CHAPTER 2.2.2.

INFECTION WITH *APHANOMYCES ASTACI* (CRAYFISH PLAGUE)

1. Scope

Infection with *Aphanomyces astaci* means infection with the pathogenic agent *A. astaci*, Phylum Oomycota. The disease is commonly known as crayfish plague.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Aphanomyces astaci is a water mould. The Oomycetida or Oomycota, are considered protists and are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

Five groups (A–E) of *A. astaci* have been described based on random amplification of polymorphic DNA polymerase chain reaction (RAPD PCR) (Dieguez-Uribeondo *et al.,* 1995; Huang *et al.,* 1994; Kozubikova *et al.,* 2011). Additional geno- or haplotypes are still being detected using molecular methods (Di Domenico *et al*., 2021). Group A (the so called *Astacus* strains) comprises strains isolated from several European crayfish species. These strains are thought to have been in Europe for a long period of time. Group B (*Pacifastacus* strains I) includes isolates from several European crayfish species and from the invasive *Pacifastacus leniusculus* in Europe as well as Lake Tahoe, USA. Imported to Europe, *P. leniusculus* probably introduced this genotype of *A. astaci* and infected the native European crayfish. Group C (*Pacifastacus* strains II) consists of a strain isolated from *P. leniusculus* from Pitt Lake, Canada. Another strain (Pc), isolated from *Procambarus clarkii* in Spain, sits in group D (*Procambarus* strains). This strain shows temperature/growth curves with higher optimum temperatures compared with groups A and B (Dieguez-Uribeondo *et al*., 1995). *Aphanomyces astaci* strains that have been present in Europe for many years (group A strains) appear to be less pathogenic than strains introduced with crayfish imports from North America since the 1960s. North American host species spiny-cheek crayfish (*Orconectes limosus*) has been shown to be a carrier of Group E (Kozubíková *et al.,* 2011).

2.1.2. Survival and stability in processed or stored samples

Aphanomyces astaci is poorly resistant against desiccation and does not survive long in decomposing hosts. Any treatment of the crayfish (freezing, cooking, drying) will affect the survival of the pathogen (Oidtmann *et al.,* 2002). Isolation from processed samples is not possible, however they may be suitable for molecular methods used for pathogen detection.

2.1.3. Survival and stability outside the host

Outside the host *Aphanomyces astaci* is found as zoospores that remain motile for up to 3 days and form cysts that can survive for 2 weeks in distilled water. As *A. astaci* can go through three cycles of encystment and zoospore emergence, the maximum life span outside of a host could be several weeks. Spores remained viable in a spore suspension in clean water kept at 2°C for 2 months (Unestam, 1966). Survival time is probably shorter in natural waters.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

The recommendations in this chapter apply to all species of crayfish in all three crayfish families (Cambaridae, Astacidae and Parastacidae).

[**Note**: an assessment of species that meet the criteria for listing as susceptible to infection with *A. astaci* in accordance with Chapter 1.5. has not yet been completed]

2.2.2. Species with incomplete evidence for susceptibility

[Under study]

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

The host species susceptible to infection with *A. astaci* fall largely into two categories: those that develop clinical disease and experience mortality, and those that are infected but do not display any significant clinical disease or experience mortality. All life stages of susceptible species are considered susceptible to infection with *A. astaci*.

Species that develop clinical disease and experience mortality include 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 Danube crayfish (*Pontastacus leptodactylus*) of eastern Europe and Asia Minor (e.g. Holdich *et al*., 2009). Australian species of freshwater crayfish are also considered vulnerable to clinical disease and mortality.

Species that can be infected but do not normally develop clinical disease include North American crayfish species such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Faxonius* spp. All North American crayfish species that have been investigated have been shown to be susceptible to infection, demonstrated by the presence of the pathogen in host cuticle (reviewed by Svoboda *et al*. 2017).

Clinical disease outbreaks caused by infection with *A. astaci* are generally known as 'crayfish plague' outbreaks. In such outbreaks, moribund and dead crayfish of a range of sizes (and therefore ages) can be found.

The only non-crayfish crustacean species known to be susceptible to infection by *A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*) (Schrimpf *et al.,* 2014).

2.2.4. Distribution of the pathogen in the host

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 European crayfish species, which are prone to development of clinical disease, 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 haemal sinuses; however, all tissues may be affected.

In North American crayfish species, infection is usually restricted to the cuticle. Based on PCR results, the tailfan (consisting of uropods and telson) and soft abdominal cuticle appear to be frequently infected (Oidtmann *et al.,* 2006; Vralstad *et al.,* 2011).

2.2.5. Aquatic animal reservoirs of infection

North American crayfish species act mostly as reservoirs of the infection without showing clinical signs. However, some strains of *A. astaci,* especially from group A, show lowered virulence, enabling European crayfish to act as reservoirs as well (see review by Svoboda *et al*., 2017).

Colonisation of habitats by North American crayfish species carrying *A. astaci* is likely to result in an epizootic if crayfish species that are prone to expression of clinical disease are present.

2.2.6. Vectors

None known.

2.3. Disease pattern

2.3.1. Mortality, morbidity, and prevalence

When the infection first reaches a naïve population of crayfish species that are prone to clinical disease, high levels of mortality are usually observed within a short space of time. In areas with high crayfish densities the bottoms of lakes, rivers and streams become covered with dead and dying crayfish. A band of mortality will spread quickly from the initial outbreak site downstream, whereas upstream spread is slower. Lower water temperatures are associated with a lower rate of mortalities and a greater range of clinical signs in affected animals (Alderman *et al*., 1987). Observations from Finland suggest that at low water temperatures, noble crayfish (*Astacus astacus*) can be infected for several months without any noticeable mortalities (Viljamaa-Dirks *et al*., 2013).

On rare occasions, single specimens of species that are prone to clinical disease have been found after a wave of infection with *A. astaci* 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 or 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 epizootic). However, low virulent strains of *A. astaci* have been described to persist in a waterway, kept alive by a weak infection in the remnant population (Viljamaa-Dirks *et al*., 2011). Although remnant populations of susceptible crayfish species remain in many European watersheds, the dense populations that existed 150 years ago are now heavily diminished (Souty-Grosset *et al*., 2006; Holdich *et al*., 2009). Populations of susceptible crayfish may re-establish, but once population density and geographical distribution is sufficient for susceptible animals to come into contact with spores, new outbreaks of infection with *A. astaci* and large-scale mortalities may occur.

In European crayfish species, which are prone to clinical disease, exposure to *A. astaci* spores usually leads to infection and eventually to death. Prevalence of infection within a population in the early stage of an outbreak may be low (few animals in a river population may be affected). However, the pathogen amplifies in affected animals and is subsequently released into the water; usually leading to 100% mortality in a contiguous population. The rate of spread from initially affected animals depends on several factors, one being water temperature. 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%. Data from a noble crayfish population in Finland that experienced an acute mortality event due to infection with *A. astaci* in 2001 suggest that in sparse noble crayfish populations, spread of disease throughout the host population may take several years (Viljamaa-Dirks *et al.,* 2011).

2.3.2. Clinical signs, including behavioural changes

Susceptible species prone to clinical disease

Gross clinical signs are variable and depend on challenge severity and water temperatures. The first sign of an outbreak may be the appearance of crayfish during daylight (crayfish are normally nocturnal), some of which may show loss of co-ordination, falling onto their backs and remaining unable to right themselves. Occasionally, the infected animals can be seen trying to scratch or pinch themselves.

Often, however, the first sign of an outbreak may be the presence of large numbers of dead crayfish in a river or lake (Alderman *et al.,* 1987).

Infection with *A. astaci* may cause mass mortality of crayfish. However, investigation of mortality event should consider other causes such as environmental pollution (e.g. insecticides such as cypermethrin have been associated with initial misdiagnoses).

Susceptible species that do not normally develop clinical disease

Infected North American crayfish may be subclinical reservoirs. Controlled exposure to a highly virulent strain has resulted in mortality in juvenile stages of *Pacifastacus leniusculus* as well as behavioural alterations in adults (Thomas *et al.,* 2020).

2.3.3 Gross pathology

Susceptible species prone to clinical disease

Depending on a range of factors, the foci of infection in crayfish may be seen by the naked eye or may not be discernible despite careful examination. Infection foci are best viewed under a low power stereo microscope and are recognisable by localised whitening of the muscle beneath the cuticle. In some cases, a brown colouration of cuticle and muscle may occur, or hyphae may be visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle. Sites for examination include the intestinal 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, eyestalks and finally the gills.

Susceptible *species that do not normally develop clinical disease*

Infected North American crayfish do not usually show signs of disease. However, populations with high levels of infection can show abnormally high levels of cuticular damage in individual animals, such as missing legs and claws due to deteriorated joints.

2.3.4. Modes of transmission and life cycle

Transmission from crayfish to crayfish occurs through the release of zoospores from an infected animal and attachment of the zoospores to naïve crayfish. 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, upon encountering a susceptible host, attach and germinate to produce invasive vegetative hyphae. The zoospores of *A. astaci* swim actively in the water column and have been demonstrated to show positive chemotaxis towards crayfish (Cerenius & Söderhäll, 1984). Zoospores are capable of repeated encystment and re-emergence, extending the period of their infectivity (Soderhall & Cerenius 1999). Growth and sporulation capacity is strain-and temperature-dependent (Dieguez-Uribeondo *et al*., 1995).

The main routes of spread of the pathogen are through 1) movement of infected crayfish, 2) movement of spores with contaminated water or equipment, or 3) through colonisation of non-native habitats by invasive North American crayfish species.

The main route of spread of *A. astaci* in Europe between the 1960s and 2000 was through the active stocking of North American crayfish into the wild or escapes from crayfish farms. Subsequent spread occurred through expanding populations of invasive North American crayfish, accidental co-transport of specimens, and release of North American crayfish into the wild by private individuals (Holdich *et al.,* 2009).

Transportation of finfish may facilitate the spread of *A. astaci* through the presence of spores in the transport water or co-transport of infected crayfish specimens (Alderman *et al*., 1987; Oidtmann *et al*., 2002). There is also circumstantial evidence of spread by contaminated equipment (e.g., nets, boots, clothing, traps) (Alderman *et al*., 1987).

2.3.5. Environmental factors

Under laboratory conditions, the preferred temperature range at which the *A. astaci* mycelium grows varies slightly depending on the strain. In a study, which compared several *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 (Dieguez-Uribeondo *et al*., 1995).

Field observations show that outbreaks of infection with *A. astaci* occur over a wide temperature range, and at least in the temperature range 4–20°C. The rate 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 epizootic is enhanced by higher water temperatures.

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 pH 6.0–7.5, with a maximum range between pH 4.5 and 9.0 (Unestam, 1966).

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 (Cerenius *et al*., 1988).

2.3.6. Geographical distribution

In Europe the reports of large mortalities of crayfish go back to 1860. The reservoir of the original infections in the 19th century was never established. *Faxonius* (*Orconectes*) spp. were not known to have been introduced into Europe until the 1890s, but the post-1960s extensions are largely linked to more recent introductions of North American crayfish for farming (Alderman, 1996; Holdich *et al*. 2009). *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe.

In recent years, crayfish plague has been reported in Asia and also in North- and South America (see e.g. references in Di Domenico *et al.* 2021). The distribution of *A. astaci* in North America is likely to be much wider than reported (Martín-Torrijos *et al.,* 2021).

See WOAH WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No vaccines are available.

2.4.2. Chemotherapy including blocking agents

No treatments are currently known.

2.4.3. Immunostimulation

No immunostimulants are currently known.

2.4.4. Breeding resistant strains

A few studies suggest that there might be differences in resistance between populations of crayfish species that are prone to clinical disease (reviewed by Martín-Torrijos *et al*., 2017; Svoboda *et al*., 2017).

2.4.5. Inactivation methods

Aphanomyces astaci, both in culture and in infected crayfish, is inactivated by a short exposure to temperatures of 60°C or to temperatures of –20°C (or below) for 48 hours (or more) (Oidtmann *et al*., 2002). Sodium hypochlorite at 100 ppm, free chlorine and iodophors at 100 ppm available iodine, are effective for disinfection of contaminated equipment. Equipment must be cleaned prior to disinfection since organic matter decreases the effectiveness of disinfectants (Alderman & Polglase, 1985). Thorough drying of equipment (>24 hours) is also effective as *A. astaci* is not resistant to desiccation (Rennerfelt, 1936).

2.4.6. Disinfection of eggs and larvae

No information available.

2.4.7. General husbandry

If a farm for crayfish species that are prone to clinical disease is being planned, it should be carefully investigated whether North American crayfish species are in the vicinity of the planned site or present upstream. If North American crayfish are present, there is a high likelihood that susceptible farmed crayfish will eventually become infected.

In an endemic area where species prone to expression of clinical disease are being farmed, the following biosecurity recommendations should be followed to avoid an introduction of *A. astaci* onto the site:

1. General biosecurity should be in place (e.g. controlled access to premises; disinfection of boots when entering the site; investigation of mortalities if they occur; introduction of live susceptible species or vectors only from sources known to be free from infection with *A. astaci*).

2. Prevent movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other items that might carry the pathogen from an infected to an uninfected site holding susceptible species.

3. Do not transfer of susceptible species or vectors from streams or other waters that harbour potentially infected crayfish (either susceptible crayfish populations that are going through a current outbreak of infection with *A. astaci* or North American crayfish species).

4. North American crayfish should not be brought onto the site.

5. Finfish obtained from unknown freshwater sources or from sources, where North American crayfish may be present or a current outbreak of infection with *A. astaci* may be taking place, must not be used as bait or feed for crayfish, unless they have been subject to a temperature treatment to kill *A. astaci* (see Section 2.4.5. *Inactivation methods*).

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

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

For a suspected outbreak of infection with *A. astaci* in a population of crayfish species sampled crayfish should ideally consist of: a) live crayfish showing signs of disease, and b) live, apparently healthy crayfish. Freshly dead crayfish may also be suitable, although this will depend on their condition.

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

Crayfish should be transported in a moist environment, 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.

Should only dead animals be found at the site of a suspected outbreak, freshly dead animals should be selected for diagnosis. Dead animals 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 3.5. *Preservation of samples for submission*), or c) placed in freezer at –20°C to avoid further decay and transported frozen.

When testing any population outside an acute mortality event for the presence of crayfish plague, as many individuals as possible should be inspected visually for signs of cuticular damage. Crayfish that have melanized spots or missing limbs should be selected in the first place for further analysis.

3.2. Selection of organs or tissues

The tissue recommended for sampling is the soft abdominal cuticle, which can be found on the ventral side of the abdomen. Any other soft part of the exoskeleton and eyestalks can be included as well.

If any melanised spots or whitened areas are detected, these should be included in the sampling. From diseased animals, samples should be aseptically collected from the soft abdominal cuticle. For identification of reservoirs, samples should be aseptically collected from soft abdominal cuticle, and telson and uropods, separately.

In the North American crayfish species, sampling of soft abdominal cuticle, uropods and telson are recommended. Any other soft part of the exoskeleton can be included as well. If any melanized spots are detected, these should be included in the sampling.

3.3. Samples or tissues not suitable for pathogen detection

Autolysed material is not suitable for analysis.

3.4. Non-lethal sampling

A non-destructive sampling method that detects *A. astaci* DNA in the microbial biofilm associated with the cuticle of individual crayfish through vigorous scrubbing has been described (Pavic *et al.,* 2020), and could be considered in case of testing vulnerable populations.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

3.5. Preservation of samples for submission

The use of non-preserved crayfish is preferred, as described above. If transport of recently dead or moribund crayfish cannot be arranged, crayfish may be frozen or fixed in ethanol (minimum 70%). However, fixation may reduce test sensitivity. The crayfish:ethanol ratio should ideally be 1:10 (1 part crayfish, 10 parts ethanol).

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0 *General information* (diseases of crustaceans)

3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (time since collection and time in storage). Isolation is best attempted from crayfish with clinical signs delivered alive (see Section 3.1.). Fresh specimens should be kept chilled and preferably sent to the laboratory within 24 hours of collection.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.3. Samples for histopathology

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2 of Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.4. Samples for other tests

Sensitive molecular methods can be used to detect *A. astaci* DNA directly from water samples (Strand *et al*. 2011, 2012). These methods require validation for diagnostic use.

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose is only recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found

to be suitable. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore, larger animals should be processed and tested individually. Small life stages such as PL, can be pooled to obtain the minimum amount of material for molecular detection.

4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

Ratings for purposes of use. For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ =	Methods are most suitable with desirable performance and operational characteristics.
++ =	Methods are suitable with acceptable performance and operational characteristics under most circumstances.
+ =	Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
Shaded boxes =	Not appropriate for this purpose.

Validation stage. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

WOAH Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to the WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts						+	+	NA				
Histopathology						+	+	NA				
Culture						+	+	NA				
Real-time PCR	++	++	++	1	++	++	++	1	++	++	++	1
Conventional PCR	+	+	+	1	++	++	++	1				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
***In-situ* hybridisation**												
Bioassay												
Immunohistochemistry												
Ab-ELISA												
Ag-ELISA												
Other methods[3]												

LV = level of validation, refers to the stage of validation in the WOAH Pathway (chapter 1.1.2); NA = not available. PCR = polymerase chain reaction; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.

[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose

4.1. Wet mounts

Small pieces of soft cuticle excised from the regions mentioned above (Section 2.3.3 *Gross pathology*) and examined under a compound microscope using low-to-medium power will confirm the presence of aseptate fungal-like hyphae 7–9 μm wide. The hyphae can usually be found pervading the whole thickness of the cuticle, forming a three-dimensional network of hyphae in heavily affected areas of the cuticle. The presence of host haemocytes and possibly some 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.

4.2. Histopathology

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. A histological staining technique, such as the Grocott silver stain counterstained with conventional haematoxylin and eosin, can be used to visualise *A. astaci* in tissues of crayfish species prone to clinical disease. However, such material does not prove that any hyphae observed are those of *A. astaci*, especially when the material comes from animals already dead by sampling.

See also Section 4.1 *Wet mounts*.

4.3. Culture for isolation

Isolation is not recommended as a routine diagnostic method (Alderman & Polglase, 1986; Cerenius *et al*., 1987; Viljamaa-Dirks, 2006). 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 PCR. Isolation of *A. astaci* by culture from apparently healthy crayfish is challenging and molecular methods are recommended. A detailed description of this test is available from the Reference Laboratory [1].

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 '*Use of molecular and antibody-based techniques for confirmatory testing' and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate. Shrimp tissues may be used as negative controls.

Live crayfish can be killed using chloroform, electric current or by mechanical destroying the nerve cords. If live or moribund animals are not available, only recently dead animals should be used for DNA extraction. The soft abdominal cuticle is the preferred sample tissue for DNA extraction. Any superficial contamination should first be removed by thoroughly wiping the soft abdominal cuticle with wet (using autoclaved H_2O) clean disposable swabs. The soft abdominal cuticle is then excised and 30–50 mg of tissue is homogenised using standard physical methods

Several PCR assays have been developed with varying levels of sensitivity and specificity. Two assays are described here. Both assays target the ITS (internal transcribed spacer) region of the nuclear ribosomal gene cluster within the *A. astaci* genome.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling conditions[a]
Method 1*: Vralstad *et al.,* 2009; GenBank Accession No.: AM947024			
Aphanomyces astaci & *A. fennicus*/ ITS	Fwd: AAG-GCT-TGT-GCT-GGG-ATG-TT Rev: CTT-CTT-GCG-AAA-CCT-TCT-GCT-A Probe: 6-FAM-TTC-GGG-ACG-ACC-C-MGBNFQ	500 nM 500 nM 200 nM	50 cycles of: 95°C/15 sec and 58°C/60 sec
Method 2: Strand *et al*., 2023; GenBank Accession No.: AM947024			
Aphanomyces astaci/ITS	Fwd: TAT-CCA-CGT-GAA-TGT-ATT-CTT-TAT Rev: GCT-AAG-TTT-ATC-AGT-ATG-TTA-TTT-A Probe: FAM-AAG-AAC-ATC-CCA-GCA-C-MGBNFQ	500 nM 500 nM 100 nM	50 cycles of: 95°C/15 sec and 60°C/30 sec

*These ITS-based methods have been found to give positive results for the species *Aphanomyces fennicus* (Viljamaa-Dirks & Heinikainen 2019).
[a]A denaturation step prior to cycling has not been included.

The absolute limit of detection of method 1 was reported as approximately 5 PCR forming units (= target template copies) (Vralstad *et al*., 2009). Another study reported consistent detection down to 50 fg DNA using this assay (Tuffs & Oidtmann, 2011).

Analytical test specificity has been investigated (Tuffs & Oidtmann, 2011; Vralstad *et al*., 2009) and no cross-reaction was observed in these studies. However, a novel species, *Aphanomyces fennicus*, isolated from noble crayfish was reported in 2019 (Viljamaa-Dirks & Heinikainen, 2019) that gave a positive reaction in this test at the same level as *A. astaci*. Due to this problem in specificity, the assay has been modified according to the alternative method 2 (Strand *et al*., 2023). Owing to the repeated discovery of new *Aphanomyces* strains, sequencing is required to determine the species of *Aphanomyces* in the case of an amplification product in the real-time PCR assay result. This requires separate amplification of a PCR product (see Section 4.5 *Amplicon sequencing*).

4.4.2. Conventional PCR

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling conditions[a]
Method 1*: Oidtmann *et al.,* 2006; GenBank Accession No.: AY310499; amplicon size: 569 bp			
Aphanomyces astaci & *A. fennicus*/ ITS	Fwd: GCT-TGT-GCT-GAG-GAT-GTT-CT Rev: CTA-TCC-GAC-TCC-GCA-TTC-TG-	500 nM 500 nM	40 cycles of: 1 min/96°C, 1 min/59°C and 1 min/72°C

*This ITS-based method has been found to give positive results for the species *Aphanomyces fennicus* (Viljamaa-Dirks & Heinikainen 2019).
[a]A denaturation step prior to cycling has not been included.

Confirmation of the identity of the PCR product by sequencing is required as a novel species, *A. fennicus*, isolated from noble crayfish was reported in 2019 (Viljamaa-Dirks & Heinikainen, 2019) that gave a positive reaction in this assay.

The assay consistently detects down to 500 fg of genomic target DNA or the equivalent amount of ten zoospores submitted to the PCR reaction (Tuffs & Oidtmann, 2011).

4.4.3. Other nucleic acid amplification methods

Several genotype-specific molecular methods have been developed that, instead of requiring a pure growth as sample material like the RAPD-PCR assay, can be used to analyse crayfish tissue directly (Di Domenico *et al*., 2021; Grandjean *et al*., 2014; Makkonen *et al.,* 2018; Minardi *et al*., 2018; 2019). Detection of a known genotype group combined with a positive result by a recommended conventional or real-time

PCR can be used as a confirmative test in geographical areas where crayfish plague is known to be present. However, the current knowledge of the genotype variation is mostly limited to a few original host species and new genotypes or subtypes are expected to be found. Thus, the suitability of these methods is limited for initial excluding diagnosis or as confirmative tests in geographical areas not known to be infected.

PCR targeting mitochondrial DNA with *A. astaci* genotype specific primers have been shown to detect the known genotypes of *A. astaci*, but these assays may also provide positive results for some other oomycete genera (Casabella-Herrero *et al.,* 2021).

4.5. Amplicon sequencing

Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

4.6. *In-situ* hybridisation

Not available.

4.7. Immunohistochemistry

Not available

4.8. Bioassay

No longer used for diagnostic purposes (see Cerenius *et al*., 1988).

4.9. Antibody- or antigen-based detection methods

Not available.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The recommended method for surveillance is real-time PCR utilising the modified assay by Strand *et al*. (2023).

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for suspect and confirmed cases have been developed to support decision-making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [2]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

2 For example transboundary commodities.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with *Aphanomyces astaci* shall be suspected if at least one of the following criteria is met:

i) Positive result by real-time PCR

ii) Positive result by conventional PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *Aphanomyces astaci* is considered to be confirmed if the following criterion is met:

i) Positive result by real-time PCR and positive result by conventional PCR followed by amplicon sequencing

6.2 Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *Aphanomyces astaci* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality

ii) Visual observation of hyphae indicative of *A. astaci* in wet mounts

iii) Observation of hyphae indicative of *A. astaci* in stained histological sections

iv) Culture and isolation of the pathogen

v) Positive result by real-time PCR

vi) Positive result by conventional PCR

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *Aphanomyces astaci* is confirmed if the following criterion is met:

i) Positive result by real-time PCR and positive result by conventional PCR and amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *Aphanomyces astaci* are provided in Tables 6.3.1. and 6.3.2 (no data are currently available for either). Data are only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR	Distinguish between *A. astaci* and *A. finnicus*		Mycelium, tissue samples	*Astacus astacus*		Only detected *A. astacus*		Strand *et al.*, 2023

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of animals used in the validation study.

6.3.2. For surveillance of apparently healthy animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR	Distinguish between *A. astaci* and *A. finnicus*		Tissue samples, environmental DNA	*Astacus astacus*		Only detected *A. astacus*		Strand *et al.*, 2023

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of animals used in the validation study.

7. References

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

ALDERMAN D.J. & POLGLASE J.L. (1985). Disinfection for crayfish plague. *Aquacult. Fish. Manage.,* **16**, 203–205.

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

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

CASABELLA-HERRERO G., MARTÍNEZ-RÍOS M., VILJAMAA-DIRKS S., MARTÍN-TORRIJOS L. & DIÉGUEZ-URIBEONDO J. (2021). *Aphanomyces astaci* mtDNA: insights into the pathogen's differentiation and its genetic diversity from other closely related oomycetes. *Fungal Biol.,* **125**, 316–325. doi: 10.1016/j.funbio.2020.11.010. Epub 2020 Dec 2.

CERENIUS L. & SÖDERHÄLL K. (1984). Chemotaxis in *Aphanomyces astaci*, an arthropodparasitic fungus. *J. Invertabr. Pathol.,* **43**, 278–281.

CERENIUS L., SÖDERHÄLL K. & FULLER M.S. (1987). *Aphanomyces astaci* and *Aphanomyces* spp. *In:* Zoosporic fungi in teaching and research, Fuller M.S. & Jaworski A., eds. South-Eastern Publishing Corp., Athens, Georgia, USA. pp 64–65.

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

DI DOMENICO M., CURINI V., CAPRIOLI R., GIANSANTE C., MRUGAŁA A., MOJŽIŠOVÁ M., CAMMA C. & PETRUSEK A. (2021). Real-Time PCR assays for rapid Identification of common *Aphanomyces astaci* genotypes. *Front. Ecol. Evol.,* **9**, art 597585 doi:10.3389/fevo.2021.597585.

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

GRANDJEAN F., VRÅLSTAD T., DIÉGUEZ-URIBEONDO J., JELIĆ M., MANGOMBI J., DELAUNAY C., FILIPOVÁ L., REZINCIUC S., KOZUBÍKOVÁ-BALCAROVA E., GYONNET D., VILJAMAA-DIRKS S. & PETRUSEK A. (2014). Microsatellite markers for direct genotyping of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes) from infected host tissues. *Vet. Microbiol*., **170**, 317–324.

HOLDICH D.M., REYNOLDS J.D., SOUTY-GROSSET C. & SIBLEY P.J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ec.*, **394–395**, 1–46.

HUANG T.S., CERENIUS L. & SÖDERHÄLL K. (1994). Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. *Aquaculture*, **126**, 1–10.

KOZUBÍKOVÁ E., VILJAMAA-DIRKS S., HEINIKAINEN S. & PETRUSEK A. (2011). Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague agent *Aphanomyces astaci*. *J. Invertebr. Pathol.,* **108**, 214–216.

MAKKONEN J., JUSSILA J., PANTELEIT J., KELLER N.S., SCHRIMPF A., THEISSINGER K., KORTET R., MARTÍN-TORRIJOS L., SANDOVAL-SIERRA J.V., DIÉGUEZ-URIBEONDO J. & KOKKO H. (2018). MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology,* **145**, 1210–1218. https:// doi.org/10.1017/S0031182018000227.

MARTÍN-TORRIJOS L., CAMPOS LACH M., POU ROVIRA Q. & DIÉGUEZ-URIBEONDO J. (2017). Resistance to the crayfish plague, *Aphanomyces astaci* (Oomycota) in the endangered freshwater crayfish species, *Austropotamobius pallipes*. *PLoS ONE*, **12** (7), e0181226. https://doi.org/10.1371/journal. pone.0181226

MARTÍN-TORRIJOS L., MARTÍNEZ-RÍOS M., CASABELLA-HERRERO G., ADAMS S.B., JACKSON C.R. & DIÉGUEZ-URIBEONDO J. (2021). Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci,* to the Southeastern United States. *Sci. Rep.,* **11**, 9332.doi: 10.1038/s41598-021-88704-8.

MINARDI D., STUDHOLME D.J., OIDTMANN B., PRETTO T. & VAN DER GIEZEN M. (2019). Improved method for genotyping the causative agent of crayfish plague (*Aphanomyces astaci*) based on mitochondrial DNA. *Parasitology*, 146, 1022–1029, doi:10.1017/S0031182019000283

MINARDI D., STUDHOLME D.J., VAN DER GIEZEN M., PRETTO T. & OIDTMANN B. (2018). New genotyping method for the causative agent of crayfish plague (*Aphanomyces astaci*) based on whole genome data. *J. Invertebr. Pathol.*, **156**, 6–13.

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.

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

PAVIC D., ČANKOVIĆ M., PETRIĆ I., MAKKONEN J., HUDINA S., MAGUIRE I., VLADUŠIĆA T., ŠVER L., HRAŠĆANA R., ORLIĆ K., DRAGIČEVIĆ P. & BIELEN A. (2020) Non-destructive method for detecting *Aphanomyces astaci*, the causative agent of crayfish plague, on the individual level. *J. Invertebr. Pathol.*, **169**, 107274. https://doi.org/10.1016/j.jip.2019.107274

RENNERFELT E. (1936). Untersuchungen uber die Entwicklung und Biologie des Krebspestpilzes Aphanomyces astaci Schikora. Report of the Institute of Freshwater Research (Drottningholm, Sweden), **10**, 1–21.

SCHRIMPF A., SCHMIDT T. & SCHULZ R. (2014). Invasive Chinese mitten crab (*Eriocheir sinensis*) transmits crayfish plague pathogen (*Aphanomyces astaci*). *Aquatic Invasions*, **9**, 203–209. 10.3391/ai.2014.9.2.09.

SODERHALL K. & CERENIUS L. (1999) The crayfish plague fungus: history and recent advances. *Freshwater Crayfish*, **12**, 11-34.

SOUTY-GROSSET C., HOLDICH D.M., NOEL P.Y., REYNOLDS J.D. & HAFFNER P. (eds) (2006). Atlas of Crayfish in Europe. Muséum National d'Histoire Naturelle, Paris (Patrimoines naturels, 64), 188 p.

STRAND D.A., HOLST-JENSEN A., VILJUGREIN H., EDVARDSEN B., KLAVENESS D., JUSSILA J. & VRÅLSTAD T. (2011). Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. *Dis. Aquat. Org.*, **95**, 9–17.

STRAND D.A., JINNEROT T., ASPÁN A., VILJAMAA-DIRKS S., HEINIKAINEN S., ROLÉN E. & VRÅLSTAD T. (2023). Molecular detection of *Aphanomyces astaci* – An improved species specific qPCR assay. *J. Invertebr. Pathol.,* **201**, 108008. doi: 10.1016/j.jip.2023.108008. Epub 2023 Oct 18. PMID: 37863282.

STRAND D.A., JUSSILA J., VILJAMAA-DIRKS S., KOKKO H., MAKKONEN J., HOLST-JENSEN A., VILJUGREIN H. & VRÅLSTAD T. (2012). Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. *Vet. Microbiol.,* **160**, 99–107.

SVOBODA J., MRUGAŁA A., KOZUBÍKOVÁ-BALCAROVÁ E. & PETRUSEK A. (2017). Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *J. Fish Dis.*, **40,**127–140. https://doi.org/10.1111/jfd.12472

THOMAS J.R., ROBINSON C.V., MRUGAŁA A., ELLISON A.R., MATTHEWS E., GRIFFITHS S.W., CONSUEGRA S. & CABLE J. (2020). Crayfish plague affects juvenile survival and adult behaviour of invasive signal crayfish. *Parasitology*, **1–9**, https://doi.org/10.1017/S0031182020000165

TUFFS S & OIDTMANN B (2011). A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci*. *Vet. Microbiol.,* **153**, 345–353.

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

VILJAMAA-DIRKS S. (2006). Improved detection of crayfish plague with a modified isolation method. *Freshwater Crayfish*, **15**, 376–382.

VILJAMAA-DIRKS S. & HEINIKAINEN S. (2019) A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. *J. Fish Dis*., **42**, 413–422. https://doi.org/10.1111/jfd.12955

VILJAMAA-DIRKS S., HEINIKAINEN S., NIEMINEN M., VENNERSTRÖM P. & PELKONEN S. (2011). Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population – a case report. *Bull. Eur. Assoc. Fish Pathol*., **31**, 182–188.

VILJAMAA-DIRKS S., HEINIKAINEN S., TORSSONEN H., PURSIAINEN M., MATTILA J. & PELKONEN S. (2013). Distribution and epidemiology of genotypes of the crayfish plague *Aphanomyces astaci* from noble crayfish *Astacus astacus* in Finland. *Dis. Aquat. Org*., **103**, 199–208.

VRALSTAD T., JOHNSEN S.I., FRISTAD R., EDSMAN L. & STRAND D.A. (2011). Potent infection reservoir of crayfish plague now permanently established in Norway. *Dis. Aquat. Org.,* **97**, 75–83.

VRALSTAD T., KNUTSEN A.K., TENGS T. & HOLST-JENSEN A. (2009). A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Vet. Microbiol.,* **137**, 146–155.

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NB: There is a WOAH Reference Laboratory for infection with *Aphanomyces astaci* (crayfish plague) (please consult the WOAH web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on infection with *Aphanomyces astaci* (crayfish plague)

NB: FIRST ADOPTED IN 1995; MOST RECENT UPDATES ADOPTED IN 2024.