CRAYFISH PLAGUE (*APHANOMYCES ASTACI*)

1. Scope

For the purpose of this chapter, crayfish plague is considered to be infection of crayfish with *Aphanomyces astaci* Schikora.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent of crayfish plague is *Aphanomyces astaci*. *Aphanomyces astaci* is a member of a group of organisms commonly known as the water moulds. Although long regarded to be fungi, this group, the Oomycetida, are now considered protists and are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

There is evidence that established older *A. astaci* strains that have been present in Europe for many years are less aggressive than newer strains introduced with crayfish imports from North America since the 1960s. Some isolates from warmer waters in Spain have temperature/growth curves with higher optimum temperatures compared with those from northern Europe (13).

2.1.2. Survival outside the host

Although *A. astaci* is not an obligate parasite and will grow well under laboratory conditions on artificial media (5, 11), in the natural environment it does not survive well for long periods in the absence of a suitable host.

Observations on the longevity of the various life stages of *A. astaci* have been presented (26, 28). The authors observed that *A. astaci* cysts survive for 2 weeks in distilled water, and that zoospores remained motile for up to 3 days (28). As *A. astaci* can go through three cycles of zoospore emergence, the maximum life span outside of a host could be several weeks. Unestam found still viable spores in a spore suspension kept at 2°C for 2 months (27).

2.1.3. Stability of the agent (effective inactivation methods)

Aphanomyces astaci, both in culture and in infected crayfish, is killed by a short exposure to temperatures of 60°C or to temperatures of –20°C or less for 48 hours or more (2, 19). Sodium hypochlorite and iodophors are effective for disinfection of contaminated equipment. Equipment must be cleaned prior to disinfection, since organic matter was found to decrease the effectiveness of iodophors (4). Thorough drying of equipment (>24 hours) is also effective as *A. astaci* is not resistant to desiccation.

2.1.4. Life cycle

The life cycle of *A. astaci* is simple with vegetative hyphae invading and ramifying through host tissues, eventually producing extramatrical sporangia that release amoeboid primary spores. These initially encyst, but then release a biflagellate zoospore (secondary zoospore). Biflagellate zoospores swim in the water column and, on encountering a susceptible host, attach and germinate to produce invasive vegetative hyphae. Free-swimming zoospores appear to be chemotactically attracted to crayfish cuticle (9) and often settle on the cuticle near a wound (16). Zoospores are capable of repeated encystment and re-emergence, extending the period of their infective viability (10). Growth and sporulation capacity is strain-and temperature-dependent (13).

2.2. Host factors

2.2.1. Susceptible host species

To date, all species of freshwater crayfish have to be considered as susceptible to infection with *A. astaci*. The outcome of an infection varies depending on species. All stages of European crayfish species, including the Noble crayfish (*Astacus astacus*) of north-west Europe, the white clawed 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 highly susceptible (1, 7, 22, 29, 30, 32). Laboratory challenges have demonstrated that Australian species of crayfish are also highly susceptible (30). North American crayfish such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Orconectes* spp. are infected by *A. astaci*, but under normal conditions the infection does not cause clinical disease or death. All North American crayfish species investigated to date have been shown to be susceptible to infection (18, 29, 32) and it is therefore currently assumed that this is the case for any other North American species.

The only other crustacean known to be susceptible to infection by *A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*) but this was reported only under laboratory conditions (8).

2.2.2. Susceptible stages of the host

All live stages need to be considered as susceptible to infection.

2.2.3. Species or subpopulation predilection (probability of detection)

Highly susceptible species: in natural outbreaks of crayfish plague, moribund and dead crayfish of a range of sizes (and therefore ages) can be found.

North American crayfish species: the prevalence of infection tends to be lower in animals that have gone through a recent moult (B. Oidtmann, unpublished data). However, large scale systematic studies have not been undertaken to corroborate these observations. Juvenile crayfish go through several moults per year, whereas adult crayfish usually moult at least once per year in temperate climates. Therefore, animals in which the last moult was some time ago may show higher prevalence compared to animals that have recently moulted.

2.2.4. Target organs and infected tissue

The tissue that becomes initially infected is the exoskeleton cuticle. Soft cuticle, as is found on the ventral abdomen and around joints, is preferentially affected. In the highly susceptible European crayfish species, the pathogen often manages to penetrate the basal lamina located underneath the epidermis cell layer. From there, *A. astaci* spreads throughout the body primarily by invading connective tissue and blood vessels; however, all tissues may be affected.

In North American crayfish species, infection is usually restricted to the cuticle.

2.2.5. Persistent infection with lifelong carriers

A number of North American crayfish species have been investigated for their susceptibility to infection with *A. astaci* and disease (18, 29, 32). So far, infection has been consistently shown in all North American crayfish species tested to date. Animals investigated were usually clinically healthy.

Based on the observations made in North American crayfish species, it seems reasonable to assume that all crayfish species native to the North American continent can be infected with *A. astaci* without development of clinical disease and they may therefore act as lifelong carriers of the pathogen.

2.2.6. Vectors

There is good field and experimental evidence that movements of fish from areas in which crayfish plague is active can transmit infection from one watershed to another (6, 19).

Fomites: A. astaci can also be spread by contaminated equipment (nets, boots, clothing etc.).

2.2.7. Known or suspected wild aquatic animal carriers

A number of studies have shown that tested species native to North America can act as carriers of *A. astaci* (e.g. signal crayfish, spiny cheek crayfish, red swamp crayfish (3, 18). Since all North American species tested to date have been shown to be potential carriers of the disease, it is also assumed that North American species not tested to date are likely to act potentially as carriers of *A. astaci*.

2.3. Disease pattern

2.3.1. Transmission mechanisms

The main routes of spread of the pathogen are through 1) movement of infected crayfish (either the highly susceptible species or North American crayfish species), 2) movement of spores with contaminated water

or equipment, as may occur during fish movements, or 3) through colonisation of habitats by North American crayfish species.

Transmission from crayfish to crayfish occurs, in short, through the release of zoospores from an infected animal and attachment of such zoospores to a naïve crayfish. The zoospores of *A. astaci* swim actively in the water column and have been demonstrated to show positive chemotaxis towards crayfish (9).

The main route of spread of crayfish plague in Europe between the 1960s and 2000 was through the active stocking of North American crayfish into the wild or escapes from crayfish farms (1, 12). Nowadays, spread mainly occurs through expanding populations of North American crayfish, accidental co-transport of specimens, and release of North American crayfish into the wild by private individuals (14, 21).

Colonisation of habitats, initially occupied by highly susceptible species, by North American crayfish species carrying *A. astaci* is likely to result in an epidemic among the highly susceptible animals. The velocity of spread will depend, among other factors, on the prevalence of infection in the population of North American crayfish.

Fish transports may facilitate the spread of *A. astaci* in a number of ways, such as through the presence of spores in the transport water, *A. astaci* surviving on fish skin, co-transport of infected crayfish specimens, or a combination of all three (6, 19). There is also circumstantial evidence of spread by contaminated equipment (nets, boots clothing, etc.).

2.3.2. Prevalence

In the highly susceptible European crayfish species, exposure to *A. astaci* spores is considered to lead to infection and eventually to death. The minimal infectious dose has still not been established, but it may be as low as a single spore per animal (B. Oidtmann, unpublished data). Prevalence of infection within a population in the early stage of an outbreak may be low (only one or a few animals in a river population may be affected). However, the pathogen is amplified in affected animals and subsequently released into the water; usually leading to 100% mortality in a contiguous population. The velocity of spread from initially affected animals depends on several factors, one being water temperature (21). Therefore, the time from first introduction of the pathogen into a population to noticeable crayfish mortalities can vary greatly and may range from a few weeks to months. Prevalence of infection will gradually increase over this time and usually reach 100%.

Prevalence levels in North American crayfish appear to vary greatly. Limited studies suggest prevalence levels ranging from anywhere between 0 and 100% are possible (18).

2.3.3. Geographical distribution

First reports of large crayfish mortalities go back to 1860 in Italy (15, 24). These were followed by further reports of crayfish mortalities, where no other aquatic species were affected, in the Franco-German border region in the third quarter of the 19th century. From there 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, in 1907, to Sweden. In the 1960s, the first outbreaks in Spain were reported and in the 1980s further extensions of infection to the British Isles, Turkey, Greece and Norway were reported (1). The reservoir of the original infections in the 19th century was never established; *Orconectes* spp. were not known to have been introduced until the 1890s, but the post-1960s extensions are largely linked to movements of North American crayfish introduced more recently for purposes of crayfish farming (1). Escapes of such introduced species were almost impossible to prevent and *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe.

Since North American crayfish serve as a reservoir of *A. astaci*, any areas where North American crayfish species are found have to be considered as areas where *A. astaci* is present (unless shown otherwise).

Australia has not experienced any outbreaks of crayfish plague to date and is currently considered free of the disease (OIE WAHID website, accessed April 2009).

2.3.4. Mortality and morbidity

When the infection first reaches a naïve population of highly susceptible crayfish species, high levels of mortality are usually observed within a short space of time, so that the bottoms of lakes, rivers and streams are covered with dead and dying crayfish. A band of mortality will spread from the initial outbreak site downstream almost at the speed of the flow of the river, whereas upstream spread is slower. Where population densities of susceptible crayfish are low fewer zoospores will be produced, the spread of

infection will be slower and evidence of mortality less dramatic. Water temperature has some effect on the speed of spread and this is most evident in low-density crayfish populations where animal-to-animal spread takes longer and challenge intensity will be lower. Lower water temperatures and reduced numbers of zoospores are associated with slower mortalities and a greater range of clinical signs in affected animals (6).

On rare occasions, single specimens of the highly susceptible species have been found after a wave of crayfish plague has gone through a river or lake. This is most likely to be due to lack of exposure of these animals during an outbreak (animals may have been present in a tributary of a river/lake or in a part of the affected river/lake that was not reached by spores, or crayfish may have stayed in burrows during the crayfish plague wave).

Although remnant populations of susceptible crayfish species remain in many European watersheds, the dense populations that existed 150 years ago are now heavily diminished (1, 25). Characteristically, populations of susceptible crayfish may re-establish, but once population density and geographical distribution is sufficient for susceptible animals to come into contact with infection, new outbreaks of crayfish plague in the form of large-scale mortalities will occur.

2.3.5. Environmental factors

Under laboratory conditions, the preferred temperature range at which the *A. astaci* mycelium grows slightly varies depending on the strain. In a study, which compared a number of *A. astaci* strains that had been isolated from a variety of crayfish species, mycelial growth was observed between 4 and 29.5°C, with the strain isolated from *Procambarus clarkii* growing better at higher temperatures compared to the other strains. Sporulation efficiency was similarly high for all strains tested between 4 and 20°C, but it was clearly reduced for the non-*P. clarkii* strains at 25°C and absent at 27°C. In contrast, sporulation still occurred in the *P. clarkii* strain at 27°C. The proportion of motile zoospores (out of all zoospores observed in a zoospore suspension) was almost 100% at temperatures ranging from 4–18°C, reduced to about 60% at 20°C and about 20% at 25°C in all but the P. clarkii strain. In the *P. clarkii* strain, 80% of the zoospores were still motile at 25°C, but no motile spores were found at 27°C (13).

Field observations show that crayfish plague outbreaks occur at a wide temperature range, and at least in the temperature range from 4–20°C. The velocity of spread within a population depends on several factors, including water temperature. In a temperature range between 4 and 16°C, the speed of an epidemic is enhanced by higher water temperatures. At low water temperatures, the epidemic curve can increase very slowly and the period during which mortalities are observed can be several months (B. Oidtmann, unpublished data).

In buffered, redistilled water, sporulation occurs between pH 5 and 8, with the optimal range being pH 5–7. The optimal pH range for swimming of zoospores appears to be in a pH range from 6.–7.5, with a maximum range between pH 4.5 and 9.0 (27).

Zoospore emergence is influenced by the presence of certain salts in the water. $CaCl_2$ stimulates zoospore emergence from primary cysts, whereas $MgCl_2$ has an inhibitory effect. In general, zoospore emergence is triggered by transferring the vegetative mycelium into a medium where nutrients are absent or low in concentration (10).

2.4. Control and prevention

Once *A. astaci* has been introduced into a population of highly susceptible crayfish species in the wild, the spread within the affected population cannot be controlled. Therefore, prevention of introduction is essential. To avoid the main pathways of introduction, the following measures are necessary:

1. Movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other item that might carry the pathogen from an infected to an uninfected site holding susceptible species should be prevented.

2. When fish transfers are being planned, it should be considered whether the source water may harbour infected crayfish (including North American carrier crayfish).

3. Any fish movements from the site of a current epidemic of crayfish plague carries a high risk of spread and should generally be avoided.

4. If fish movements from a source containing North American crayfish are being planned, fish harvest methods at the source site need to ensure that: a) crayfish are not accidentally co-transported; b) the transport water does not carry *A. astaci* spores, and, c) equipment is disinfected between use; d) the consignment does not become contaminated during transport.

5. The release of North American crayfish into the wild should be prevented.

6. Aquaculture facilities for the cultivation of crayfish are very rarely suitable for preventing the spread of crayfish from such sites. Therefore, careful consideration needs to be given, as to whether such facilities should be established.

Certain pathways of introduction, such as the release of North American crayfish by private individuals are difficult to control.

2.4.1. Vaccination

Currently, there is no evidence that vaccines offer long-term protection in crustaceans and even if this were not to be the case, vaccination of natural populations of crayfish is impossible.

2.4.2. Chemotherapy

No treatments are currently known that can successfully treat the highly susceptible crayfish species, once infected.

2.4.3. Immunostimulation

No immunostimulants are currently known that can successfully protect the highly susceptible crayfish species against infection and consequent disease due to *A. astaci* infection.

2.4.4. Resistance breeding

In the 125 years that crayfish plague has been recognised in Europe, no evidence of resistant populations of European crayfish has been found. However, the fact that North American crayfish are not very susceptible means that selection for resistance may be possible and laboratory studies using *A. astaci* strains attenuated for virulence might be successful. However, no such study has yet been undertaken.

2.4.5. Restocking with resistant species

North American crayfish have been used in various European countries to replace the lost stocks of native crayfish. However, since North American crayfish are potential hosts for *A. astaci*, restocking with North American crayfish would further the spread of *A. astaci*. Given the high reproduction rates and the tendency of several North American crayfish species to colonise new habitats, restocking with North American crayfish species would largely prevent the re-establishment of the native crayfish species.

2.4.6. Blocking agents

No data available.

2.4.7. Disinfection of eggs and larvae

Limited information is available on the susceptibility of crayfish eggs to infection with *A. astaci*. Unestam & Söderhäll mention that they experimentally exposed *Astacus astacus* and *P. leniusculus* eggs to zoospore suspensions and were unable to induce infection (31). However, the details of these studies have not been published.

Although published data are lacking, disinfection of larvae, once infected, is unlikely to be successful, since *A. astaci* would be protected from disinfection by the crayfish cuticle, in which it would be present.

2.4.8. General husbandry practices

If a crayfish farm for highly susceptible species is being planned, it should be carefully investigated whether North American crayfish species are in the vicinity of the planned site or whether North American crayfish populations may be present upstream (for sites that are "online" on a stream or abstracting water from a stream), even if at a great distance upstream. If North American crayfish are present, there is a high likelihood that susceptible farmed crayfish will eventually become infected.

In an established site, where the highly susceptible species are being farmed, the following recommendations should be followed to avoid an introduction of *A. astaci* onto the site:

1. Movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other item that might carry the pathogen from an infected to an uninfected site holding susceptible species must be prevented.

2.   If fish transfers are being planned, these must not come from streams or other waters that harbour potentially infected crayfish (either susceptible crayfish populations that are going through a current outbreak of crayfish plague or North American carrier crayfish).

3.   North American crayfish must not be brought onto the site.

4.   Fish obtained from unknown freshwater sources or from sources, where North American crayfish may be present or a current outbreak of crayfish plague may be taking place, must not be used as bait or feed for crayfish, unless they have been subject to a temperature treatment that will kill *A. astaci* (see Section 2.1.3).

5.   Any equipment that is brought onto site should be disinfected.

6.   General biosecurity measures should be in place (e.g. controlled access to premises; disinfection of boots when site is entered; investigation of mortalities if they occur; introduction of live animals (crayfish, fish) only from sources known to be free of crayfish plague).

3.   Sampling

3.1. Selection of individual specimens

In the case of a suspected outbreak of crayfish plague in a population of highly susceptible crayfish species, the batch of crayfish selected for investigation for the presence of *A. astaci* should ideally consist of: a) live crayfish showing signs of disease, b) live crayfish appearing to be still healthy, and, c) dead crayfish that may also be suitable, although this will depend on their condition.

Live crayfish should be transported using polystyrene containers equipped with small holes to allow aeration, or an equivalent container. The temperature in the container should not exceed 16°C.

The container should provide insulation against major temperature differences outside the container. In periods of hot weather, freezer packs should be used to avoid temperatures deleterious to the animals. These can be attached at the inside bottom of the transport container. The crayfish must however be protected from direct contact with freezer packs. This can be achieved using, for instance, cardboard or a several layers of newspaper.

Crayfish should be transported in a moist atmosphere, for example using moistened wood shavings/wood wool, newspaper or grass/hay. Unless transport water is sufficiently oxygenated, live crayfish should not be transported in water, as they may suffocate from lack of oxygen.

The time between sampling of live animals and delivery to the investigating laboratory should not exceed 24 hours.

Should only dead animals be found at the site of a suspected outbreak, these might still be suitable for diagnosis. Depending on the condition they are in, they can either be: a) transported chilled (if they appear to have died only very recently), or, b) placed in non-methylated ethanol (minimum concentration 70%; see Section 3.2).

Animals showing advanced decay are unlikely to give a reliable result, however, if no other animals are available, these might still be tested.

3.2. Preservation of samples for submission

The use of non-preserved crayfish is preferred, as described above. If, for practical reasons, transport of recently dead or moribund crayfish cannot be arranged quickly, crayfish may be fixed in ethanol (minimum 70%). However, fixation may reduce test sensitivity. Only non-methylated ethanol should be used for fixation as other types of alcohol might be detrimental for subsequent analysis. The crayfish:ethanol ratio should be at least 1:3 (1 part crayfish, 3 parts ethanol), although the relative proportion of ethanol may be increased.

3.3. Pooling of samples

Not recommended.

3.4. Best organs or tissues

In highly susceptible species, the tissue recommended for sampling is the soft abdominal cuticle, which can be found on the ventral side of the abdomen.

In the North American crayfish species, sampling of soft abdominal cuticle and telson are recommended.

3.5. Samples/tissues that are not suitable

Autolytic material is not suitable for analysis.

4. Diagnostic methods

Until recently, the diagnosis of crayfish plague from highly susceptible species strictly required the isolation and characterisation of the pathogen, *A. astaci*, using simple mycological media fortified with antibiotics to control bacterial contamination (6). Isolation is only likely to be successful before or within 24 hours of the death of infected crayfish. However, there is no other disease or pollution effect that is known to 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. However, the publication of molecular methods has now rendered diagnosis easier (17, 18, 20).

4.1. Field diagnostic methods

4.1.1. Clinical signs

Highly susceptible species

Gross clinical signs are extremely variable and depend on challenge severity and water temperatures. The first sign of a crayfish plague mortality may be 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 remain unable to right themselves. Often, however, unless waters are carefully observed, the first sign that there is a problem will be the presence of large numbers of dead crayfish in a river or lake (6)

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 50 km may lose all their crayfish in less than 21 days from the first observed mortality (D. Alderman, pers. comm.). Crayfish plague has unparalleled severity of effect, since infected susceptible crayfish generally do not survive and 100% mortality is the norm. It must be emphasised, however, that the presence of large numbers of dead crayfish, even in crayfish plague-affected watersheds, is not on its own sufficient for diagnosis. 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 such as cypermethrin have been associated with initial misdiagnoses).

North American crayfish species

None available.

4.1.2. Behavioural changes

Highly susceptible species

Infected crayfish of the highly susceptible crayfish species may leave their hides during daytime (which is not normally seen in crayfish), have a reduced escape reflex, and progressive paralysis. Dying crayfish are sometimes found lying on their backs. The animals are often no longer able to upright themselves. Occasionally, the infected animals can be seen trying to scratch or pinch themselves.

North American crayfish species

Infected North American crayfish do not show any behavioural changes (B. Oidtmann, unpublished data).

4.2. Clinical methods

4.2.1. Gross pathology

Highly susceptible species

Depending on a range of factors, the foci of infection in crayfish may be seen by the naked eye or may not be discernable 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 colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles 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 abdomen, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.

North American crayfish species

Infected North American crayfish can sometimes show melanised spots in their soft cuticle, for example the soft abdominal cuticle. However, it must be stressed that these melanisations can be caused by mechanical injuries or infections with other water moulds and are very unspecific. Conversely, visible melanisation is not always associated with carrier status. Infected animals can appear completely devoid of visible melanisations.

4.2.2. Clinical chemistry

No suitable methods available.

4.2.3. Microscopic pathology

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. Additionally, such material does not prove that any hyphae observed are those of the primary pathogen. A histological staining technique, such as the Grocott silver stain counterstained with conventional haematoxylin and eosin, can be used.

See also Section 4.2.4.

4.2.4. Wet mounts

Small pieces of soft cuticle excised from the regions mentioned above (Section 4.2.1) and examined under a compound microscope using low to medium power will confirm the presence of aseptate fungal hyphae 7–9 µm wide. The presence of host haemocytes and melanisation closely associated with and encapsulating the hyphae give good presumptive evidence that the hyphae represent a pathogen rather than a secondary opportunist invader. In some cases, examination of the surface of such mounted cuticles will demonstrate the presence of characteristic *A. astaci* sporangia with clusters of encysted primary spores (see Section 4.3).

4.2.5. Smears

Not suitable.

4.2.6. Electron microscopy/cytopathology

Not suitable.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

As indicated above (Section 4.2.4.), presumptive identification of *A. astaci* may be made from the presence of hyphae and sporangia of the correct morphological types (see below) on the surface of crayfish exoskeletons.

4.3.1.1.2. Smears

Not suitable.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Highly susceptible species

Care should be taken that animals to be used for isolation of *A. astaci* via cultivation are not exposed to desiccation.

Isolation methods are as described by (5). 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.

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 is defined as any natural river or lake water, as opposed to demineralised water.

Any superficial contamination should first be removed from the soft intersternal abdominal cuticle or any other areas from which cuticle will be excised by thoroughly wiping the cuticle with a wet (using autoclaved H_2O) clean disposable paper towel. Simple aseptic excision of infected tissues, which are then placed as small pieces (1–2 mm^3) 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 discernable 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 colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles 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 water and there further cut into small pieces with sterile instruments for transfer to isolation medium (IM). Suitable instruments for such work are cataract knives and fine electron microscope or instrument-grade forceps and scissors.

Inoculated agar can be incubated at temperatures between 16°C and 24°C. The Petri dishes should be sealed with a sealing film (e.g. Parafilm[1]) to avoid desiccation.

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 (5). 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 river water (natural water with available cations encourages sporulation better than 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 river water in a sterile stainless steel sieve, before transfer into fresh sterile river 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 river water relative to the amount of fungus being transferred (100:1). Sporangia are myceloid, terminal or intercalary, developing from undifferentiated vegetative hyphae. The 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.

---

1   Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

Within developing sporangia, the cytoplasm cleaves into a series of elongate units (10–25 × 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 that encysts at the sporangial orifice and probably represents the suppressed saprolegniaceous primary zoospore. No evidence has been found 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 for the majority of European isolates of *A. astaci* are between 16 and 24°C (5). For some isolates, particularly from Spanish waters, slightly higher optimal temperatures may prevail (12). 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. *A. astaci* does not survive at –5°C and below for more than 24 hours in culture, although –20°C for >48 hours may be required in infected crayfish tissues, nor does it remain viable in crayfish tissues that have been subject to normal cooking procedures (2, 19).

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 the spore shape changes gradually from spherical to reniform. Flagellar attachment is lateral (23); zoospores are typical saprolegniaceous secondary zoospores measuring 8 × 12 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 48 hours (5).

Test sensitivity and specificity of the cultivation method can be very variable depending on the experience of the examiner, but in general will be lower than the polymerase chain reaction (PCR).

North American crayfish species

Isolation of *A. astaci* by culture following the methods described for the highly susceptible species usually fails. Currently, the recommended method for detecting infection in such species is by PCR.

4.3.1.2.2. Antibody-based antigen detection methods

None available.

4.3.1.2.3. Molecular techniques

The PCR method described follows the method of (18). Amplification of a 569 bp fragment of *A. astaci* DNA is performed using primers targeting the ITS region (internal transcribed spacer) of the protist: 5'-GCT-TGT-GCT-GAG-GAT-GTT-CF-3' (primer 42) and 5'-CTA-TCC-GAC-TCC-GCA-TTC-TG-3' (primer 640).

Animals

Whenever possible, moribund or recently dead (<24 hours) crayfish should be used for analysis using the PCR method. Live crayfish have to be killed, which can be done using chloroform. When the only

animals available are animals that have died a few days prior to DNA extraction, they can be tested, but as degradation of DNA may have occurred, a negative result must be interpreted with caution. If circumstances prevent delivery of crayfish to the specialist laboratory within 24 hours, fixation in 70% ethanol (≥3:1 ethanol to crayfish tissue) is possible, but may result in a reduction of the DNA yield.

DNA extraction from crayfish soft abdominal cuticle

Any superficial contamination should first be removed from the soft intersternal abdominal cuticle by thoroughly wiping the soft abdominal cuticle with a wet (using autoclaved H_2O) clean disposable paper towel. It is then excised and 30–50 mg ground in liquid nitrogen to a fine powder using a pestle and mortar (alternative grinding techniques may be used, but should be compared with the liquid nitrogen method before routine use). DNA is then extracted from the ground soft abdominal cuticle using a proteinase K-based DNA extraction method (e.g. DNeasy tissue kit; Qiagen, Hilden, Germany; protocol for insect tissue) following the manufacturer's instructions.

4.3.1.2.3.1. PCR

Highly susceptible species

The PCR is carried out in a 50 µl reaction volume containing 1 × PCR buffer (75 mM Tris/HCl, pH 8.8, 20 mM [NH$_4$]2SO$_4$, 0.01% (v/v) Tween 20), 1.5 mM MgCl$_2$, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, 0.5 µM of each primer, and 1.25 units of DNA polymerase (e.g. Thermoprime Plus DNA Polymerase; AB Gene, Epsom, UK) or equivalent Taq polymerase and 2 µl DNA template. The mixture is denatured at 96°C for 5 minutes, followed by 50 amplification cycles of: 1 minute at 96°C, 1 minute at 59°C and 1 minute at 72°C followed by a final extension step of 7 minutes at 72°C. Amplified DNA is analysed by agarose gel electrophoresis. Confirmation of the identity of the PCR product by sequencing is recommended.

The diagnostic test sensitivity largely depends on the quality of the samples taken. The test sensitivity in animals that died from crayfish plague within 12 hours prior to sampling or in live crayfish showing clear clinical signs of disease is expected to be near 100%. Studies to investigate the effect of sensitivity loss caused by deteriorating sample quality (for instance because of delayed sampling, processing or unsuitable storage of samples) have not been carried out. Diagnostic test specificity has been investigated (18) and, when protocols for DNA extraction and PCR are followed using primers 42 and 640 as described in ref. 18, should be 100%.

North American crayfish species

For carrier identification, 30–50 mg tissue from each soft abdominal cuticle and telson are sampled, DNA is extracted and submitted to PCR separately (18). The PCR protocol described for susceptible crayfish species is used but the diagnostic test has not gone through a formal validation process. However, based on current knowledge, when protocols for DNA extraction and PCR are followed using primers 42 and 640 as described in ref. 18, the test sensitivity for PCR is estimated to be near 100%.

4.3.1.2.3.2. Sequencing

Amplified DNA (569 bp) is analysed by agarose gel electrophoresis. PCR products are excised from the gel and purified (e.g. using the Freeze n' Squeeze DNA purification system, Anachem, Luton, UK). Sequencing follows the method of Sanger. Both DNA strands should be sequenced using the primers used in the initial amplification. The consensus sequence is generated using the sequence analysis software and submitted to a blast search (genbank). If 100% identify with submitted *A. astaci* sequences is found, the amplified product is *A. astaci*. Alternatively, primers ITS-1 (5'-TCC-GTA-GGT-GAA-CCT-GCG-G-3') and ITS-4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3') (33) can be used to generate an amplicon of 757 bp and provide a sequence in the same region, but expanded at both ends beyond the sequence generated by primers BO42 and BO640.

4.3.1.2.4. Agent purification

None available.

4.3.2. Serological methods

None available.

5. Rating of tests against purpose of use

The methods currently available for diagnosis of clinical diseases of crayfish plague (*Aphanomyces astaci*) in highly susceptible species are listed in Table 5.1. Methods for targeted surveillance to demonstrate freedom from disease in highly susceptible species are displayed in Table 5.2.

Clinical disease is extremely rare in North American crayfish. Therefore a rating of methods for diagnosing clinical disease in these species is not provided. However, methods for targeted surveillance to demonstrate freedom from infection in North American crayfish are listed in Table 5.3.

The designations used in the tables indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Crayfish plague diagnostic methods in highly susceptible crayfish species*

Method	Presumptive diagnosis	Confirmatory diagnosis
Gross and microscopic signs	b	c
Isolation and culture	b	b
Histopathology	d	d
PCR	a	b
Sequencing of PCR products	n/a	a
Transmission EM	d	d
Antibody-based assays	n/a	n/a
DNA probes – *in situ*	n/a	n/a

PCR = polymerase chain reaction; EM = electron microscopy; n/a = not applicable or not available

Table 5.2. *Methods for targeted surveillance in highly susceptible crayfish species to declare freedom from crayfish plague*

Method	Screening method	Confirmatory method
Inspection for gross signs and mortality	a	c
Microscopic signs (wet mounts)	c	c
Isolation and culture	c	b
Histopathology	d	d
PCR	a	b
Sequencing of PCR products	n/a	a
Transmission EM	d	d
Antibody-based assays	n/a	n/a
DNA probes – *in situ*	n/a	n/a

PCR = polymerase chain reaction; EM = electron microscopy; n/a = not applicable or not available

Table 5.3. *Methods for targeted surveillance in North American crayfish species to declare freedom from crayfish plague*

Method	Screening method	Confirmatory method
Gross and microscopic signs	d	d
Isolation and culture	c	c
Histopathology	d	d
PCR	a	b
Sequencing of PCR products	n/a	a
Transmission EM	d	d
Antibody-based assays	n/a	n/a
DNA probes – *in situ*	n/a	n/a

PCR = polymerase chain reaction; EM = electron microscopy; n/a = not applicable or not available

6. Test(s) recommended for targeted surveillance to declare freedom from crayfish plague (*Aphanomyces astaci*)

6.1. Highly susceptible species

Crayfish farms keeping susceptible crayfish would need to be inspected at a frequency outlined in Chapter 2.2.0. A history of no mortalities (this does not include losses due to predation) occurring within the population over a period of at least 12 months combined with absence of clinical signs, as well as gross and microscopic pathology at the time of inspection are suitable methods for this purpose. Surveillance of wild crayfish stocks presents greater problems, especially where the species concerned is endangered. As movements of fish stocks from infected waters present a risk of disease transmission, monitoring the status of crayfish populations to confirm that they remain healthy, is necessary.

In a farm setting, an infection with crayfish plague should be noticed relatively quickly, due to a relatively quick onset of mortalities in the farmed crayfish population.

To undertake targeted surveillance, regular inspections are recommended, where samples are collected if there is any suspicion of mortality or disease. If moribund or dead animals are found, it is recommended that samples are analysed using PCR and if amplicons of the expected size are amplified, that these (or PCR products generated using primers ITS-1 and -4) are sequenced and the sequences analysed.

6.2. North American crayfish species

In North American crayfish species, animals would need to be sampled and analysed using the PCR assay described above. This applies to both farmed and naturalised stocks, and surveillance programmes need to take into account the risks of indirect transmission by movements of fish.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Highly susceptible crayfish species

Any extensive mortality solely of the highly susceptible species of freshwater crayfish where all other aspects of the flora and fauna, particularly other aquatic crustaceans, are normal and healthy.

North American crayfish species

Any population of North American crayfish is generally to be regarded as potentially infected with *A. astaci*.

7.2. Definition of confirmed case

Highly susceptible crayfish species

Confirmation of presence of *A. astaci* by PCR and sequencing of PCR products.

North American crayfish species

Confirmation of presence of *A. astaci* by PCR and sequencing of PCR products.

8. References

1. ALDERMAN D.J. (1996). Geographical spread of bacterial and fungal diseases of crustaceans. *Rev sci. tech. Off. int. Epiz.,* **15** (2), 603–632.

2. ALDERMAN D.J. (2000). Summary final report: effects of exposure to high and low temperatures on the survival of the crayfish plague fungus *A. astaci in vitro* and *in vivo.* Australian Quarantine and Inspection Service, Canberra, Australia.

3. ALDERMAN D.J., HOLDICH D. & REEVE I. (1990). Signal crayfish as vectors in crayfish plague in Britain. *Aquaculture*, **86**, 3–6.

4. ALDERMAN D.J. & POLGLASE J.L. (1985). Disinfection for crayfish plague. *Aquaculture and Fisheries Management,* **16**, 203–205.

5. ALDERMAN D.J. & POLGLASE J.L. (1986). *Aphanomyces astaci:* isolation and culture. *J. Fish Dis.*, **9**, 367–379.

6. ALDERMAN D.J., POLGLASE J.L. & FRAYLING M. (1987). *Aphanomyces astaci* pathogenicity under laboratory and field conditions. *J. Fish Dis.*, **10**, 385–393.

7. ALDERMAN D.J., POLGLASE J.L., FRAYLING M. & HOGGER J. (1984). Crayfish plague in Britain. *J. Fish Dis.*, **7**, 401–405.

8. BENISCH J. (1940). Kuenstlich hervorgerufener *Aphanomyces* Befall bei Wollhandkrabben. *Zeitschrift fuer Fischerei*, **38**, 71–80.

9. CERENIUS L. & SÖDERHÄLL K. (1984). Chemotaxis in *Aphanomyces astaci*, an arthropod-parasitic fungus. *J. Invertebr. Pathol.,* **43**, 278–281.

10. CERENIUS L. & SÖDERHÄLL K. (1984). Repeated zoospore emergence from isolated spore cysts of *Aphanomyces astaci. Exp. Mycol.,* **8**, 370–377.

11. CERENIUS L, SÖDERHÄLL K, PERSSON M, AJAXON R (1988). The crayfish plague fungus, *Aphanomyces astaci* – diagnosis, isolation and pathobiology. *Freshw. Crayfish*, **7**, 131–144.

12. DEHUS P., BOHL E., OIDTMANN B., KELLER M., LECHLEITER S. & PHILLIPSON S. (1999). German conservation strategies for native crayfish species with regard to alien species. *In:* Crustacean Issues 11: Crayfish in Europe as Alien Species (How to Make the Best of a Bad Situation?), Gherardi F. & Holdich D.M., eds. A.A Balkema, Rotterdam, The Netherlands, pp. 149–160.

13. DIEGUEZ URIBEONDO J., HUANG T.-S., CERENIUS L. & SODERHALL K. (1995). Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii. Mycol. Res.*, **99**, 574–578.

14. EDSMAN L. (2004) The Swedish story about import of live crayfish. *Bulletin Français de la Pêche et de la Pisciculture,* **372–373**, 281–288.

15. NINNI A.P. (1865). Sulla mortalita dei gambari (*Astacus fluviatilis* L.) nel veneto e piu particalarmenta nella provincia trevigiana. *Alti Inst. Veneto Ser. III,* **10**, 1203–1209.

16. NYHLEN L. & UNESTAM T. (1980). Wound reactions and *Aphanomyces astaci* growth in crayfish cuticle. *J. Invertebr. Pathol.*, **36**, 187–197.

17. OIDTMANN B., BAUSEWEIN S., HÖLZLE L., HOFFMANN R. & WITTENBRINK M. (2002). Identification of the crayfish plague fungus *Aphanomyces astaci* by polymerase chain reaction and restriction enzyme analysis. *Vet. Microbiol.,* **85**, 183–194.

18. OIDTMANN B., GEIGER S., STEINBAUER P., CULAS A. & HOFFMANN R.W. (2006). Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. *Dis. Aquat. Org.,* **72**, 53–64.

19. OIDTMANN B., HEITZ E., ROGERS D. & HOFFMANN R.W. (2002). Transmission of crayfish plague. *Dis. Aquat. Org.,* **52**, 159–167.

20. OIDTMANN B., SCHAEFERS N, CERENIUS L., SÖDERHÄLL K. & HOFFMANN R.W. (2004). Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. *Vet. Microbiol.*, **100**, 269–282.

21. OIDTMANN B., THRUSH M., ROGERS D. & PEELER E. (2005). Pathways for transmission of crayfish plague, Aphanomyces astaci, in England and Wales. Meeting of the Society for Veterinary Epidemiology and Preventive Medicine. Nairn, Inverness, Scotland, 30.03.-01.04.05, Poster.

22. RAHE R. & SOYLU E. (1989). Identification of the pathogenic fungus causing destruction to Turkish crayfish stocks (*Astacus leptodactylus*). *J. Invertebr. Pathol.,* **54**, 10–15.

23. SCOTT W.W. (1961). A monograph of the genus *Aphanomyces. Virginia Agricultural Research Station Technical Bulletin*, **151**, 1–95.

24. SELIGO A. (1895). Bemerkungen ueber die Krebspest, Wasserpest, Lebensverhaeltnisse des Krebses. *Zeitschrift fuer Fischerei und deren Hilfswissenschaften*, **3**, 247–261.

25. SOUTY-GROSSET C, HOLDICH DM, NOEL. PY, REYNOLDS JD AND HAFFNER P (Eds) (2006) Atlas of Crayfish in Europe. Series: Collection Patrimoines Naturels 64, 188 pages, colour photos, maps. Muséum National d'Histoire Naturelle

26. SVENSSON E. & UNESTAM T. (1975). Differential induction of zoospore encystment and germination in *Aphanomyces astaci*, Oomycetes. *Physiologia Plantarum*, **35**, 210–216.

27. UNESTAM T. (1966). Studies on the crayfish plague fungus *Aphanomyces astaci*. II. Factors affecting zoospores and zoospore production. *Physiologia Plantarum*, **19**, 1110–1119.

28. UNESTAM T. (1969). On the adaptation of *Aphanomyces astaci* as a parasite. *Physiologia Plantarum*, **22**, 221–235.

29. UNESTAM T. (1969). Resistance to the crayfish plague in some American, Japanese and European crayfishes. *Report of the Institute of Freshwater Research*, 202–206.

30. UNESTAM T. (1976). Defence reactions in and susceptibility of Australian and New Guinean freshwater crayfish to European-crayfish-plague fungus. *The Australian Journal of Experimental Biology and Medical Science*, **53**, 349–359.

31. UNESTAM T. & SÖDERHÄLL K. (1977). Specialisation in crayfish defence and fungal aggressiveness upon crayfish plague infection. *Freshw. Crayfish*, **3**, 321–331.

32. UNESTAM T. & WEISS D.W. (1970). The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. *J. Gen. Microbiol.,* **60**, 77–90.

33. WHITE T.J., BRUNS T., LEE S. & TAYLOR J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* PCR Protocols: A Guide to Methods and Applications, Innis M.A., Gelfand D.H., Sninsky J.J., White T.J., eds. Academic Press, San Diego, California, USA, pp. 315–322.

*
* *

NB: There are OIE Reference Laboratories for Crayfish plague (*Aphanomyces astaci*) (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).