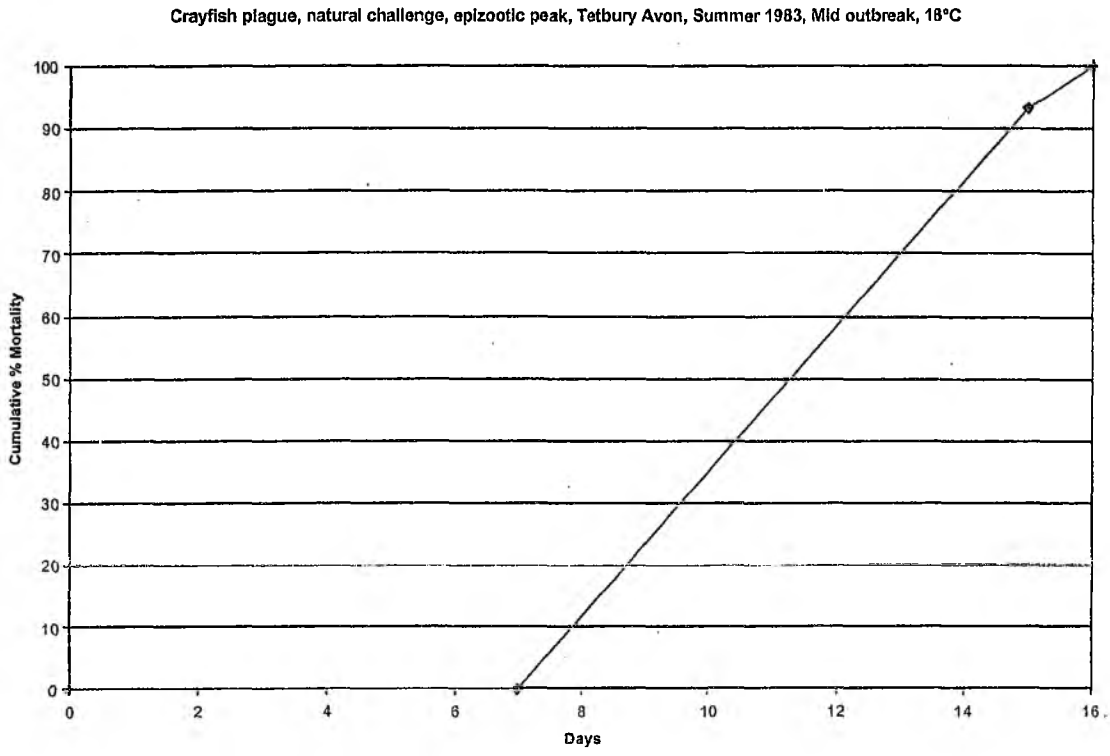

Figure 25 Crayfish plague, natural challenge, epizootic peak, Tetbury Avon, Summer 1983, Mid outbreak, 18°C

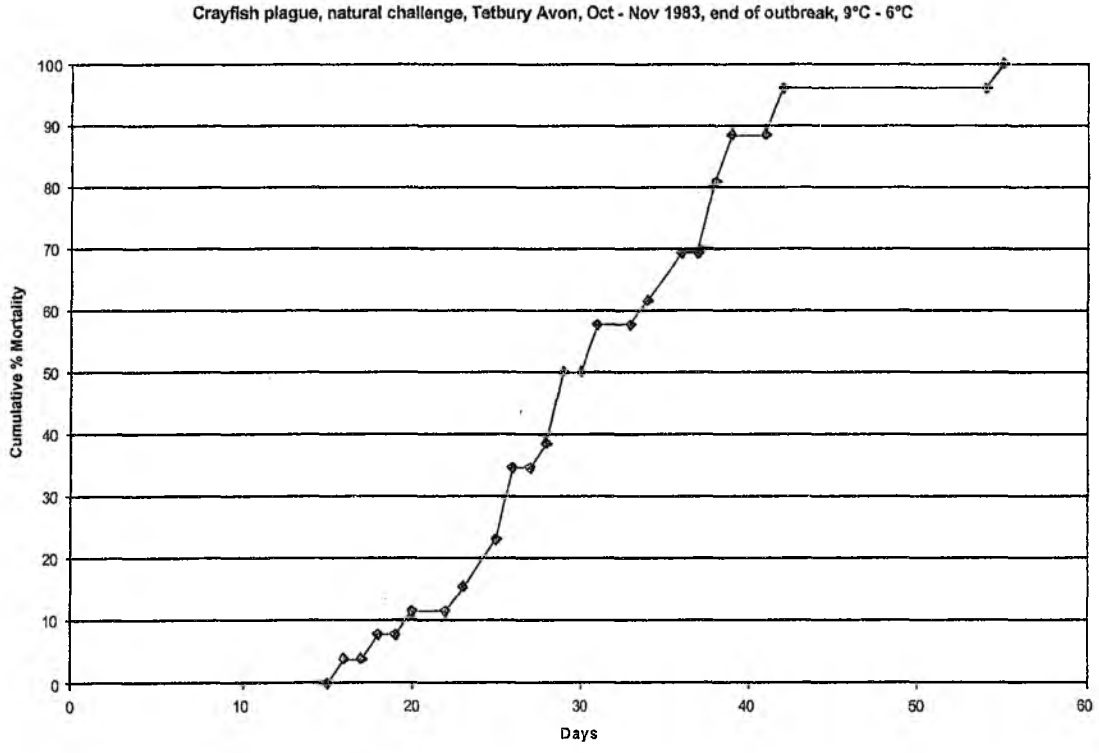

Figure 26 Crayfish plague, natural challenge Oct - Nov 1983, by the end of the outbreak, water temperatures had fallen from 9°C to 6°C

The *A. pallipes* exposed to the natural challenge in August 1983 at the height of a natural crayfish plague mortality in the river (when many crayfish were dying in surrounding waters), were rapidly infected. During the first 7 days no behavioural abnormalities were noted. At the end of an unavoidable 8-day lapse in observations, all but one animal was found to be dead (Figure 25). Examination of these animals showed the simpler gross signs of crayfish plague, including localised-to-extensive patches of muscle necrosis and a visible surface patterning of soft exoskeletal areas due to the presence of extra-matrical fungal hyphae. The surviving animals showed both gross and behavioural signs of crayfish plague and died within 24 h. The water temperature was then 20°C.

A further experiment was conducted at the same site, commencing in late September 1983. At this time, no live crayfish could be found surviving in the immediate vicinity of the experimental site which was only 4 km from the source of the stream. Water temperature was 12°C at this time and maintained 9°C for most of October, finally falling to 6°C by mid-November. The resultant mortality rate was much lower (Figure 26), with behavioural abnormalities occurring for up to 3 days prior to death with the last crayfish surviving for 55 days before succumbing to the infection. This prolonged, low mortality rate was accompanied by an increased range of gross signs visible on the infected animal, including limb autotomy, extensive melanisation plus blackening due to secondary bacterial infection.

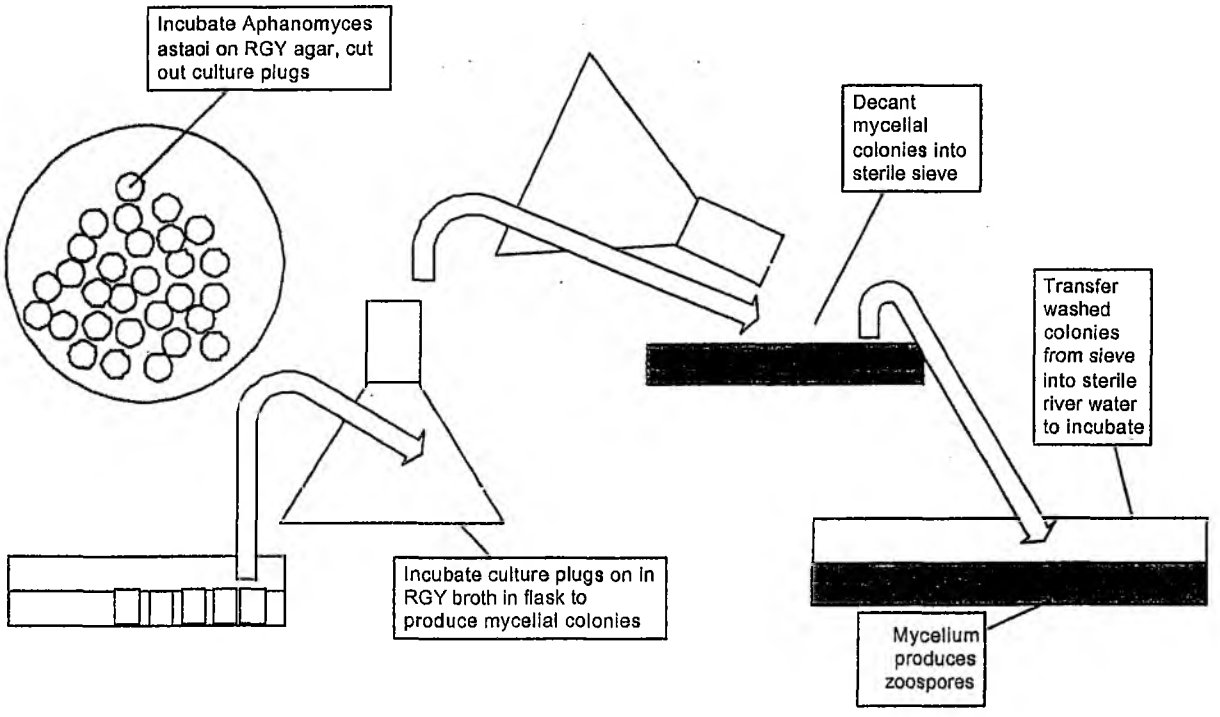

Figure 27 Procedure for production of zoospores of *Aphanomyces astaci*

To produce zoospores for laboratory challenges standard 90mm plastic petri dishes containing RGY agar were inoculated by placing inverted 4mm diameter plugs of actively growing culture of *A. astaci* at their centres. These plates were then incubated at 15°C until the new colony had a diameter of between 40 and 60mm. At this time, using a sterile punch the whole growing surface of the culture plate was "converted" to fresh plugs of inoculum. Such plugs were seeded into 250ml of RGY broth in 1 litre conical flasks (10 plugs per flask) and incubated at 15°C for 5 to 7 days (Figure 27).

The mycelial colonies so produced were then harvested by decanting the flasks into sterile stainless steel sieves through which 500 ml of sterile river water was then flushed to wash away remaining nutrients. The washed mycelium was then dispensed using aseptic techniques into petri dishes of sterile river water and left overnight to sporulate. Zoospore containing water was then added to the challenge tanks, either at full strength or diluted. Aliquots (1ml) of the challenge dose were sampled, plated on RGY agar and incubated for 48h at 15°C. Resultant colonies were then counted to determine challenge dose used.

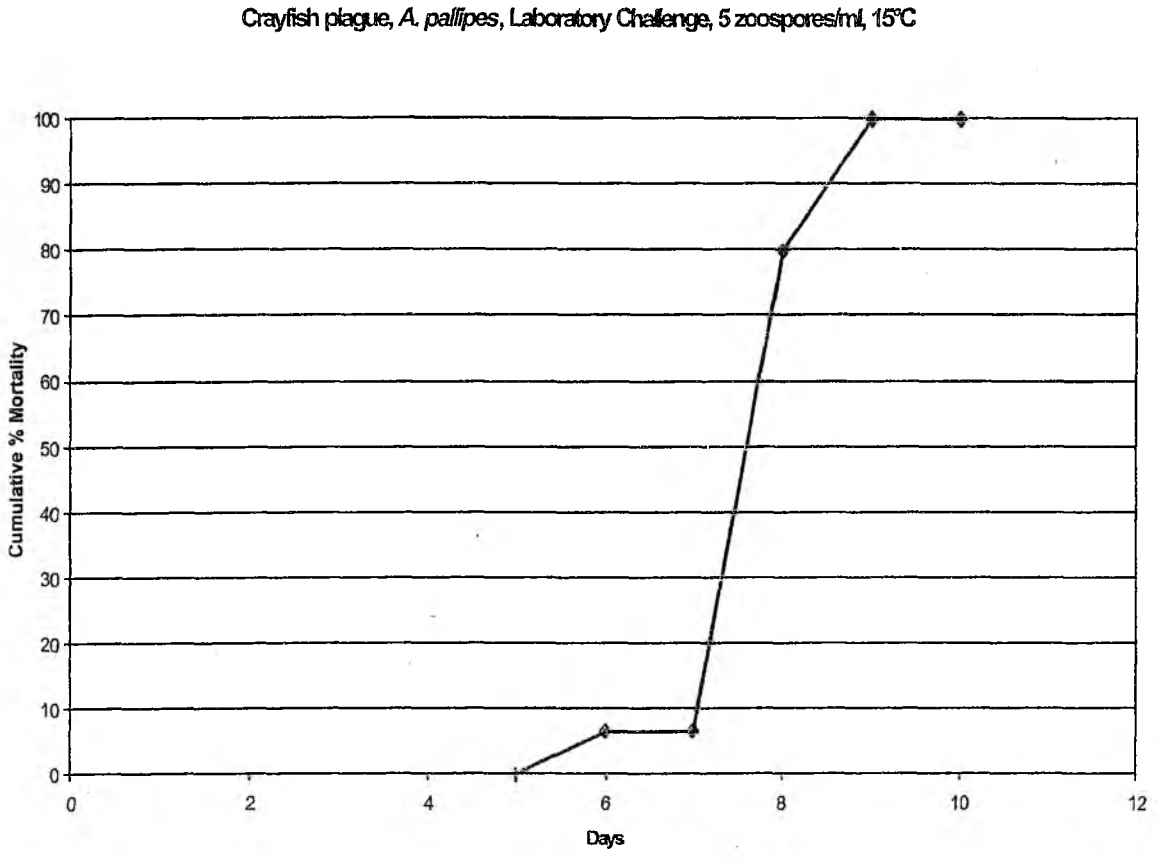

Figure 28 Crayfish plague, *A. pallipes*, Laboratory Challenge, 5 zoospores/ml, 15°C.

The majority of the laboratory challenge experiments were made using *A. leptodactylus* since, with crayfish plague present in the UK, *A. pallipes* populations were regarded as under threat. A single experiment (

Figure 28) with *A. pallipes* was carried out to demonstrate the pathogenicity of *A. astaci* under controlled conditions. At 5 zoospores/ml at 15°C, *A. pallipes* was confirmed to be extremely susceptible, with the first mortalities occurring at 6 days post-challenge and reaching 100% at 8 days post-challenge.

Using *A. leptodactylus,* a simple variation in spore challenge produced a marked difference in mortality pattern (Figure 29). With a high challenge (13 zoospores/ml, 20°C), crayfish died quickly (mortalities from 5 to 12 days post-challenge) but with one-tenth the number of spores (1.3 zoospores/ml, 20°C) the mortality was delayed, commencing at day 11 and continuing slowly until day 40. The effect of challenge size was further investigated in experiments to demonstrate the influence of temperature on the pathogenicity to crayfish (Figure 30 & Figure 31). With a relatively high spore challenge (12 zoospores/ml), decreasing water temperature produced only a slight increase in host survival time. With a lower zoospore challenge (1.4

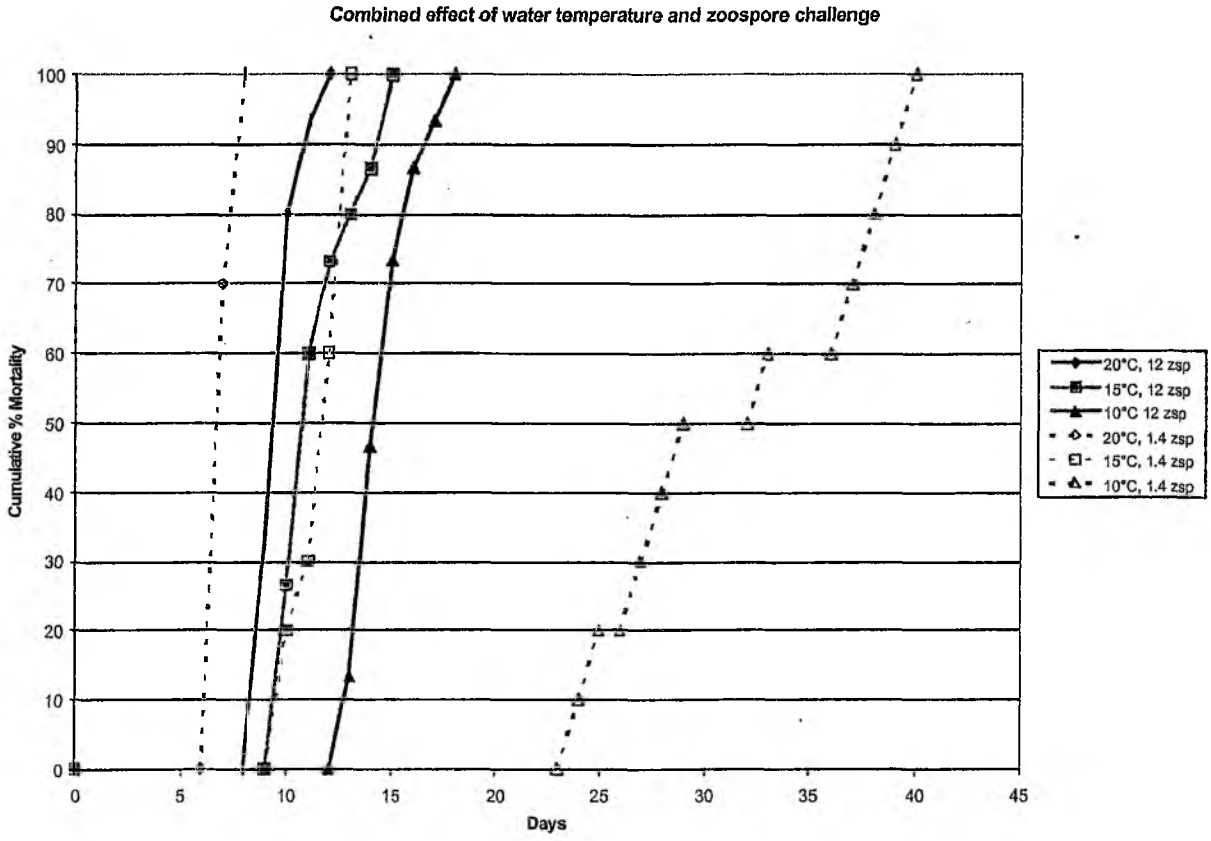

Figure 32 Comparison of effects of challenge dose and water temperature

Irrespective of the challenge dose, the mortality pattern of crayfish plague is consistent. After exposure there is a long post-challenge incubation period during which the behaviour of the infected animals remains normal, followed by a short period of abnormal behaviour and death. The duration of the two phases is altered by temperature and challenge sizes, but the overall pattern is not. At the highest challenge doses reported, the incubation period from challenge to first mortality is 5-8 days, followed by a rapid onset of mortality with 90% or more animals dying within the following 48 h. With challenge of this severity, the effects of temperature between 10 and 20°C on the duration of the mortality phase are limited. Moderate or low-dose challenges result in a markedly extended incubation period, extended duration of abnormal behaviour, and a greater spread in time of death. Under those conditions, water temperature then plays a major role in the spread of the incubation and mortality phases of the infection, so that identical challenges produce widely different mortality duration.

These mortality patterns may be used to give some explanation of the results obtained from the field trials. In August, with an active natural mortality providing a high spore challenge, and with higher water temperatures, a typical short incubation prior to rapid mortality resulted, whilst in October, with only a few surviving crayfish dying in the river, a presumed low-spore challenge and low water temperatures produced an extended survival phase and a much prolonged mortality.

In the laboratory challenge experiments described above, no mortalities occurred in any of the experimental control tanks, even in the 3 months of the transmission experiment. In each experiment, all challenged animals died eventually, although low-challenge doses presented the most variable mortality pattern with an extended incubation phase followed by anything between

an extended mortality phase and a rapid mortality phase. Reasons for this variability can be sought in secondary infection, from *A. astaci* sporulating on infected animals. Sporulation may vary from a few zoospores developing on a localised infection on one animal, to many spores from a number of lightly-infected animals or to many spores from one heavily-infected, dying animal. On this variation will depend the pattern of subsequent mortality produced by the secondary infections. Results obtained by Unestam & Weiss (1970) with an initial challenge of 2.5 zoospores/ml are thus readily explicable. Severe primary infections produced the seven mortalities within 14 days mean time to death whilst the 28-day and 36-day mortalities will have been due either to light, primary infections or to secondary infections derived from animals dying earlier in the same tank.

Animals subject to intermediate levels of challenge can exhibit clearly visible "trails" of brown melanisation which track the presence of hyphae of *A. astaci* in the intersternal and articular soft cuticle, and in the connective tissues immediately below. This is rarely observed in crayfish from natural epizootics, but is not uncommon in laboratory experiments.

The areas of the crayfish body affected include the intersternal soft cuticle of the abdominal region and of the joints of the pereiopods, particularly in the proximal joint, that between the basipodite and coxopodite (Figure 34). Infection of pereiopods was occasionally associated with autotomy of the affected limb, but this rarely appeared to improve the outcome of the infection. Additionally, many infections were associated with the soft cuticle of the perianal region (Figure 39), these often seeming to be the more chronic in which severe pathology was associated with a slow death. Other areas vulnerable include the gills and eyes (Figure 38). In most animals, these gross signs appeared largely to be confined to one, often limited, area of the body.

10.3 Microscopical observations on fresh preparations

In addition to low power examination of intact gross specimens of crayfish plague infected crayfish, the examination of fresh preparations of cuticle and of muscle from such animals provided further information on the process of infection and acted as a valuable tool to aid to give a presumptive diagnosis of plague and also aided in the selection of suitable tissues from which the isolation and culture of the pathogen could be attempted.

Soft intersternal or interarticular cuticle from above the site of whitening necrotic muscle was carefully excised and mounted in water on a slide. Small excised blocks of muscle and lengths of ventral nerve cord were also be examined in the same way. The cuticular mount normally consisted of the cuticle itself, plus the epidermal and connective tissues with which it is intimately associated. Infected areas of such cuticle under bright field or phase contrast microscopy showed fungal hyphae ramifying through epithelial and connective tissues below the cuticle. Stages of host response could be seen in the form of haemocytes aggregating along the hyphae and the development of melanisation and encapsulation (Figure 40). Although this encapsulation affected significant lengths of hyphae, containment was not achieved and normally the hyphae could be observed growing on beyond the area of encapsulation, unaffected by it. Numerous hyphae unaffected by any host response were also present. In many specimens, hyphae could be observed growing out through the cuticle to sporulate on the surface Such typical narrow zoosporangia with clumps of encysted primary spores at their tips provided presumptive evidence of the presence of *A. astaci* (Figure 36).

Animals from laboratory experiments tended to be much cleaner in appearance microscopically than those from natural mortalities where a wide range of epibiotic fouling organisms including bacteria, other fungi, protozoans and algae were intermingled with the hyphae, zoosporangia and spores of the pathogen on the cuticular surface.

10.4 Histopathology

The variability of gross pathological signs in crayfish plague infections was found to be reflected in the considerable range of histopathology encountered even in moribund animals. As with gross signs, in animals dying and newly dead from a high zoospore challenge at warmer water temperatures, it was often difficult or impossible to find evidence of hyphae of *A. astaci* in the tissues and evidence of host response was equally sparse, whilst in animals with chronic lesions, hyphae were locally abundant and tissues were heavily infiltrated with haemocytes accompanied by varying development of melanisation and encapsulation.

10. PATHOLOGY

The comments in this section derive from a manuscript in preparation (Alderman and Polglase) on the detailed pathology of crayfish plague

10.1 Behavioural Effects

Crayfish infected with *Aphanomyces astaci* show two different behavioural abnormalities. Clinically infected animals can be found in open water in full daylight and their movement is staggering and uncoordinated and has been described as "walking on stilts" (Schäperclaus, 1979). Both evidently reflect a loss of normal co-ordination.

Observation of both natural crayfish plague epizootics and experimental laboratory infections, supports the view that these behavioural signs of infection reported in the literature are a normal occurrence in infected crayfish in the last 24h before death. Animals wander in open water in an increasingly disoriented way, co-ordination of limb movements is lost, the pereiopods tend to be held straighter, rather than curved in under the body which is thus lifted higher off the substrate, hence "walking on stilts". Animals loose balance and fall onto their backs. The crustacean "tail flip" is lost. Once overturned crayfish rapidly deteriorate, with uncoordinated limb movements until death intervenes.

In natural mortalities most animals died in the open, the majority did so, and dying crayfish are soon predated upon by other aquatic animals and in particular by birds. This forms a possible route of transmission via contaminated feathers and feet.

10.2 Gross Pathology

Gross signs of crayfish plague have long been reported to be highly variable, so much so that they have induced confusion in investigators to the extent that some have thought that more than one disease might be involved. The description presented here is confined to the signs observed on susceptible crayfish, both *A. pallipes* in natural and experimental epizootics and *A. leptodactylus* in experimental ones and does not consider resistant N. American species such as *P. leniusculus.* The considerable variability of gross pathology was interpreted as a direct reflection of the effects of challenge severity and water temperature discussed in the author's previous publication on the pathogenicity of *A. astaci* (Alderman & Polglase, 198x). Animals subjected to a high challenge dose at warmer water temperatures were there noted as dying quickly and presenting few gross signs whilst conversely a range of clearly evident gross pathological signs resulted from low challenges and lower temperatures.

At the high challenge, warmer temperature end of the pathological range, a careful external low power stereo microscope examination of moribund crayfish frequently fail to find evidence of infection. At most, areas of muscle can be seen through the soft intersternal and articular cuticle in which a whitening and increased opacity of the normal greyish translucency of muscle indicates localised muscle necrosis associated with the invasion of fungal hyphae (Figure 33).

With few *A. astaci* spores and at lower water temperature, moribund animals presented with major areas of gross whitened muscle necrosis, associated with brown to brown black melanisation, both focal and general in the tissues. Again, gross lesions are only readily visible below the soft intersternal cuticle. The external surface of the cuticle in such regions has a rough or "fluffy" appearance (Figure 37) resulting from the presence of numerous extramatrical hyphae growing out through the cuticle. Secondary bacterial attack of such lesions produces a dark necrosis different to the brown colour of melanisation.

The hyphae of *A. astaci* are thin walled and were found to be very difficult to discern in crayfish tissues with routine histological stains such as haematoxylin eosin. Equally, the normal counter stain, light green, employed with fungal silver stains such as Grocott, which would stain the hyphae clearly, did not provide adequate differentiation of the surrounding crayfish tissues. Therefore a combined Grocott silver with haematoxylin and eosin as counter stain was employed which gave good definition of the *A. astaci* hyphae and of the detail of the crayfish tissues (Figure 43, Figure 47).

Even with a stain which could readily demonstrate the presence of *A. astaci* hyphae in tissues, when areas for examination were selected on the basis of the presence of gross external signs, *A. astaci* proved difficult to detect in histological sections of infected animals. Except in moribund animals and those subject to chronic infections, the distribution of hyphae proved to be very limited. After careful examination of an extensive range of infected animals, the principal tissues which were found to be invaded were

i) peripheral connective tissue between the exoskeleton and the body musculature

ii) connective tissue around the ventral nerve cord with little or no penetration into the nerve cord itself. (Figure 43)

iii) connective tissues surrounding blood vessels and blood sinuses including the dorsal aorta, followed by invasion of the vessels and sinuses themselves.

iv) external surfaces of muscle blocks with a few hyphae penetrating between segments of those blocks along the connective tissue sheaths.

In acute infections in the primary area of infection, a few hyphae could be observed penetrating into and causing significant lysis of muscle, with the smaller peripheral muscle blocks being affected first.

Figure 33 White muscle necrosis, natural infection

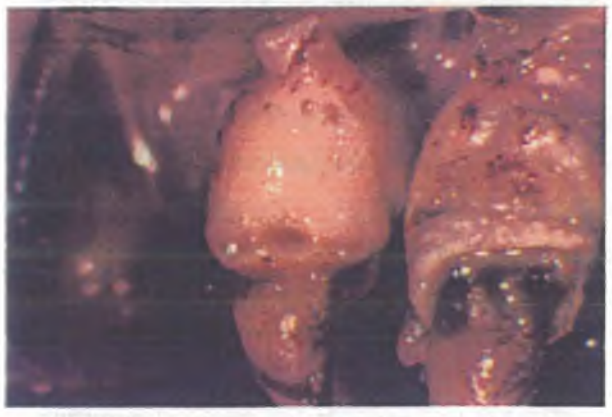

Figure 34 Infected proximal periopod joints

Figure 35 Melanisation and extramatrical hyphae

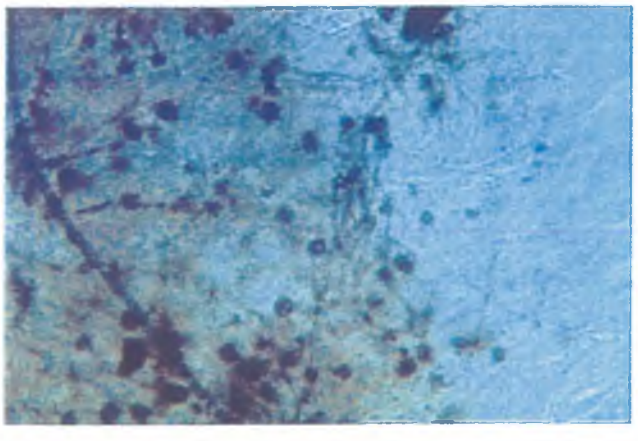

Figure 36 *A. astaci* primary spores on exoskeletal surface

Figure 37 Melanisation and "fluffy" surface

Figure 38 Infected right eye

Figure 39 Perianal region infected

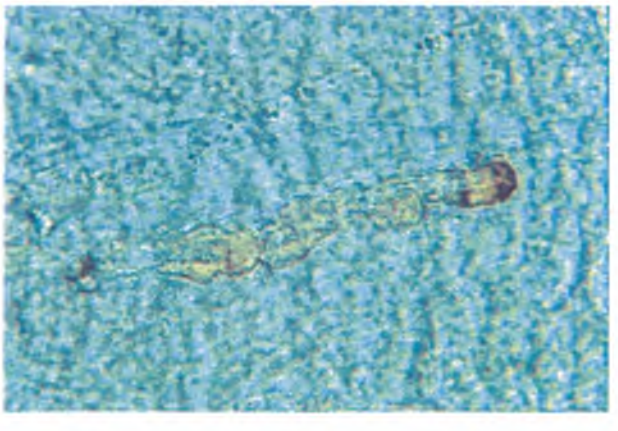

Figure 40 Encapsulation of *A. astaci* hypha in soft cuticle

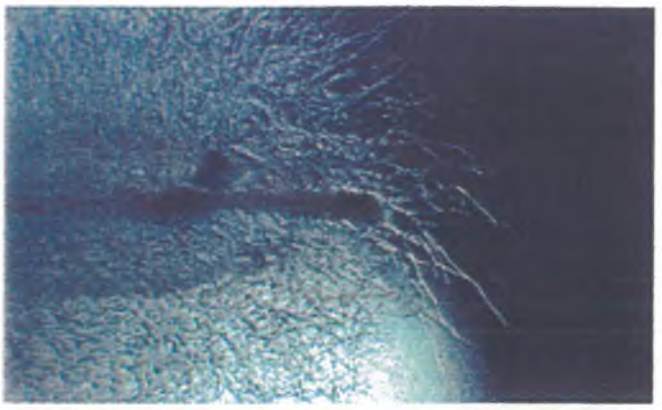

Figure 41 Isolation of *A. astaci* from ventral nerve cord

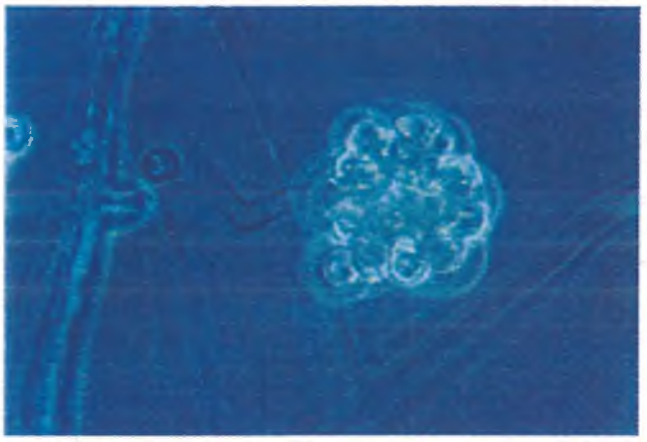

Figure 42 Primary spores encysted at sporangial tip

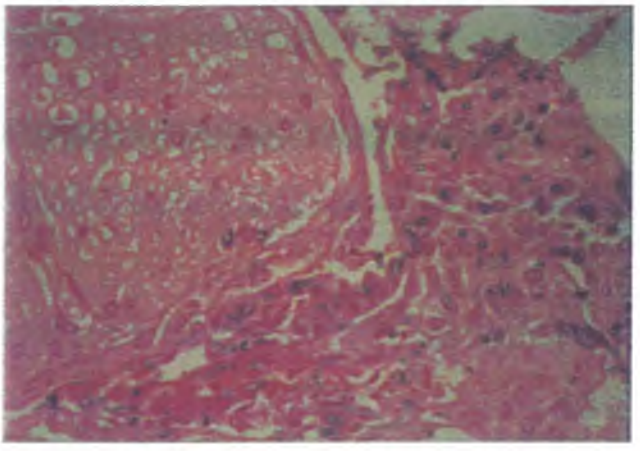

Figure 43 Hyphae in connective tissue around nerve cord

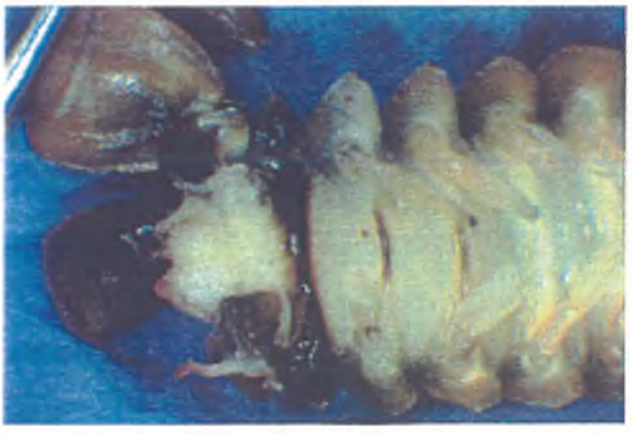

Figure 44 Severe chronic pathology, animal still living.

Figure 45 A. astaci hyphae (short) on crayfish periopod

Figure 46 Spore germlings

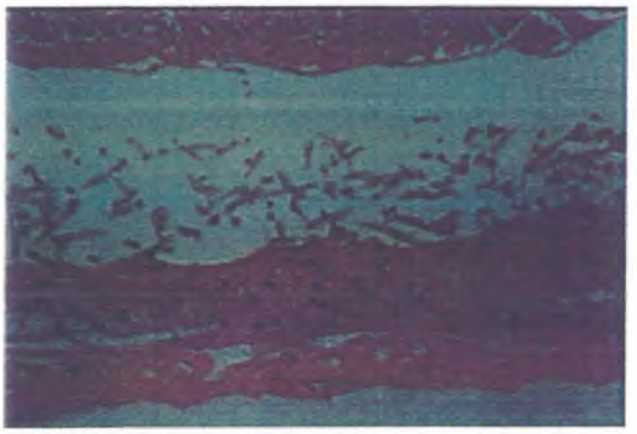

Figure 47 Hyphae in connective tissue

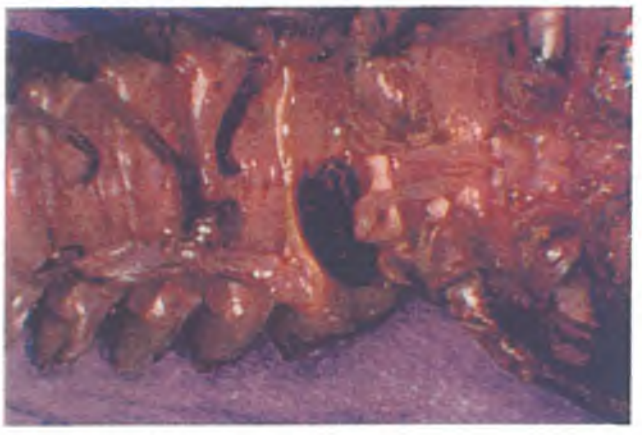

Figure 48 *A. astaci* Lesion on Signal crayfish

11. OTHER DISEASES

Although crayfish plague is the most important disease of crayfish in Europe, there are a number of other diseases and parasites known from British waters that will be described briefly with an indication of distribution, prevalence and severity

11.1 Other fungi

11.1.1 *Aphanomyces* spp.

Other members of the genus *Aphanomyces* other than *A. astaci* have been found associated with crayfish, particularly *A. laevis*. The presence and isolation of a member of the genus is not therefore diagnostic of crayfish plague and of *A. astaci*. Most such isolates, even from suspect plague cases can be persuaded to produce sexual stages that allow identification to species. The only *Aphanomyces* species for which sexual stages are so far undescribed is *A. invadens* the aetiologic agent of Epizootic Ulcerative Necrosis a highly invasive and virulent pathogen of tropical freshwater fish known from Japan, Australasia and SE Asia. It is a common characteristic of Oomycetes parasitic on aquatic animals that sexual stages are absent or suppressed.

11.1.2 *Saprolegnia* sp.

Saprolegnia spp are very common in freshwater environments in the UK. Best known for infestation of salmonid eggs in fish farm hatcheries and of stressed and immunosupressed adult salmonids (mainly males), *Saprolegnia* spp are also associated with crayfish. In particular they are frequently found as secondary invaders in crayfish dying from primary crayfish plague. The much broader hyphae (20μm) of *Saprolegnia* spp are unlikely to be confused with those *A. astaci* (9-10μm).

11.1.3 *Fusarium* spp.

The genus *Fusarium* comprises aero-aquatic hyphomycetes and that are the imperfect (asexual) stages of members of the Ascomycota. Their hyphae are hyaline and sepatate and produce large numbers of small, generally banana shaped septate conidia. This widespread genus is well recognised as producing infections in marine and freshwater crustaceans, including lobster and crayfish. They appear to be opportunist wound invaders rather than primary pathogens.

Once infection is established, host response produces a grossly evident melanised encapsulation that limits the extent of the infection (Figure 53). However encapsulated penetrations into subcuticular tissues can prevent molting and thus be lethal over long periods. The appearance of *Fusarium* lesions is very similar to that induced by *A. astaci* in plague resistant N. American crayfish. In the marine environment most cases reported have been attributed to *Fusarium solani*, but *F. tabacinum* and *F. melanchlorum* have been reported from freshwater crayfish (Alderman D.J. & Polglase J.L., 1984; Vey, 1978). Identification of isolates to species (Figure 54) requires experience and is best left to experts in the genus (e.g. IMI).

11.2 Porcelain disease

A microsporidian, *Thelohania contejeani*
spores. Infected animals present with a "bright" whitening of the musculature (Figure 49, Figure 52), clearly visible through the thin uncalcified intersegmental cuticle of the abdomen and in advanced cases through the calcified exoskeleton as well.

Distribution world wide, present in most crayfish waters in the UK. Foci of high levels of infection have been observed in rivers. Transmission not studied, but thought to be oral. Believed to be lethal over a period of months, infected animals should therefore be culled. The white appearance of infected crayfish muscle can be mistaken for the effects of crayfish plague by the uninitiated, but porcelain disease really does result in a "bright" white coloration which is not easily confused once seen.

11.3 *Psorospermium haekeli*

P. haekeli (Figure 51)is, like *T. contejeani* widely distributed in crayfish in Europe and has been reported from as far afield as Australia and in most crayfish species, European, N. American and Australian. It has been observed in occasional specimens in the UK. It has long been regarded as an organism of uncertain taxonomic affinity. Authors, without good basis have referred to it as a "fungus" and a nematode which are clearly inappropriate. The protists *Anurofeca*, *Ichthyophonus*, and *Psorospermium*, are now regarded as belonging taxonomically near the animal-fungal divergence. These last two genera have been included, together with *Dermocystidium*, in the newly described DRIPs (*Dermocystidium*, rosette agent, *Ichthyophonus,* and *Psorospermium*) clade, recently named Mesomycetozoa. The clade constitutes the most basal branch of the metazoa; or in some analyses that incorporate less well-aligned sequence regions, an alternative topology it diverges immediately before the animal-fungal dichotomy Ragan et al. (1996), Figueras et al., (2000).

11.4 Branchiobdellids

The Branchiobdellids (Figure 50) are a round worm like but distinct Phylum essentially confined to crayfish. They have been observed in the UK in particularly on *A. pallipes* in the Thames. Some are true parasites browsing on the gills within the gill chambers of their hosts.

Figure 49 Porcelain disease, left animal infected, right animal normal

Figure 52 Porcelain disease

Figure 50 Branchiobdellids in gill chamber of A. pallipes

Figure 53 *Fusarium* infection of gills

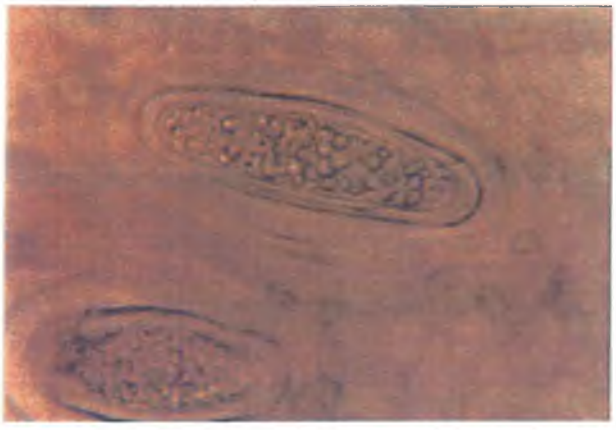

Figure 51 *Psorospermium haekeli* in *A. leptodactylus*

Figure 54 *Fusarium tabacinum*

PART II
SPECIFICATION FOR FURTHER WORK AND OUTLINE EXPERIMENTAL DESIGNS

12. BACKGROUND

The literature of crayfish plague has been extensively reviewed in the first part of this report. It is clear that the pathogen *A. astaci* is an aggressive pathogen that has been present in continental Europe since the last quarter of the 19th Century. It is also clear that the pathogen was introduced from N. America at that time and that 20th Century imports of the signal crayfish *Pacifastacus leniusculus* and the Louisiana Swamp crayfish *Procambarus clarkii* for farming and restocking have led to further introductions of new strains of *A. astaci*. In the UK introductions of *P. leniusculus* which commenced in or about 1978 have been responsible for introduction of crayfish plague into the waters of England and Wales where large populations of the native white claw crayfish were long established and highly susceptible.

Both experimental and field evidence shows that N American crayfish are highly resistant to *A. astaci* and it is theorised that this developed in N America as the host species and pathogen co-existed together over many years. Resistant N American crayfish can carry infection and under multiple stresses can become susceptible to clinical disease. The introduction of crayfish plague into naïve populations of European crayfish had the inevitable result that always happens, massive mortalities. Unlike most diseases the virulence of *A. astaci* to susceptible crayfish has been such that no survivors have been recorded and it has been suggested that one single zoospore can be a minimum infective dose with the final outcome of infection being death. The outcome has been the disappearance of susceptible European crayfish from most waters that have become infected.

High infective doses produce rapid mortalities and high populations of susceptible crayfish will of course produce high doses of spores to infect their neighbours. In resistant crayfish little fungal growth can occur and most is heavily encapsulated by host response. Relatively little sporulation and few spores may therefore be expected. Introduction of infected signal crayfish may therefore take some time to produce evident effect even if introduced into areas with good populations of native species. In some cases a lag period of 2 to 4 years appears to have separated introduction and outbreak of mass plague mortalities.

When a crayfish plague outbreak is over, *A. astaci* will not normally survive for more than a few months after the disappearance of its host. However experimental infections have been performed in laboratory conditions. In the real environment, rivers, lakes, ponds and other waters are complex bodies and not all crayfish will have been in a situation to be exposed to heavy infection, some may have only been reached by a few spores, others may escape infection completely and start to repopulate. Low level infection is likely to remain and eventually these regenerating populations will again be exposed to infection. If introduced resistant species are present and most populations introduced for farming purposes have resulted in a lot of escapees followed by establishment in the wild, then a continuing reservoir of infection will be present.

The Prohibition of Keeping of Live Fish (Crayfish) Order 1996 has provided the ability to prevent further importations and further introductions of signal or other exotic crayfish into new sites. The would be "crayfish farming industry" has now lost momentum, so that the major cause of spread of infection can now be controlled, unfortunately rather later than would have been desirable. England and Wales now is in the situation of having a heavily decreased number of populations of a highly susceptible native crayfish species and a number of known locations of infectivity associated with past or recent crayfish plague mortalities. There are however a number of other potential sources of infection such as populations of signal crayfish of unknown disease history. Except for those signal crayfish populations that have successfully co-existed with native crayfish for at least 5 years, all must be regarded as probable sources of infection. Such crayfish can no longer be moved, but if they are infected they must be regarded as presenting a risk as sources of contamination for further spread of infection.

The major sources of risk of further transfers of crayfish plague can therefore be listed as follows:-

1) In N American crayfish by deliberate transfer for farming
2) In N American crayfish by release e.g. from restaurant, wholesaler, pet shop or pet owner.
3) On contaminated equipment - professional EA or other workers
4) On contaminated equipment - anglers, canoes etc
5) By fish transfers in the transport water and tanks from waters in which potentially infected signal crayfish are present
6) By fish transfers on fish scale (attached or loose), mucus or skin from waters in which potentially infected signal crayfish are present
7) By inter-regional transfer pumping of water from waters in which potentially infected signal crayfish are present

Table 1 attempts to place these risks in order of severity. Routes 1 and 2 are controlled by the Prohibition of Keeping of Live Fish (Crayfish) Order and as far as practicable have been closed. Risks 3 and 4 can also be completely controlled. Routine disinfection is a Standard Operating Procedure for all CEFAS Fish Health Inspectors and researchers as it is (or should be) for all Environment Agency and English Nature etc. staff. Bodies such as these should ensure that proper disinfection is not only practised by biology and fisheries staff, but by engineers etc as well.

It should be borne in mind that another quite significant source of risk may come from academic researchers and every opportunity should be taken to emphasise good disinfection practice to aquatic biologists, ecologists etc.

The outbreak of crayfish plague in the Irish midland lakes has been attributed to contaminated fishing tackle. Certainly the need for disinfection of fishing tackle, protective clothing etc needs to be emphasised to anglers and others such as canoeists as much as it does for EA staff. This has certainly been done in the case of the recent Ribble mortality, although it is probably only practical to keep public attention for short periods during major mortalities.

Setting aside risks associated with long distance pumping of water, the remaining risks relate to movements of fish and fish transport water from putative infected sites to presumed disease free sites.

Table 1 Risk matrix for further spread of crayfish plague

Route	From site with current plague outbreak	From plague positive site	From plague indeterminate site*	From site with signals and natives co-existing for several years
	To sites with populations of susceptible crayfish			
1) In N American crayfish by deliberate transfer for farming (or deliberate release with the intention of future harvesting)	Certain	Certain	Moderate	Slight
2) In N American crayfish by release e.g. from restaurant, wholesaler, pet shop or pet owner.	Potentially High since risk level of source cannot be determined or controlled			
3) On contaminated equipment - professional EA or other workers	High	High	Low	Low
4) On contaminated equipment - anglers, canoes etc	High	High	Low	Low
5) By fish transfers in the transport water and tanks from waters in which potentially infected signal crayfish are present	High	Moderate	Low	Low
6) By fish transfers on fish scale (attached or loose), mucus or skin from waters in which potentially infected signal crayfish are present	High	Moderate	Low	Low
7) By inter-regional transfer pumping of water from waters in which potentially infected signal crayfish are present (includes canals)	High	Moderate	Low	Low

*i.e. from a site with no history of plague and no records of presence of susceptible crayfish that might act as indicators of level of risk, but where signal or other N., American crayfish are known to be present.

Risk levels are defined at the level that would result if no attempt to control or mitigate risk were to be undertaken

12.1 Can fish movements results in the transfer of crayfish plague?

Crayfish mortalities in the R. Wey and more recently in the R. Ribble have been linked (circumstantially) with fish stocking movements. In the case of the R. Wey at least the movement is believed to have taken place during or shortly after an outbreak of crayfish plague, the circumstances relating to the R. Ribble are at the moment, less well defined. Hall and Unestam (1981) demonstrated that *A. astaci* will grow for at least for a short period on detached fish scales and Alderman et al., (1987) by experimentally creating a crayfish plague mortality, exposing fish to this and then carrying out a mock transport movement showed that viable *A. astaci* propagules would survive the transport process and produce infection in a naïve population of susceptible crayfish. When fish and water were transferred in the water taken from the plague outbreak tank, rapid acute infection and mortality resulted in the challenged crayfish. When the fish were netted from the tank into clean water and transported in that, infection was much slower and the incubation period to the first mortality was extended. Once clinical disease appeared however, its progress was as acute as with the fish plus water transfer. Although these trials were conducted to represent the "worst case" scenario, it is quite clear from experimental and from field data that fish movements can and do result in the transfer of crayfish plague if susceptible crayfish are present in the receiving waters.

Although the only British isolate of *A. astaci* that has been examined by RAPD-PCR has been found to belong to the Group B "strain" associated with the Swedish signal crayfish introductions, there is (anecdotal) evidence that signal crayfish have also been imported directly from the USA into UK waters. It is surprising that, having developed the RAPD PCR method, the Uppsala group have not so far extended their studies to attempt to determine where in the New World the old Group A crayfish plague "strain" may have originated With direct and indirect (i.e. via Sweden) introductions of signal crayfish into the UK, the possibility must exist that more than one "strain" of *A. astaci* may be present in British waters. Movements of fish therefore offer the hazard of introducing different "strains" of *A. astaci* into already infected areas as well as introducing or re-introducing the pathogen to new waters

Powers exist to prevent movements from waters in which current or recent crayfish plague outbreaks are known, no matter whether or not the recipient site has crayfish populations or for that matter, whether plague is already present at the recipient site. Given the evidence outlined above and the history of the disease, the exercise of such powers to prevent further transfer under these circumstances cannot reasonably be disputed.

The remainder of this report will therefore concentrate upon the grey marked low risk cells of Table 1. These are the risks of movements from sites of unknown (or unconfirmed) disease history, sites with few signal crayfish or where such crayfish are geographically distant or sites where evidence suggests that only non carrier signal crayfish may be present. It is from stocking sites in locations such as this that farmers may well feel that there is no hard evidence to show why their businesses should be affected. In many cases these may be stocking farms with a long history of supplying fish to potentially susceptible sites with no evidence that any problem has been created, either before or after the arrival of signal crayfish in the neighbourhood of their farms. In a few cases the introduced exotic crayfish may be as a result of the action of the farmer, but in many cases the introductions may come from the actions of a third party.

12.2 Risks of fish movements from sites of uncertain status

Where there are no crayfish in a watershed and for ecological or other reasons never have been, then there should be no risk from fish from such waters acting as carriers of crayfish plague

infection. Where crayfish occur in a watershed from which fish stock producers wish to supply fish for restocking or on-growing a number of possible scenarios exist:

1. The crayfish may be uninfected signal (or other) crayfish.
2. The crayfish may be infected carrier signal (or other introduced species) crayfish.
3. The crayfish may be susceptible native crayfish undergoing or recovering from a crayfish plague epizootic.
4. The crayfish may be susceptible native crayfish in healthy condition.
5. The crayfish may be both native and signal (or etc) crayfish.

With scenario 4, there is no risk of transfer, crayfish plague is absent. However given the resumption of spread of the disease in the last two years, the health status of such crayfish could change rapidly (in fact to scenario 3) and as has been demonstrated fish movements from current and recent plague epizootic sites present significant risks of transfer. Scenario 5 is very similar provided that the signal crayfish are well established. Evidence suggests that several years from introduction of carrier signal crayfish may be required before the infection dynamics reach the point of induction of acute disease. The length of the lag period will be heavily dependent on the infection frequency and severity in the signal crayfish and the population densities of both species. Again the same caveat in regard to not making assumptions that the disease status cannot change rapidly applies here as much as it does for pure native crayfish populations and hence for the risks associated with fish movements from such sites.

Scenarios 1 and 2 present quite different risks, scenario 1 presents no risk of transfer of crayfish plague, scenario 2 presents a significant risk, although much lower than scenario 3. The problem is to be able to determine whether a fish farm is in scenario 1 or in scenario 2, i.e. to determine whether or not potential carrier crayfish are or are not actual carriers of infection. This is the central problem with all attempts to control spread of disease by control of movements of aquatic animals, particularly where a carrier state is known to exist. A diagnostic method that is both sensitive, reliable and robust is needed to establish presence or absence of infection.

12.3 Potential diagnostic methods

Three approaches are possible, molecular, immunological and traditional isolation and culture. Each has strengths and each has weaknesses.

All four basic methods require validation of method and sensitivity and with molecular and immunological methods validation to confirm lack of cross reactivity is also essential. These needs are summarised in Table 3 and some further suggestions of how these might be satisfied experimentally are given in the final recommendations on research needed at the end of this report. The "canary" method which depends on caging native crayfish with the suspect signal crayfish, is almost certainly not worth pursuing further, even if stocks of *A. leptodactylus* could be exploited as "canaries". This approach in practice could only be used in the laboratory because, if used under field conditions, there would be a significant risk of escape.

In the medium term it is likely that molecular methods will replace traditional isolation and culture. Sensitivities will be higher and the diagnostic skills needed will be less. Once a suitable diagnostic method has been developed, whilst full validation is underway, it should already be possible to exploit it to aid in reducing transfer risks. The method described by Oidtmann et al., 2002 is close to that stage now, but because it still requires isolation and culture as a first stage cannot be applied to crayfish tissues as yet. By combination of improved diagnostic methods and the risk reduction approaches defined below, the risks of transfer of infection from fish movement could be reduced to acceptable levels.

Table 2 Strengths and weaknesses of available diagnostic tools

	Strengths	Weaknesses
Traditional isolation	Verifiable viable pathogen which can be subjected to a battery of additional tests including rival methods	Difficult, requires technical skills. Can be relatively slow. Good for presence of pathogen, false negatives possible
Traditional canary method	Verifiable viable pathogen isolable if the canaries die which can be subjected to a battery of additional tests including rival methods	Difficult, requires technical skills and uses endangered native species. Very slow. Good for presence of pathogen, false negatives possible
Immunological, includes ELISA, MAb	Two main types polyclonal and monoclonal antibody based. Polyclonals fairly straightforward, monoclonals less easy but can be very specific and very quick, possibly usable in field, but rarely robust enough for this.	Does not confirm the presence of viable pathogen, simply signal that a component of the pathogen is present. Even specific monoclonals may cross react with closely related pathogens e.g. *A. astaci* and *A. invadens.*
Molecular e.g. RAPD-PCR	Once established, robust and rapid	Method is robust but specificity less so, as with immunological methods simply gives signal that an element of the pathogen is present and demonstrating lack of cross reactivity difficult

Table 3 Improvements needed in diagnostic tools

	Main improvement needed	Approach
Traditional isolation	Increase sensitivity	With signal crayfish try to develop method to break down carrier status to clinical disease
Immunological, includes ELISA, MAb	Improve or validate specificity	Test against a range of Oomycetes *in vitro*, carry out *in vivo* trials to determine ratio of false positive to false negatives under laboratory conditions
Molecular e.g. RAPD-PCR	Improve or validate specificity, use in clinical cases	Test against a range of Oomycetes *in vitro*, carry out *in vivo* trials to determine ratio of false positive to false negatives under laboratory conditions

Once a diagnostic method is established, two further points will require consideration and decisions.

The first decision is to define the limit of detection required. In aquatic disease normally a statistical sampling level is selected. To give a 95% probability of detecting a 2% prevalence level of infection requires a sample size of 130 animals. Samples of 30 animals give lower assurance levels. Assuming RAPD PCR which has the potential for speed, economy and robustness, can be developed to give an adequately validated method that is reliable in crayfish tissues, then the rough costs of running PCR on a 30 animal sample would be in the region of £250 and a 130 animal sample, £700 (these are CEFAS rates).

The second decision needed relates to test frequency. Should tests be one off, annual or repeated only when there is reason to believe that the situation has changed? Should the first test be on a large sample and if repeats were required could the smaller sample size and cost be acceptable? If charged to the farm the cost of a PCR would seem acceptable as a one off test for stocking farms, but repeated tests might be affordable even if required for all stocking farms regardless of risk.

The situations considered above relate to sites at which crayfish are available in the immediate neighbourhood that can be used to establish the site's disease status. In the absence of crayfish the situation becomes more difficult. Without indicator animals, how can the risk be assessed? The recent case of the R. Darenth indicates that infection can remain in a watershed for ten years or more, presumably cycling at a low level either in sparse populations of surviving native species or in populations of signal crayfish. If signal crayfish can be foundIf no crayfish (white claw or signal) are available to diagnose presence of plague, levels of viable *A. astaci* spores must be low. By implementing the risk reduction methods described in the next section, reduction of any undefinable risk to acceptable levels should not present a problem. There is therefore no good reason why movements from crayfish free sites should be subjected to more than risk reduction procedures.

13. RISK REDUCTION

We have to accept that an absolute guarantee that any source fish farm site is free of all possibilities of crayfish plague contamination is not possible and that total application of precautionary principles may also not be legally or practically possible. The risk will always exist that there are ponds in gardens, farm irrigation ponds etc. on any watershed with signal crayfish in them that have not been detected. The possibility of new stocking of such ponds at any time also exists (regardless of the legality of such introductions). Therefore any fish stocking source could potentially be or become a risk without the knowledge or control of the owners of such farms. With disease as with many scientific problems, proving the negative, absence is almost impossible, proving the positive, presence of disease is usually easier, but with crayfish plague as described above reliable and robust methods for this are not as yet available. Consideration therefore needs to be given to developing risk reduction procedures that can be implemented to reduce risk of transfer of disease from sites with low infection risk potential.

13.1 Transport Water

Examining the only experimental fish transmission study so far published, it is clear that a very simple way in which the risk of transmission of crayfish plague can be reduced, is to ensure that the transport water does not come from a potentially infected river source. Transport tanks should be disinfected with hypochlorite and then rinsed several times with chlorinated tap water before filling with transport water. The simplest approach for transport water will be to use tap water, aerated or equilibrated overnight to ensure that chlorine has dispersed before the fish are placed in the transport tanks. Some action may be necessary to ensure that the transport water temperature is adjusted, ice or warmed water would be appropriate.

"Dilution" stages can also be introduced to prevent transfer of *A. astaci*. During loading of the fish transport tanks, each net of fish should be allowed to drain for several seconds and only the fish and not the net should enter the tank water. The nets of fish should also be briefly hosed with clean tap water as a further dilution of risk stage. At the receiving end, clean nets belonging to the receiving site should be used to remove the fish from the tanks, draining time for the nets of fish should again allowed and only fish, not nets should enter the receiving waters. Nets could also be hosed with clean tap water at this stage before transferring the fish to the receiving water. Nets should then be disinfected before further use on the receiving site. Water should either be retained on board and discharged back at the exporting site or after hypochlorite disinfection if discharged elsewhere.

13.2 Water and Fish

Hall and Unestam (1981) and Alderman et al. (1987) demonstrated that even in a worse case situation the use of an effective fungicide would prevent transmission of crayfish plague when moving fish. In those studies malachite green was employed. Malachite green bioaccumulates in fish and results in persistent tissue residues and as a potential carcinogen its use is no longer acceptable. The use of a fungicidal compound to prevent transmission of crayfish plague on fish falls into a difficult legal area. The problems associated with the legal constraints on use are considered in the following section whilst the principles of using a fungicide to further reduce the risk from fish stocking transfers are dealt with in this section.

Perhaps the first question should be what are we trying to apply the fungicidal treatment to, what are we trying to disinfect? It is clear that the use of a really effective fungicidal compound can kill all stages of *A. astaci* on the external surfaces of fish and in their transport water. This will

eliminate the risk associated with transfer of fish, even were the fish movement to be from the site of a full blown crayfish plague epizootic to an SSSI with good populations of *A. pallipes*.

Perhaps the only uncertainty in this is whether or not viable *A. astaci* propagules can pass through the gut of fish and be capable of infecting fish afterwards. Malachite green could ensure disinfection of water, external fish surfaces and even of fish gut because it bioaccumulates, is effective at low concentrations and is in part hepatically excreted and therefore is present in all tissues and in the gut contents. Other fungicidal compounds may not be effective systemically, they offer the possibility of "disinfecting" transport water and fish external surfaces, but may not be effective in the fish gut.

Perhaps the most extreme risk case of this type would be the rather remote possibility that one of the fish to be transported manages to catch and eat an infected crayfish immediately before transport. Even if free in water stages of *A. astaci* do not pass through fish gut and remain viable, there must be a higher probability that stages in crayfish exoskeleton may survive and remain infectious.

However, the standard procedure when transporting fish is that they be starved prior to transport, salmonid guts are short and food has a short transit time within. Therefore a requirement for pre transport starvation periods, followed by antifungal disinfection would seem to offer satisfactory levels of protection against transfer of *A. astaci* on or in fish and fish transport water. Non salmonids in general will have longer guts and therefore more extended pre transport starvation periods may be necessary for such species. Such pre transport starvation should of course preferably be in a situation / location where there is no access to crayfish since starved fish would be more likely to take a crayfish than well fed fish! Further reassurance could be provided by fairly simple tests to predict the ability of the fungus to survive gut passage in a viable state.

13.3 Use of veterinary medicines

If the use of a fungicide were to prevent or control a fungal infection in the fish itself the situation would be clear, the use would be a medicinal one and would fall under veterinary medicines legislation. In the present situation it might be possible to claim that the intended use was pesticidal or disinfectant. However in cases such as this, in general the Medicines Act has been ruled to be prime, and in any case the requirements and monitoring programme for veterinary medicine residues make the veterinary medicines route the best to take. At the end of treatment, the fungicidal treatment will have to be discharged and for this normal EA discharge approvals process will be required.

Under veterinary medicines legislation, with food animal species, only veterinary medicines with a Marketing Authorisation for a specific use may be used i.e. as a fisheries fungicide. Exceptions under the cascade prescription system are possible but have no practical relevance in the present situation. Trout (species irrelevant) and salmon are regarded as food species by definition. To protect consumers an appropriate withdrawal period between treatment and slaughter must be enforced to ensure that unacceptable residues are not present in the edible tissues of the fish at slaughter. In fish, edible tissues are defined as muscle and skin in normal proportions. The withdrawal period is determined from data generated by the pharmaceutical supplier / manufacturer applying for the Marketing Authorisation. In the case of stocking fish, the controllable withdrawal period would be very short, since in theory fish could be caught within minutes of stocking. The basic requirement in regard to residues is imposed by Regulation 2377/90/EEC that requires that no component of a veterinary medicine for use in food animal species may receive a Marketing Authorisation unless a Maximum Residue Limit (MRL) has been set. Substances may be entered in one of four Annexes to the Regulation. Most important is Annex II that contains those substances that are regarded as safe with no specific

MRL level set, Annex IV contains substances that must not be used in food species under any circumstances.

Regulation 2377/90/EEC was the first stage of regulation of veterinary medicines on the European scale, the second main component is Directive 96/23/EC which required that all Member States introduce an expanded veterinary residues monitoring programme to include fish meat and to ensure consumes were protected from unacceptable residues. Malachite green is specifically included in that Directive as a substance to be monitored for. In the UK that programme is operated by the Veterinary Medicines Directorate of DEFRA. In England and Wales samples are collected on their behalf by the CEFAS Fish Health Inspectorate who would be obliged to draw to VMD's attention any use of fungicides on fish; sampling would then result. Therefore only a substance with MRL and appropriate authorisation can be employed, particularly in recommendations from official bodies such as EA, DEFRA etc.

Fortunately there are two candidate compounds that fit this regulatory straightjacket. These are formalin, which although not approved specifically for this type of purpose does not result in a residue problem and will not encounter regulatory barriers. Its toxicity to fish may however be a discouragement. Pyceze™ is a new fisheries fungicide that is at an advanced stage of development by Novartis Animal Vaccines and well down the route in the process of obtaining a Marketing Authorisation. Although not yet tested on *A. astaci* it may be expected to be as effective against *A. astaci* as it is against *Saprolegnia*. Novartis is aware of the crayfish plague problem and may be expected to have a positive approach to investigations into the efficacy of the product for this market. Because Pyceze™ active has an Annex II MRL, zero withdrawal periods present no problem, any residues are regarded as consumer safe.

Finally, it must be emphasised once more that:

1. Any product used to prevent transfer of fungi on fish may be expected to be regarded legally as being used for a medicinal purpose.
2. Thus only products approved for such a purpose are acceptable.

The costs of generating the data required to gain MRL assessment, not to mention the other safety and efficacy data requirements for veterinary medicinal use approval in a food animal species is such [perhaps a minimum of £2 million] that there is no point in considering or wishing for substances that do not already meet these regulatory standards.

14. RECOMMENDATIONS: DIAGNOSTIC METHODS

Outline experimental designs of specific research objectives for selecting diagnostic methods to determine whether signal crayfish populations are carriers of *A. astaci*. To ensure adequate and defensible validation of method, wherever possible the principles of Good Laboratory Practice or Good Clinical Practice should be employed.

1. Obtain signal crayfish stock and infect with range of challenge doses of *A. astaci*. Use modifications of the methods described in the first part of this report for this. Initial disease status of this stock at entry into study is not important. Proper containment will be essential for such studies.
2. Use RAPD-PCR, isolation, and possibly MAb to culture to determine recovery of infection from animals that should now all be infected.
3. Attempt to improve isolation sensitivity by use of methods such as injection of â-glucans to attempt to break carrier status into clinical disease. Söderhäll has suggested this approach as effective, but to the best of the author's understanding, no quantified data has been published.
4. If â-glucans work, incorporate into method since the ability to produce culture is robust evidence. Current molecular methods rely on isolation and are not yet usable on clinical cases.
5. Confirm absence of cross-reaction between selected method and other *Aphanomyces* species as far as possible. Only about 6 of 30 described species are currently available from culture collections.
6. Determine sensitivity and robustness of method and set the level of infection and probability wanted to determine sample size (e.g. a 95% probability of detecting a 2% level of infection gives a sample size of 130 from a population)
7. Use these results to produce a validated method of determining infection status by mixing artificially infected and uninfected signal crayfish (presumably obtainable from sites where signals and natives have cohabited for some time) in a blinded study [double blind if possible].
8. Then apply the method to a minimum of 5 wild stocks of signal crayfish, including at least one believed to be healthy and one believed to be carriers [blind this study].

15. RECOMMENDATIONS: RISK REDUCTION AND FISH MOVEMENTS

Recommendations and outline experimental design for specific research objectives to develop methods of risk reduction for fish movements:

To ensure adequate and defensible validation of method, wherever possible the principles of Good Laboratory Practice or Good Clinical Practice should be employed.

1. Confirm that the legally usable fungicides, formalin and / or Pyceze™ are effective against all stages of *A. astaci* (zoospores, spore cysts and mycelium) at concentrations and exposures that are safe for fish. This would be an *in vitro* study and can use modifications of the published methods for fungicide tests described in the first part of this report. Consider the influence of water temperature on this efficacy at at least 2 temperatures relevant to normal water temperatures in the U.K. Also examine the effect of water temperature on the toxicity of the test compound to the fish.
2. Develop data to support recommendations for "antifungal disinfection" of fish and water at transport. Initially studies should be *in vitro,* but should be confirmed by experimentally exposing fish to high levels of *A. astaci,* employing the selected disinfection procedure and then introducing susceptible crayfish. Develop from the published methods described in the first part of this report.
3. From these results develop a practical protocol for "disinfecting" fish in transport to provide a method that is both effective and without harm to the fish. This protocol should form an expansion of the risk mitigating procedures already defined in this report.
4. Determine whether [and under what conditions] viable *A. astaci* can pass through salmonid gut and remain viable as a) naked fungus b) protected fungus [e.g. in agar plugs] c) possibly in infected crayfish exoskeleton fed to fish. The experimental procedures can again be built on the published methods described in this report.
5. If 3a), b) or c) are viable, determine whether the fungicides are effective on these forms and determine whether a practical "disinfection" protocol can be developed.

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Appendix 1 Crayfish Plague in Europe, 1870 to 1945

The chain of spread of crayfish plague mortalities which continues across Europe to this day can be traced back to the area between Morvan and Lorraine in France in 1874. There is however an earlier discrete series of crayfish mortalities which may well have also been due to crayfish plague. These took place in Lombardy, northern Italy commencing in the summer of 1859, although earlier mortalities in the Sarnico region of Italy may also be associated (Cornalia, 1860).

In 1859 crayfish began to die in numbers in Lombardy (Figure 56) the summer and the affected area spread eastwards towards the Veneto reaching Gambara, Isocella and Verona in September and the provinces of Veronese and Trevigiano by December. (Cornalia, 1860). In 1860 mortalities were reported near Brescia. Further extensions in rivers of the Verona region were reported in the spring of 1861, including the R. Benaco. East of the Adige the waters of Zevio, Persacco, Palu, Vallese, Raldon, San Giovanni Lupatoto, Buttapietra, Oppeano, Upper Menago and the Bongiovanni were all affected and in December 1861 the first dead crayfish were found in Lake Garda (Martinati 1862). To the west of the Adige infection spread to Belfiore Diporcile and Bionde amongst others and appeared in the Upper Fibbilo, the Antanello, the Fossa Balbi and spread downstream from the Ferrazze de San Martino. It also appeared in the Dugal Fontaine, the Sarega, the Lower Tartoro and in the Cerea and Casaleone valleys (Martinati 1862). By 1864 the disease reached the source of the R. Sile in December at Casacorba (Ninni, 1865). In February 1865 infection spread further in the Sile and Botteniga catchments and then to the rivers Storga, Melma, Limbraga and Magnagola. By April of that year it had spread to the rivers Musestre, Musestrelle, Pero, Valilo and other lesser waterways (Ninni, 1865). So far written reports of mortalities have been found only for streams on the north side of the Po, but at that date the river formed the boundary of Austrian territory before the unification of Italy. Political boundaries may thus have influenced investigations, reports and therefore the apparent distribution of mortalities. The severity, extent and rapidity of spread of the mortality indicates an infectious disease and the only known crayfish disease of such severity is crayfish plague, but there is no hard evidence to support this hypothesis and this series of outbreaks is separate, physically and temporally, from the main chain of spread of crayfish plague in Europe. It was also suggested that these mortalities coincide in time with the widespread introduction of the use of copper sulphate into viticulture (De Luise, 1989). Certainly there have been no more reported crayfish plague mortalities from Italy, either from the Po valley or elsewhere, but the way in which the mortality reported by Ninni (1865) spread has all the characters of plague. Certainly Schikora (1926) was convinced that the Italian mortalities were plague and reported that Italy had made major efforts to restock the rivers of Lombardy with crayfish "shortly before her entry into the war (presumably World War I). It is of interest that Italy is the only western European country which has apparently not reported crayfish plague mortalities since 1900 and recent publications indicate good crayfish populations in the headwaters of the Po.

Although Seligo (1895) reported that there were crayfish mortalities in the R. Spree in N. Germany in 1864, he did not believe that these were due to crayfish plague. Accepting this view therefore the site of the first epizootic in Europe north of the Alps appears to have been in France

on the Plateau de Langres between Morvan and Lorraine in 1874-5 (Ravaret-Wattel, 1885, Vivier, 1951).

The Plateau de Langres is the central watershed between major rivers such as the Meuse, Seine and Rhine. Crayfish plague first appeared in 1874 on this plateau (Figure 57) in the following three years infection appeared at a large number of sites around the plateau, including the southern Nievre and Jura departments, the northern Meurthe and the Moselle and in tributaries of the Meuse, Rhine, Seine and Saone (Figure 58). From this focus, a relentless spread of mortality began which with only temporary remissions has continued to this day.

In 1877, further spread occurred in France to the departments of Aisne, Aurbe, Haute. Marne and Vosges (R. Vair) (Anon, 1879) and for the first time mortalities were noted in Germany in the vicinity of Frankfurt-am-Main (Tzukerzis, 1964) (Figure 59). The next year, in France, most of the Meuse dept apart from the Rivers Vinte and Loiven were infected before March (Figure 60). In the Marne dept the Seine basin rivers the Vitry le Francois region was affected and the rivers Marne, Cher, Viere, Bruxenelle, Germenelle, the Marne-Rhine-Seine Canal and all Seine tributaries except the Amance, Vanne, Landeon, and Brevennes. In addition the Aube, Loing, Fusin and Lunain, La Bresle and Andelle were also infected and infection had also reached the Rhone basin in the Dole dept with the La Veyle tributary of the Saone and the Seran (a Rhône tributary).

In Germany, by March 1877, infection had appeared near Strassburg (Strasbourg) and in Alsace (Figure 60). In July and August crayfish brought from the R. Hünsbruk and the Eifel to Mainz all died suggesting that those source rivers were infected (Hofer, 1906). In Hessen-Darmstadt and Baden crayfish were first infected in the summer of 1878.

Further extensions in 1879 in France included the Coney in the Vosges, all tributaries of the Seine in the Aisne dept. and the R. Therain in the Oise dept. To the east in Germany in January plague appeared in the Munich region and in September an outbreak started at Gmunden on Lake Traun, in the Klambach at Grein and in the Krems and mortalities were reported spreading along the upper Danube, all in upper Austria (Anon, 1879).

In 1880 in southern Germany, crayfish died in the Altmühl and its tributaries including the Wieseth and Sulz whilst in Belgium, Luxembourg and Alsace Lorraine mortalities occurred in the rivers Moselle, Saar, Orne, Seille and Nied (Seligo, 1895) (Figure 61). The mortalities in the Danube had spread downstream and into tributaries in the Kulpa region on the Austrian Yugoslavian border. (Franke, 1894). All of these outbreaks were associated with the original focus, but by the end of 1880 mortalities were occurring in northern Germany including the Rorsee, Thuringia, Mecklemburg and Saxony and the Brandenberg Mark (Hofer, 1906, Schäperclaus, 1927) and began to spread up the Meitzel from the Oder (Van dem Borne, 1883).

The following year (1881), infection reached up the Rhine into Switzerland at Berne. In southern Germany and upper Austria crayfish mortalities were reported from Wels and in the Eiterbach in October and November (Roch, 1881) and from the Tauber and tributaries of the Mainz (Seligo, 1895). To the east, Franke (1894) reported further extensions in the Kulpa region advancing in the next three years into the headwaters of these streams. Once established in northern Germany, more areas were affected by crayfish plague with new outbreaks in the Bober in Silesia, in the Kuddow in the Oder region, in the Ferze and Schwarzwasser in the Weichsel region of W. Prussia and at Angermunde in Brandenberg (Selio, 1895).

In 1893 the R. Altmühl (Anon, 1893) lost all crayfish and there were further mortalities in the Kuddow. To the north, the Brahe of Western Prussia was affected as were the waters of the Kamonica, Zemplona, Widlgartenfluss, Weichsel and Mischkerfleiss (Seligo, 1895). The upland areas of the Glatz province of Silesia (now the Klodzko district of Poland) were invaded up the eastern (Glatzer) Neisse (). (Figure 62).

Between 1885 and 1890 in N. Yugoslavia, half of the R. Temnica was affected (Franke, 1894) as was the Ljubljana region and plague started a ten year advance up the R. Gurk and its tributaries (Figure 63). In W. Prussia the Drewen, Ossa and Liebe (Seligo, 1895) were affected and the continuing eastward extension of infection led to mortalities in L. Doubuzhis in NE Latvia by 1886 (Tzukerzis, 1964).

The year 1890 saw further mortalities in L. Drewenz and L. Ewing in the Passer region of W. Prussia and in the next two years major extensions into Russia were reported from the R. Luga in the Leningrad region, the Volga and Lake Onega basin (Arnold, 1900) (Figure 64). In the "rushing waters of the Volga", plague covered 3000km to reach the Caspian Sea in the autumn of 1892 (Schikora, 1926). The infections in the Danube the downstream spread reached the Black Sea and devastated crayfish populations of the coastal provinces. In the Danube tributaries, information is only available from the Kulpa region where infection had apparently spread through underground streams to the R. Rinse. The Masuren in W. Prussia was infected as were the waters around Shialaai in central Latvia (Tzukerzis, 1964).

By 1893, in France, crayfish had completely disappeared from Lorraine. In the Kulpa region of the Balkans, disease had reached to Atenmarkt. The spread in Russia and the Baltic states continued on a grand scale with the Dniepr being infected down to rapids at Yekaterinoslav (Arnold, 1900) where the piles of dead crayfish produced such an unbearable smell that special efforts had to be made to bury them. In Latvia the lakes and rivers of the Niamunas (Neman) and Niaris (Vilnia) basins were affected, completely wiping out the animals in the waters of Ssviachensk, Trakaisk, Utiansk, Birzhaisk and Varniaisk. This rapid spread in Latvia has been attributed to introduction of infected crayfishing gear from Germany (Tzukerzis, 1964). By 1894 further extensions in the Baltic states brought infection to Courland (= Kurland, S. Latvia), Livonia (N. Latvia) and Estonia (Schäperclaus, 1979) (Figure 65). In Russia, the R. Kliasma, a tributary of the Volga as was the Dvina (Vitebsk district) and lakes in the Vladimir district (Arnold, 1900). The destruction of crayfish in Russian lakes and rivers continued in 1895 with losses of stocks in the R. Beresina (Dniepr tributary), the Moscow R., the R. Oka and lakes of the Suvalki district and in 1896 the Scheksna in the Jaroslav district in Russia and Embach in Livonia (Arnold, 1900), the Duna in Kurland. In N. Germany the lowland areas of the Glatz region were affected, twelve years after the uplands.

In 1897 in Russia the Tchernigov and Tver districts, lakes near Novgorod were affected. From the Volga, plague was also climbing the Kama into the Urals, "from where it was a small matter for it to reach the Ob via the Tura and there in Siberia to annihilate the easternmost crayfish colonies of the Russias" (Schikora, 1926) (Figure 66). In Livonia the R. Woo and Lake Werro through which it flows (Hofer, 1900) were hit and, in the next two years, in Russia the Poltava, Kharkov and Pskov districts (Arnold, 1900) and the R. Aa, a mortality which had evidently spread upstream from Latvia. In what are now the Baltic states the R. Dvina and Lake Peipus (from the Woo) (Arnold, 1900, Hofer, 1900) were infected, but Tzukerzis (1964) reports that after 1902 new outbreaks in Latvia ceased for some time. In other infected regions, waters that had previously escaped plague were affected including the Pansdorfer See in Silesia (Schikora, 1906).

In 1903 Shikora (1903, 1906) continuing his investigations reported outbreaks from the Nariensee (W. Germany), Mecklemburg, and from Volhynia in Russia. In Bavaria numerous outbreaks occurred including in the R. Aurach (Surbeck, 1903). The following year in Germany, plague was identified in Zamhauser See and spreading from a new focus in the Neumark (SE of the Oder and S. of Pomerania, now in Poland) to the Kloppsee and the Muckenburger See in the following spring.

Finland had been first infected in about 1900 (Figure 67) although few details have been traced, then in 1907 the disease reached Sweden, from Finland when imported infected crayfish were found to be moribund on arrival in Stockholm (Figure 68) and were jettisoned into the harbour

which lies at the entrance to the Malaren (Alm, 1929), so that deaths began next year when the most productive crayfish waters of Sweden, the Malaren and Halmaren were destroyed. By the end of 1908, all the crayfish in the Halmaren were dead, the disease spreading between the two lakes through the R. Eskiltunaa in which the crayfish also died (Alm, 1929). Controls were imposed which succeeded in confining infection in Sweden to the lakes and rivers of the Halmaren and Malaren systems (Alm, 1929). During this period however infection spread steadily though these waters.

Elsewhere large mortalities occurred in the Ukermark region of Germany (1912-1914 and 1923-25) (Schäperclaus, 1934, 1935) and in 1920, following a period of nearly twenty years in which there was little or no spread of the disease in Latvia, new outbreaks of plague were reported in Lake Obiala and Lake Dusia (Tzukerzis, 1964) and in Lithuania (Mazylis and Grigelis, 1979)

A relaxation of controls in Sweden in the late 1920's resulted in crayfish deaths in 1928 in L. Addar in Rosengen, the lower reaches of the Dalev, the west and east Gothic ends of the Gota Canal as well as the majority of confluent lakes and streams (Nybelin, 1931) (Figure 6). Additionally the Tidern, Osan and Vikern lakes were affected. Infection then in 1929 spread from the Gota Canal area to the Stang A and L. Asund. Elsewhere in Sweden crayfish deaths also occurred in Nashulta lake, the Addarn in eastern Uppland and lakes and streams in western Ostergotland. Lake Erken, the most productive crayfish lake in Sweden was devastated by plague in 1931. These mortalities in Sweden led to major new studies by Nybelin (1931, 1934, 1936) which resulted in the first isolation and culture of the pathogen.

Figure 55 Europe before crayfish plague

Figure 56 Possible Italian outbreak in 1860-65

Figure 57 First proven outbreak on Franco-German border, 1875

Figure 58 Spread in 1876

Figure 59 Spread in 1877

Figure 60 Extent in 1878

Figure 61 Extent in 1880

Figure 62 Extent in 1885

Figure 63 Extent in 1886

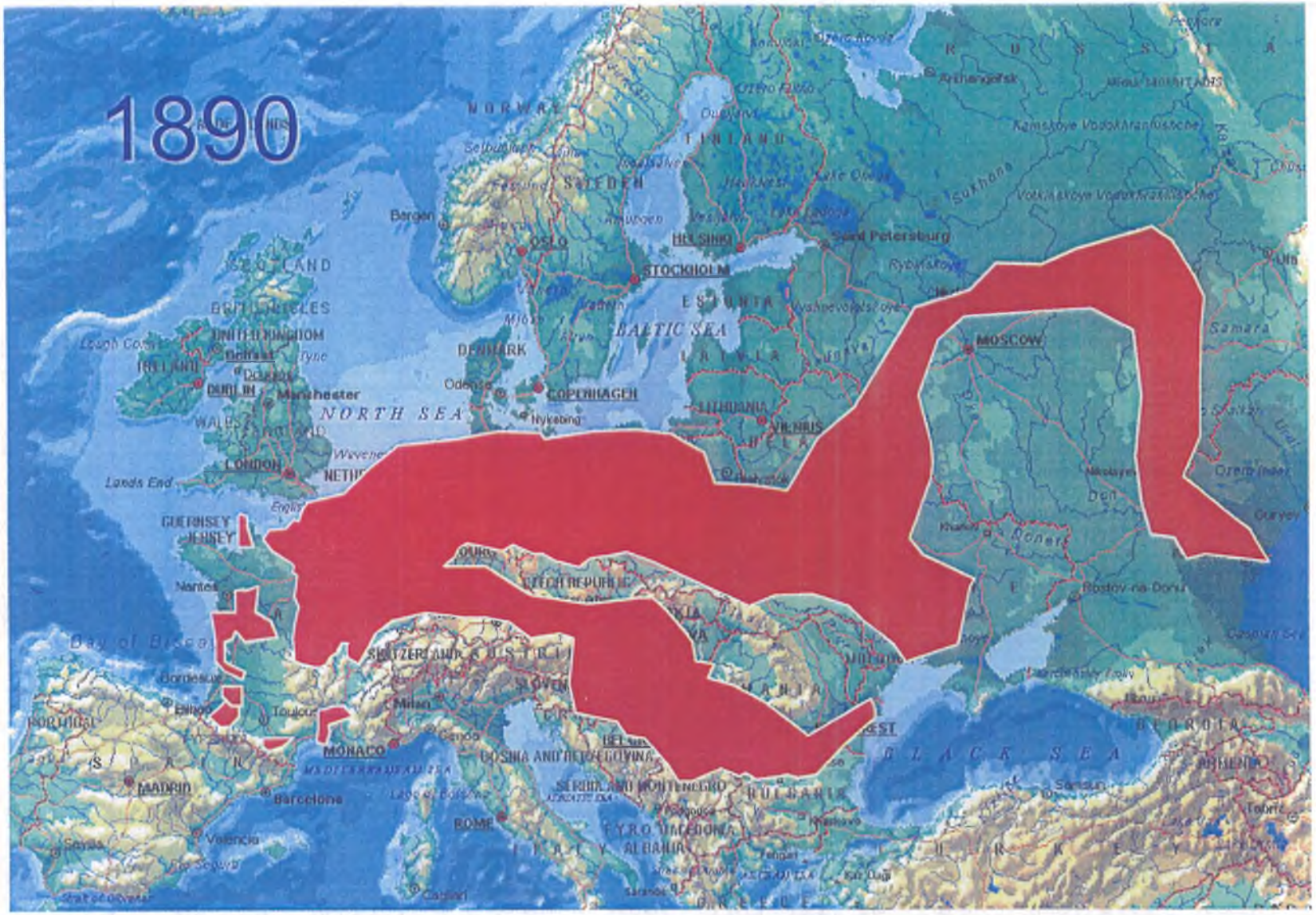

Figure 64 In 1890 into Russia

Figure 65 In 1892 reaches the Baltic States

Figure 66 By the mid 1890s has reached the Urals

Figure 67 Penetrates Finland in 1900

Figure 68 First outbreak in Sweden, 1907

Figure 69 Further spread in Sweden by 1930

Appendix 2 Crayfish Species

The distribution of the only native British crayfish species, the white claw crayfish, *A. pallipes* is indicated in Figure 70 as is the distribution of the signal crayfish, *Pacifastacus leniusculus* and *Astacus leptodactylus*, the Turkish or narrow claw crayfish in England and Wales. Introduced local populations of the noble crayfish, *Astacus astacus* and the red claw or red swamp crayfish, *Procambarus clarkii* also exist as do two populations of *Orconectes limosus*, the spiny cheeked crayfish.

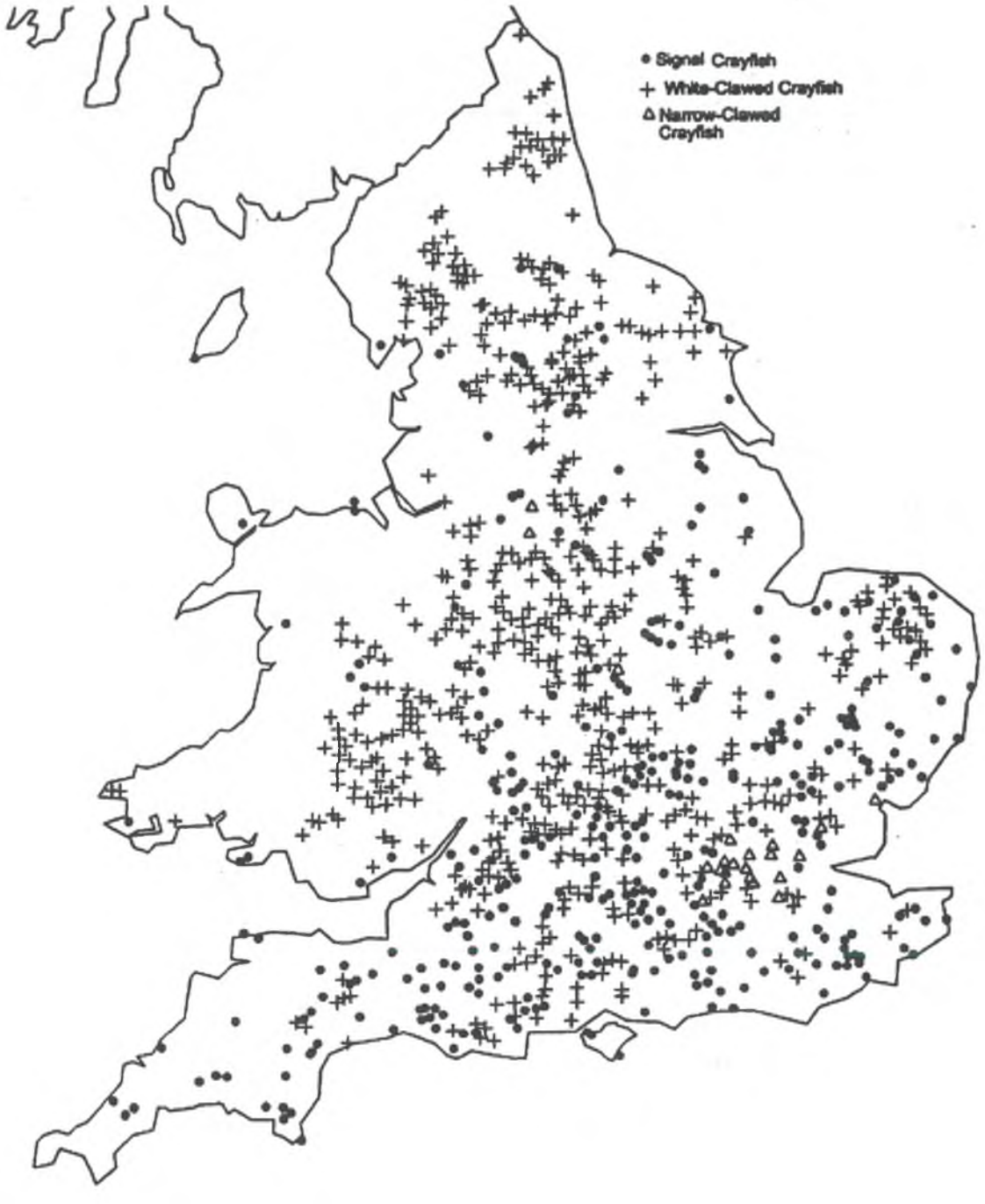

Figure 70 Distribution of crayfish species in Britain (after J. Brickland, Environment Agency)

Whilst populations of red claw, noble and spiny cheeked crayfish in England and Wales have remained very restricted in distribution, the narrow claw and the signal crayfish are now widely distributed. The narrow claw was primarily imported for food purposes although many may have been transferred from that original purpose to use as bait of for stocking would be crayfish farms. The narrow claw has proved an effective escapee and coloniser and is present in many waters, particularly around London. The signal crayfish is also an effective coloniser and was introduced from Scandinavia for purposes of crayfish farming. Most of such would be farm sites made only perfunctory attempts to prevent escape and soon most waters in proximity to such sites contained good populations of signal crayfish. Since signal crayfish are potential non clinical carriers of crayfish plague this represents a major source of infection to the native white claw that is highly susceptible to the disease. The spiny cheeked crayfish has also apparently been introduced into English waters but remains limited in distribution in contrast to the situation in Europe where after introductions into Germany and France it has now become very widely distributed throughout much of Europe excluding Scandinavia.

Table 4 Crayfish species present in U.K. waters.

Species	Common Name	Susceptibility to *A. astaci*	U.K. Distribution	Natural distribution
Austropotamobius pallipes	White claw	High	Alkaline waters of England and Wales	S and W Europe
Astacus astacus	Noble	High	One population, SW England	N and W Europe
Astacus leptodactylus	Narrow claw or Turkish	High	Ponds, canals and rivers in SE England	E Europe to Urals, Asia Minor
Pacifastacus leniusculus	Signal	Resistant	Widely distributed in ponds and rivers	California
Procambarus clarkii	Red claw	Resistant	One or two populations in S England	South Central USA
Orconectes limosus	Spiny cheeked	Resistant	Two sites eastern England	Eastern USA to Canada

Figure 71 gives a rough indication of the natural distribution of the three principal European species of crayfish before the effects of crayfish plague and the widespread introductions of crayfish took place in the 19th and 20th Centuries.

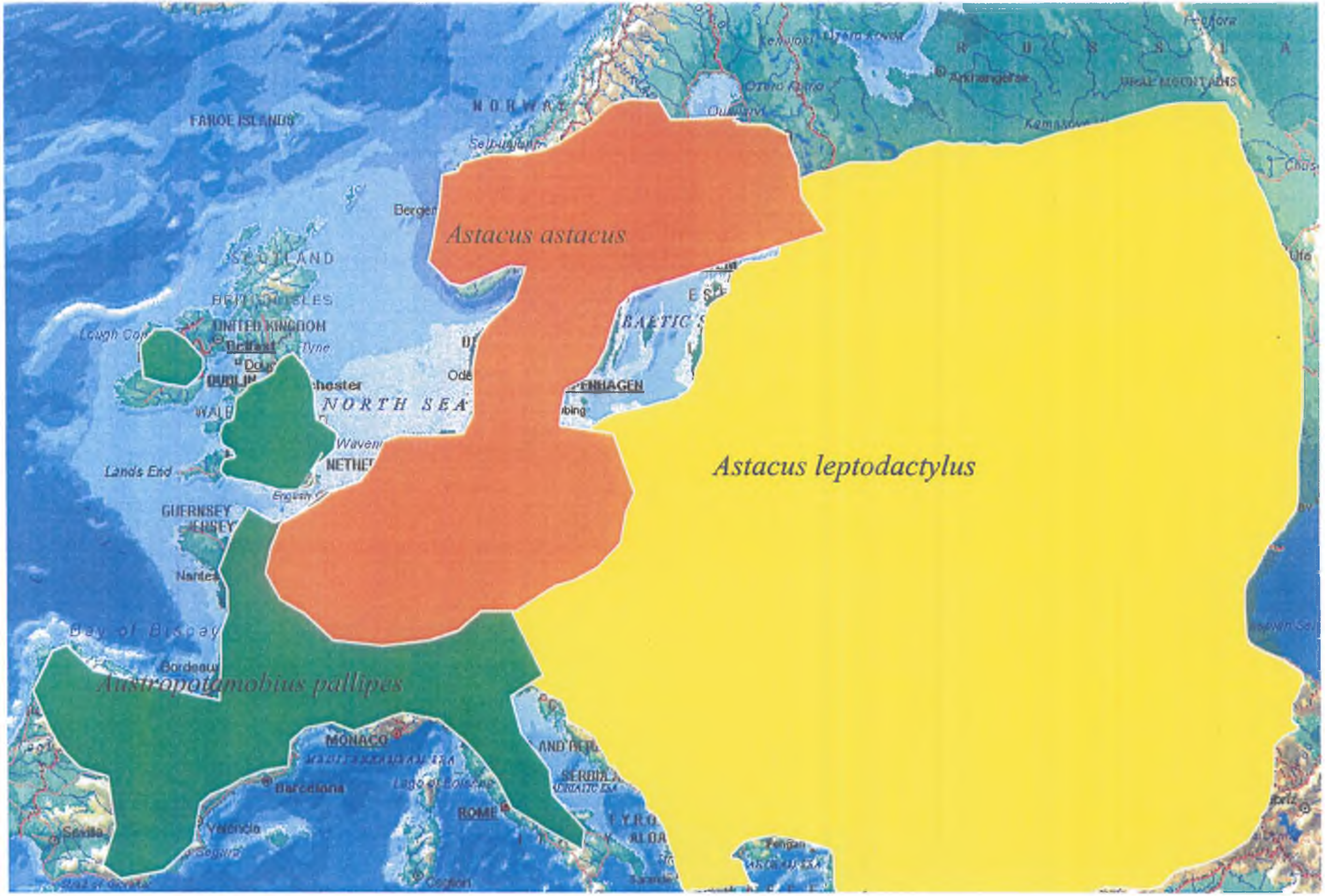

Figure 71 Approximate original distribution of European crayfish species

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