Australian Government
Department of Agriculture and Water Resources

AUSTRALIAN AQUATIC VETERINARY EMERGENCY PLAN (AQUAVETPLAN)
Whirling disease

Version 2, 2016

AQUAVETPLAN–Disease Strategy

AQUAVETPLAN is a series of manuals that outline Australia's approach to national disease preparedness and propose the technical response and control strategies to be activated in a national aquatic animal disease emergency.

National Biosecurity Committee

This disease strategy forms part of:

AQUAVETPLAN

This strategy will be reviewed regularly. Suggestions and recommendations for amendments should be forwarded to:

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AQUAVETPLAN is available on the internet at:
http://www.agriculture.gov.au/animal-plant-health/aquatic/aquavetplan

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DISEASE WATCH HOTLINE
1 800 675 888

The Disease Watch Hotline is a toll-free telephone number that connects callers to the
relevant State or Territory officer to report concerns about any potential emergency
animal disease situation. Anyone suspecting an emergency disease outbreak should
use this number to get immediate advice and assistance.

Preface

This disease strategy for the control and eradication of whirling disease is an integral part of the **Australian Aquatic Veterinary Emergency Plan**, or **AQUAVETPLAN.**

AQUAVETPLAN disease strategy manuals are response manuals and do not include information about preventing the introduction of disease.

The Department of Agriculture and Water Resources provides quarantine inspection for international passengers, cargo, mail, animals, plants and animal or plant products arriving in Australia, and inspection and certification for a range of agricultural products exported from Australia. Quarantine controls at Australia's borders minimise the risk of entry of exotic pests and diseases, thereby protecting Australia's favourable human, animal and plant health status. Information on current import conditions can be found at the Department of Agriculture and Water Resources BICON website (agriculture.gov.au/import/online-services/bicon).

This strategy sets out the disease control principles for use in an aquatic veterinary emergency incident caused by the suspicion or confirmation of whirling disease in Australia. The strategy was scientifically reviewed by the Sub-Committee on Aquatic Animal Health of the Animal Health Committee, before being endorsed by:

- the Animal Health Committee of the National Biosecurity Committee in January 2016, and the National Biosecurity Committee in June 2016.

Whirling disease is listed on Australia's *National List of Reportable Diseases of Aquatic Animal* (DA 2015a)*.* Detailed instructions for the field implementation of AQUAVETPLAN are contained in the disease strategies, operational procedures manuals and management manuals. Industry-specific information is given in the enterprise manual. The full list of AQUAVETPLAN manuals that may need to be accessed in an emergency is shown below:

Disease strategies	**Enterprise manual**
Individual strategies for each disease	Includes sections on:
	– open systems
Operational procedures manuals	– semi-open systems
Disposal	– semi-closed systems
Destruction	
Decontamination	
Management manual	
Control centres management	

Aquatic Animal Diseases Significant to Australia: Identification Field Guide (DA 2012) is a source of information about the aetiology, diagnosis and epidemiology of infection with whirling disease and should be read in conjunction with this strategy.

The first edition of this manual was prepared by Dr Paul Hardy-Smith, with the assistance of Professor Ron Hedrick, Dr Craig Stephens and Dr Mark Crane, in 2005. This revision was prepared by Dr Brendan Cowled and Dr Andy Shinn in 2016. The authors were responsible for drafting the strategy, in consultation with a wide range of stakeholders from aquaculture, recreational fishing and government sectors throughout Australia. The text, however, was amended at various stages of the consultation and endorsement process, and the policies expressed in this version do not necessarily reflect the views of the authors. The revision authors would like to thank Dr Matt Longshaw. Contributions made by others not mentioned here are also gratefully acknowledged.

The format of this manual was adapted from similar manuals in AUSVETPLAN (the Australian veterinary emergency plan for terrestrial animal diseases) and from the AQUAVETPLAN Enterprise Manual. The format and content have been kept as similar as possible to these documents, in order to enable animal health professionals trained in AUSVETPLAN procedures to work efficiently with this document in the event of an aquatic veterinary emergency. The work of the AUSVETPLAN writing teams and the permission to use the original AUSVETPLAN documents are gratefully acknowledged.

The revised manual has been reviewed and approved by the following representatives of government and industry:

Government

CSIRO Australian Animal Health Laboratory

Department of Primary Industries, New South Wales

Department of Primary Industry and Fisheries, Northern Territory

Department of Agriculture and Fisheries, Queensland

Department of Primary Industries, Parks, Water and Environment, Tasmania

Department of Fisheries, Western Australia

Department of Economic Development, Jobs, Transport and Resources, Victoria

Department of Primary Industries and Regions, South Australia

Biosecurity Animal Division, Department of Agriculture and Water Resources, Australian Government

Department of the Environment, Australian Government

Industry

National Aquatic Animal Health Industry Reference Group

The complete series of AQUAVETPLAN documents is available on the internet (http://www.agriculture.gov.au/animal-plant-health/aquatic/aquavetplan).

Contents

1   Nature of the disease

Whirling disease is a disease of freshwater salmonid fish caused by the myxozoan parasite *Myxobolus cerebralis*. The parasite has never been detected in Australia, but is present in New Zealand and areas of North America, Europe (including Iceland), Russia, Africa (Morocco and South Africa), the Middle East (Lebanon) and Asia (Blaylock & Bullard 2014; Hallett & Bartholomew 2012).

Importantly, the parasite has two hosts — salmonid fish species and a freshwater oligochaete worm, *Tubifex tubifex* (or species assemblage) — hereafter called *T. tubifex*. There are two spore stages, one released from the salmonid fish, which is infective for *T. tubifex*, and the other released from *T. tubifex* worms, which is infective for fish. Generally, the younger and smaller fish are infected when first exposed (Ryce et al. 2005), and the higher the parasite exposure dose, the greater the severity of the clinical disease. Water temperature has a significant influence on all stages of the parasite life-cycle. Fish populations are most affected at temperatures of 10–15 °C. Temperatures greater than 20 °C are not conducive to parasite development or survival.

All salmonids can be infected, but rainbow trout (*Oncorhynchus mykiss*) and cutthroat salmon (*O. clarki*) are very susceptible to clinical disease (Blaylock & Bullard 2014). The parasite caused severe hatchery losses of rainbow trout in the early stages of industry development in Europe. Early trout culture in earthen ponds provided habitat for *T. tubifex* worms. Changed culture techniques for trout aquaculture have greatly reduced the incidence of the disease in aquaculture, particularly rearing of young fish in concrete or plastic-lined raceways. In the United States, significant declines in wild trout populations have occurred in some areas. Variability in the severity of clinical disease is associated with ecological conditions and different strains of rainbow trout. Other salmonids can be subclinically infected.

Australian native freshwater fish are not salmonids, and it is therefore unlikely that they are susceptible to this disease. Both wild and farmed salmonids used in restocking programs would be susceptible. In many areas of southern Australia, trout populations are maintained or enhanced by restocking using hatchery-reared fish. These stocks are most at risk from potential *M. cerebralis* infection. Stocking with trout is no longer considered environmentally responsible in some areas, but continues in many areas of southern Australia for recreational fishery enhancement. Hatcheries also supply stock for aquaculture. The inland recreational fishery for salmonids (primarily brown and rainbow trout) in Tasmania is worth over $40M in tourism revenue alone, an important contribution to local economies (IFS 2008).

Whirling disease is a nationally reportable disease in Australia. State and territory governments, the recreational salmonid fishing industry and the salmonid aquaculture industry need to be adequately educated and prepared for the possible incursion of this disease. This will greatly minimise the impact of whirling disease on susceptible salmonid populations if the parasite is ever introduced into Australia.

1.1   Aetiology

The aetiological agent of whirling disease is the parasite *Myxobolus cerebralis* (formerly named *Myxosoma cerebralis*). Myxozoans are a diverse group of multicellular organisms with thousands of species, most of which parasitise fish at some stage of their life-cycle (Gilbert & Granath 2003; Hallett & Bartholomew 2012). Myxozoans are characterised by spore stages.

Myxobolus cerebralis belongs to the phylum Myxozoa, class Myxosporea, order Bivalvulida, suborder Platysporina and family Myxobolidae.

Myxobolus cerebralis is the most studied myxozoan and is a tiny metazoan parasite with two morphologically distinct spores (Fig 1):

- an actinomyxon spore which is released from *T. tubifex* worms; the triactinomyxon has three float cells forming a tri-radial anchor shape
- a myxospore which is released from fish (mostly after death) and is oval to lenticular in shape with valve cells encapsulating a binucleate sporoplasm and polar capsules.

Identification and diagnosis can be difficult, as several species of myxozoa share similar morphology and can cause similar clinical signs (Bentz et al. 2012; Hallett & Bartholomew 2012; Hogge et al. 2008). Traditional diagnosis has been based on tissue tropism (identification of characteristic myxospores in the cartilage of salmonids), species affected (salmonids, especially rainbow trout) and presenting clinical signs. DNA diagnostic methods are now used to confirm identification (Hogge et al. 2008).

Figure 1 Life-cycle of *M. cerebralis* (whirling disease) (courtesy of R. Hedrick, adapted from El-Matbouli et al. (1992)
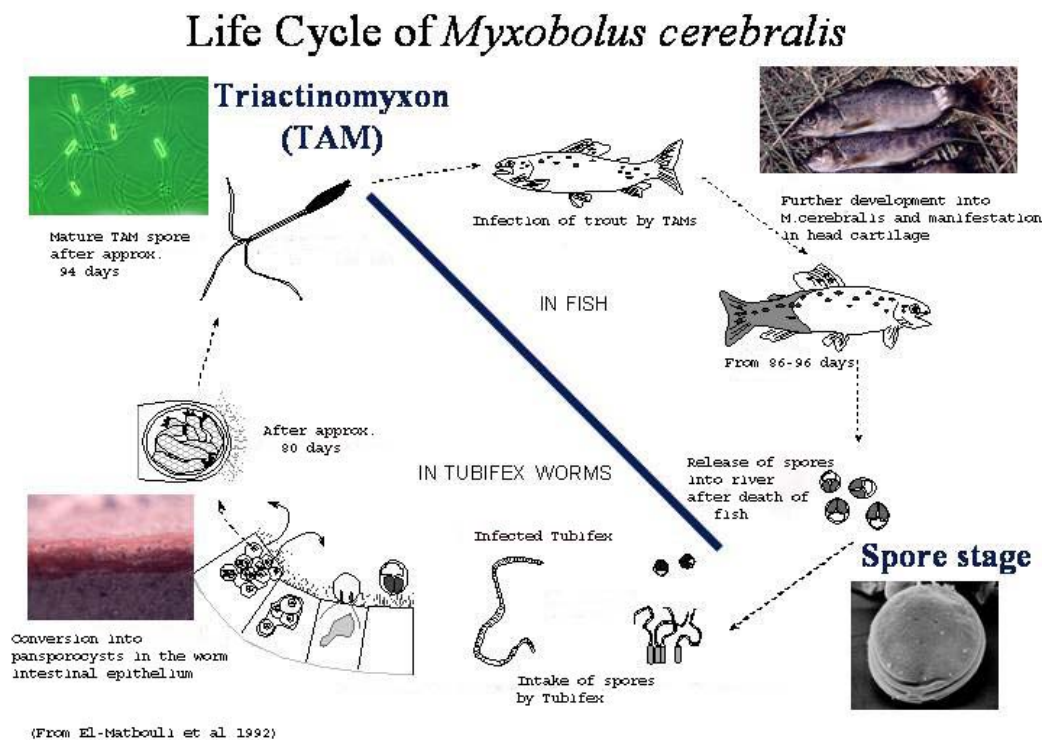
The important elements of the life-cycle of *M. cerebralis* are:

- Two-host life-cycle

  – Salmonids

    ▪ asexual replication only
    ▪ susceptible salmonids are present in southern Australia.

  – Freshwater oligochaete worms, *T. tubifex*

    ▪ sexual and asexual replication
    ▪ *T. tubifex* worms are present throughout Australia.

- Two infective stages

  - Myxospore

    - forms in the cartilage of the fish
    - infective myxospores are present in cartilage approximately 50–120 days after initial infection, depending on water temperature and salmonid species infected
    - resistant to environmental degradation, survival measured in months to years.

  - Actinospore triactinomyxon (TAM)

    - forms in the intestinal lining of the *T. tubifex* worm
    - infective TAMs present 100–170 days after initial exposure, depending on temperature and strain of *T. tubifex* worm
    - susceptible to environmental degradation, survival measured in days.

- All life-cycle stages are significantly affected by temperature

  - within limits (10–15 °C), the higher the temperature, the faster the life-cycle (see Gilbert & Granath [2003] and Hallett & Bartholomew [2012] who summarise diverse literature on temperature effects at several life stages).

- Significant variability in host susceptibility

  - both *T. tubifex* and salmonid fish display strain variability in susceptibility to infection.

1.1.1   Salmonid stage

Buoyant TAMs are released from the *T. tubifex* hosts and passively float. When a fish comes in close proximity, chemical cues, mostly in mucus, cause polar filaments to fire and anchor the TAM to the skin (Kallert et al. 2011). Firing can be stimulated by many fish species (Kallert et al. 2009). In salmonids, the amoeboid sporoplasm then emerges from between the valve cells of the TAM and enters the skin through the secretory openings in the mucous cells (El-Matbouli et al. 1999a). The parasite can also penetrate across the gills, through the fins and through the lining of the mouth.

The sporoplasm then migrates through the epithelium, peripheral nerves and central nervous system (CNS) to reach cartilage (El-Matbouli et al. 1999a).

Within 24 hours, there can be numerous parasite cells deep in the skin of the fish and, by day 4, parasite cells can be found in nervous tissue. At first, the cells are found in peripheral nerves. The cells then move through ganglia to the CNS, replicating as they go. As early as 20 days after exposure, parasite stages can be found in the cartilage of the susceptible fish. Trophozoites (plasmodial forms) in the cartilage cause pathological changes. This is dependent on water temperature; optimal temperature for development being around 10–15 °C. At this point, two parasitic cells will join to initiate sporogenesis or the formation of spores ('myxospores') in the cartilage of the fish. Cartilage throughout the body, including the cranium, spine, fins, vertebrae, ribs and operculum can be affected (Antonio et al. 1999).

Myxospores can take 52–120 days to develop in the fish at 7–17 °C (Halliday 1973a). At 16–17 °C, fully developed myxospores appear 52 days after infection. These spores are elliptical, multicellular, have thick protective shells and are approximately

10 micrometres in diameter. Myxospores remain in the cartilage and may be 'trapped' by bone as it forms around the infected cartilage.

One TAM can potentially produce 2800–7800 infective myxospores in the cartilage of a fish head and possibly more in cartilage elsewhere in the body (Hedrick et al. 1999b; Kerans & Zale 2002).

In live fish, some viable myxospores escape from destroyed cartilage and are released directly into the environment or pass out in the faeces (Nehring et al. 2002). The most significant release of myxospores occurs when the fish dies and decomposes. The decomposition of one rainbow trout can release more than a million myxospores (Hedrick et al. 1999b).

Once released, myxospores are highly persistent and are able to survive in the environment for long periods, possibly years (Hoffman 1990).

1.1.2 *Tubifex tubifex* worm stage

Tubifex tubifex is the definitive host for *M. cerebralis*. It is found only in fresh water, usually in areas of sediments rich in organic matter. It is the only species of *Tubifex* in Australia and is widespread across a range of habitats but is not commonly encountered (Pinder & Brinkhurst 2000). In Australia, *T. tubifex* is known from all mainland states and Tasmania, including being recorded as present at a trout hatchery (Pinder & Brinkhurst 2000). It is not known whether any of the five genera of the Australian Tubificinae are susceptible to *M. cerebralis*, but other tubificids (e.g. *Limnodrilus* sp.) overseas are refractory to infection (Kerans et al. 2004).

For the purposes of this manual, susceptibility of Australian *T. tubifex* worms to *M. cerebralis* should be assumed, unless otherwise demonstrated.

When an infected salmonid fish dies, its myxospores settle in sediment. Filter feeding *T. tubifex* worms may ingest myxospores while the spores are in the water column or in suspended sediment. Within *T. tubifex*, the infective sporoplasm from the myxospore is released, and migrates into the lining of the intestine where it multiplies and replicates (Hedrick & El-Matbouli 2002). Replication is both asexual and sexual. This defines *T. tubifex* as the definitive host for *M. cerebralis* (El-Matbouli & Hoffmann 1998).

TAMs are released into the lumen of the intestine as early as 74 days (at 15 °C) after ingestion of myxospores by the *T. tubifex* worm (Gilbert & Granath 2001). *Tubifex tubifex* is thought to remain persistently infected for life (Gilbert & Granath 2001). Experimentally, peak release of TAMs was found to occur at 120–170 days after exposure, but this is highly temperature dependent (Markiw 1986).

TAMs are morphologically distinct from the myxospores released from fish. They are shaped like a grappling hook, i.e. a long rod (approximately 146 micrometres) with three float cells, each approximately 193 micrometres. TAMs are produced in very high numbers from infected *T. tubifex* worms. Their maximum infective duration has not been determined but is at least 15 days, depending on temperature (the cooler the temperature, the longer the survival) (El-Matbouli et al. 1999b; Markiw 1992).

There are differences in the susceptibility of *T. tubifex* to *M. cerebralis*. Studies conducted within the United States have shown that four lineages of *T. tubifex* exist, based on mitochondrial DNA (lineages I, III, V and VI) (Hallett et al. 2009). Only lineage III worms release TAMs, and only populations dominated by this lineage amplified the parasite. Higher proportions of more susceptible lineages within a local *T. tubifex* population appear to increase the prevalence of whirling disease in local populations of susceptible salmonids (Beauchamp et al. 2005; Zielinski et al. 2011).

1.2   Susceptible species

1.2.1 Susceptible fish species

Salmonids are generally susceptible to infection by *M. cerebralis*, but there is considerable variability among salmonid species in their susceptibility to clinical disease.

1.2.2 Susceptibility to infection within susceptible fish species

It is thought that *M. cerebralis* evolved within European populations of brown trout (*Salmo trutta*), as brown trout are relatively resistant to clinical disease (Gilbert & Granath 2003; Hallett & Bartholomew 2012). Brown trout need to be exposed to very high numbers of the infective TAM stages for any clinical signs to develop (Hedrick et al. 1999b). Even at very high doses, no characteristic tail chasing swimming behaviour has been observed in brown trout.

In contrast, rainbow trout, steel head trout and cutthroat trout (native to North America) are amongst the species most susceptible to clinical disease.

Table 1 details the susceptibilities of the different species (after Hallett & Bartholomew (2012) where the original references can be found).

Table 1 Susceptibility of different salmonid species to whirling disease. Information is based on laboratory and observational studies on fish at vulnerable life stages. Adapted from Hallett & Bartholomew (2012).

Genus	Species	Common name	Suscept.[a]	Comp. suscept.[b]
Oncorhynchus	*clarki*[c]	Cutthroat trout	S-hS	1
Oncorhynchus	*gilae*	Gila	hS	1
Oncorhynchus	*gorbuscha*	Pink salmon	pR, U	3
Oncorhynchus	*keta*	Chum salmon	pR, U	3
Oncorhynchus	*kisutch*	Coho salmon	pR	3
Oncorhynchus	*masu*	Cherry salmon	pR, U	3
Oncorhynchus	*mykiss*	Rainbow trout	S-hS	1
Oncorhynchus	*mykiss*	Steelhead trout	S-hS	1
Oncorhynchus	*nerka*	Sockeye salmon	hS	1
Oncorhynchus	*tshawytscha*	Chinook salmon	S	2
Salvelinus	*confluentus*	Bull trout	pR	3
Salvelinus	*fontinalis*	Brook salmon	S	2
Salvelinus	*malma*	Dolly varden	pR, U	3
Salvelinus	*namaycush*	Lake trout	R	4

Genus	Species	Common name	Suscept.[a]	Comp. suscept.[b]
Salmo	*salar*	Atlantic salmon	S, U	2
Salmo	*trutta*	Brown trout	pR	3
Prosopium	*williamsoni*	Mountain whitefish	S	2
Thymallus	*arcticus*	Arctic grayling	R-pR	3
Thymallus	*thymallus*	European grayling	S, U	2
Hucho	*hucho*	Danube salmon	hS	1

Susc., susceptibility; Comp., comparative.

a. S, susceptible (clinical disease common at high parasite doses, e.g. > 1000 TAMs per fish, or when very young, but greater resistance to disease at low doses, i.e. 100–200). hS, highly susceptible (clinical disease common). pR, partially resistant (clinical disease rare and develops only when exposed to very high parasite doses). U, susceptibility unclear (conflicting reports or insufficient data). R, resistant (no spores develop).

b. 1 = highly susceptible, through to 4 = resistant to infection.

c. including several subspecies of cutthroat trout.

1.2.3   Within-fish species susceptibility

Considerable variation in clinical disease has been shown within susceptible species, especially in rainbow trout, and depends on several factors.

Older and larger rainbow trout are more resistant to clinical disease, although the level of skeletal ossification was not important in the development of disease (Ryce et al. 2005).

Some German rainbow trout hatchery strains (and their crosses) are resistant to clinical disease, but some United States strains are not (Fetherman et al. 2014). There is a genetic basis for this resistance (Baerwald 2013). Natural selection for resistant fish is possible in relatively few generations (Miller & Vincent 2008).

1.2.4   Susceptibility of *T. tubifex* strains

Different strains of *T. tubifex* have different susceptibility to infection with *M. cerebralis*, or may affect severity of clinical disease in local rainbow trout (Baxa et al. 2008; Beauchamp et al. 2005; Hallett et al. 2009). Despite this, the ability to reduce infection in salmonids by manipulating *T. tubifex* strains will be difficult because some susceptible strains will always remain and these will be enough to sustain transmission (Elwell et al. 2006).

1.2.5   Susceptibility of introduced populations of salmonid

Australia uses several exotic strains of salmonid fish for stocking fisheries or aquaculture, including rainbow trout, brown trout, brook trout (*Salvelinus fontinalis*), red salmon (*O. nerka*), Chinook salmon (*O. tshawytscha*) and Atlantic salmon (*Salmo salar*). These species are highly likely to be susceptible to infection, and it is likely that rainbow trout, brook trout, red salmon and Chinook salmon would be susceptible to clinical disease. Brown trout would be less likely to show clinical disease. Atlantic salmon may resist clinical disease, but are variable in their response to infection and there is insufficient evidence to be confident of their response (Hallett & Bartholomew 2012).

In general, clinical disease severity increases with increasing parasite dose (MacConnell & Vincent 2002). A low dose is considered to be 100–200 TAMs per fish, and a high dose

is 1000–2000 TAMs per fish (Hedrick et al. 1999a; Hedrick et al. 1999b). Very high doses (2000–10 000 TAMs per fish) can even cause severe clinical disease in less susceptible species such as brown trout.

Myxobolus cerebralis cannot complete its life-cycle in sea water, so farmed salmonids reared in sea cages will not be exposed. Fish infected in freshwater can remain infected in saltwater. If infected fish return to freshwater (e.g. brood stock returning to spawn), myxospores may be released and an infection could establish in *T. tubifex* worm populations.

1.2.6   Australian native fish susceptibility

There are no Australian native fish in the family Salmonidae. The two families of Australian fish most closely related to the salmonids within the order Salmoniformes are the Galaxiidae and the Retropinnidae (southern graylings) (CSIRO 2015).

A range of galaxiid species are found in New Zealand and are not susceptible to whirling disease (B. Jones, pers. comm.). To date, all fish determined to be susceptible to whirling disease are in the family Salmonidae (El-Matbouli et al. 1999a; Gilbert & Granath 2003; Nelson 1994). Although exposure trials have not been conducted, it is unlikely that Australian native fish will be susceptible to whirling disease.

1.2.7   Vectors

Myxospores are resistant to degradation in the alimentary tract of piscivorous birds and fish, and remain infectious in faecal material. Viable myxospores can therefore be widely dispersed by the movement of these animal vectors, potentially travelling some distance from the original site of infection and across catchment boundaries (El-Matbouli & Hoffmann 1991; Koel et al. 2010).

1.2.8   Not a human pathogen

Myxobolus cerebralis is not known to infect or cause clinical disease in humans. Infected fish are likely to be safe to eat, but clinically diseased fish may not be marketable due to the pathological changes associated with disease.

1.3   World distribution

Myxobolus cerebralis has never been detected in Australia. Other *Myxobolus* species, however, have been reported in Australia (Langdon 1990), and an actinosporean identified as belonging to the genus *Sphaeractinomyxon* has been isolated from a marine oligochaete in Australian waters (Hallett et al. 1995). *Myxobolus plectroplites* Johnston & Bancroft, 1918 was described from the freshwater fish golden perch (*Macquaria ambigua*) (Boreham et al. 1998). Lom & Dykova (1994) reported six *Myxobolus* species from estuarine fishes in New South Wales.

Myxobolus cerebralis was first described in Germany and was spread across Europe with transport of live rainbow trout. *Myxobolus cerebralis* subsequently spread around the world, generally with movements of salmonids such as rainbow trout for stocking, acclimatisation and farming. Additionally, widespread transport of *T. tubifex* worms for aquarium fish food and for juvenile fish food (including trout) may also have contributed to the spread of this parasite. It has been found in New Zealand and parts of North America (in 25 states), Europe (including Iceland, [Kristmundsson & Richter 2009]), Russia, Africa (Morocco and South Africa), the Middle East (Lebanon) and Asia (Japan and Korea).

Whirling disease was originally thought to be a problem confined to trout hatcheries, but in recent decades has emerged as a significant pathogen of wild salmonids in the United States, but not in New Zealand.

See Bartholomew & Reno (2002) for a historical perspective of dissemination and Hallett & Bartholomew (2012) for a review of distribution.

1.4 Diagnosis of infection with *M. cerebralis*

1.4.1 Presumptive diagnosis

A presumptive diagnosis of whirling disease relies on host characteristics, clinical signs and spore extraction (MacConnell & Bartholomew 2012).

Host characteristics

Myxobolus cerebralis myxospores infect salmonids, with clinical disease predominantly present in certain species, particularly rainbow trout. Risk factors for clinical disease include age (e.g. fingerlings and yearlings of susceptible species) and development (i.e. less developed). Suspect cases of whirling disease are therefore more likely to occur in young fish of susceptible species (See Section 1.2).

Clinical signs

Clinical signs include whirling behaviour (corkscrew swimming), black-tail, skeletal deformities, stunted growth and death (See Section 1.4.1). However clinical signs are not pathognomonic (i.e. are not specific for whirling disease) and some species and age classes of susceptible fish can be subclinically infected.

Spore extraction

Pepsin–trypsin digest and identification of spores can be used as a screening test but will not detect pre-sporogonic stages of the parasite.

1.4.2 Confirmatory diagnosis

Confirmatory diagnosis relies on histopathological examination (spores in cartilage) and parasite DNA amplification using specific polymerase chain reaction (PCR)-based tests.

Histopathological confirmation

Histopathological diagnosis relies on observation of pre-sporogonic and spore parasite stages in cartilage tissue surrounded by bone. The sensitivity of the method is high but is not 100%. For example, myxospores can simply be missed (Kelley et al. 2006) or clinical signs can be displayed before characteristic spores have formed.

Polymerase chain reaction (PCR)

Diagnosis using a PCR assay does not rely on terminal developmental stages of the parasite or on tissue damage, but instead on the amplification of parasite DNA (Kelley et al. 2006). Subclinical infection can therefore be detected before spores form. The method can be extremely sensitive and specific (Kelley et al. 2006). Purcell et al. (2011) noted the challenges in transferring PCR for research into diagnostic tests suitable for a regulated framework. That review centred on the use of qPCR, and many parallels can be drawn from it and considered in the application of other PCR-based methodologies. Current recommendations are to use qPCR (See Section 1.4.2).

Procedures for detecting subclinical infections are similar and rely on pepsin–trypsin digests, histopathology and qPCR.

A fully validated and standardised International Accreditation New Zealand (IANZ) test is detailed in Appendix 3. Further details on diagnosis can be found in the Fish Health Section Blue Book of the American Fisheries Society (MacConnell & Bartholomew 2012).

1.4.3   Case definitions

Suspect case definition

A suspect case definition should be highly sensitive (i.e. no or few false negatives) to avoid missing critical new outbreaks, yet also quite specific (i.e. few false positives). A case definition should also be easily and rapidly applied in the field to assist rapid outbreak management. A suitable suspect case definition is context specific but would include one or more of the following points:

- observance of typical clinical signs in young susceptible salmonid species (e.g. rainbow trout fingerlings or yearlings)
- a susceptible population of salmonids that has had contact or is in close proximity to a known case
- salmonids that return a positive result to a screening test (e.g. spore extraction and visualisation, or PCR test interpreted without further laboratory or epidemiological investigations)
- other epidemiological evidence strongly suggestive of infection or disease (e.g. tracing).

All suspect cases should be further investigated to confirm whether infection is present.

Confirmed case definition

A confirmed case definition is required to be highly specific and highly sensitive. The appropriate definition must depend on a mixture of evidence, such as host characteristics, clinical signs, epidemiological evidence and laboratory test results.

A confirmed case was traditionally diagnosed by detection of *M. cerebralis* spores in the cartilage from a known susceptible salmonid population displaying clinical signs or typical epidemiological patterns of whirling disease. A positive PCR test (amplification of *M. cerebralis* DNA from sample material) is currently an alternative to histopathological examination, provided the test is validated and used by an appropriate laboratory accredited to ISO/IEC 17025 and operating under a Quality Assurance System based on the ISO/IEC 17025 standard and accredited by the National Association of Testing Authorities (NATA) or its equivalent. There is a fully validated and standardised test available from the New Zealand Ministry for Primary Industries (see Appendix 3). A positive PCR test must be confirmed using other methods such as sequencing or histopathological examination.

A suitable confirmed case definition is context-specific but would include:

- confirmed identification of spores or presporogonic stages in tissue of clinically diseased fish

OR

- positive PCR test (see Appendix 3) followed by sequencing of PCR product.

All suspected exotic disease cases must be referred to the Australian Animal Health Laboratory (AAHL) for confirmatory diagnostic testing.

There are no standards detailed by the Office International des Épizooties (OIE [World Organisation for Animal Health]) or Australian New Zealand Standard Diagnostic Procedures for diagnosing *M. cerebralis* infection. An accredited qPCR and Standard Operating Procedure is shown in Appendix 3. The Fish Health Section Blue Book of the American Fisheries Society also details suitable tests (MacConnell & Bartholomew 2012).

1.4.4   Field methods: clinical signs and gross pathology

Clinical signs

In susceptible salmonids infected at an early age, clinical signs of whirling disease include erratic tail-chasing ('whirling'), blackening of the tail region, and skeletal deformities including skull depression and spinal curvatures. Infected salmonids can also show no clinical signs. Bartholomew & Reno (2002) noted that 'since clinical signs are not pathognomonic, and may be subtle, they might not be noted except when the disease reaches epizootic levels'.

The clinical signs of whirling disease will vary depending on the age of fish when first infected, the infective dose and the species and strain of salmonid.

When rainbow trout are exposed as fry:

- 'whirling' signs (rapid circular/corkscrew swimming) first appear approximately three to eight weeks after infection. Fish may die due to exhaustion and/or severe malnutrition. This swimming behaviour is considered to be due to lower brain stem and spinal cord compression and constriction (Rose et al. 2000)
- 'black tail' (caudal melanosis) due to pressure on caudal nerves controlling pigmentation (Halliday 1976). This may subside if fish are anaesthetised or after they die.

If infected fish survive, they rarely show whirling behaviour or black tail, but may have:

- skeletal deformities, e.g. skull depression, misshaped jaws, shortened operculae (gill covers), spinal curvatures; these signs can vary significantly in severity, and light infections can be difficult to detect
- opercular cysts
- decreased growth rate during clinical disease stage.

When exposed at older than nine weeks, there are very few or no clinical signs.

Other salmonid species show similar signs, depending on their susceptibility to *M. cerebralis*, but signs may vary with the age and developmental stage at which resistance to clinical disease develops.

Pathology

In susceptible fish exposed when younger than nine weeks, there may be obvious pathological changes. In fish exposed when older than nine weeks, pathological changes (gross and histological) may be minimal. There may be no obvious internal lesions in fish surviving infection.

1.4.5   Laboratory methods

Sample submission

Samples should first be submitted to the appropriate state or territory laboratory. These laboratories will provide appropriate advice on which specimens to submit. Additional detailed information on sample submission is in Appendix 3 or the Blue Book of the Fish Health Section of the American Fisheries Society (MacConnell & Bartholomew 2012).

Fish with the highest risk (i.e. susceptible salmonid species and age groups, and fish managed using practices which may increase the risk of infection) should be selected for submission and submitted live or freshly killed. High-risk groups include rainbow trout fingerlings or yearlings, and fish raised in soil-lined ponds or raceways with untreated water.

To increase the likelihood that at least partially developed spores are visible on histopathological examination, ensure some fish have experienced 1800 degree-days; this is the period over which the sum of average daily temperatures is 1800 (e.g. if mean daily temperature is 10 °C, the 1800 degree-day period would be 180 days). Alternatively, if records are poor, ensure that fish are 6 months or older. Sampling of fish should focus on those with clinical signs which are more likely to have spores in the cartilage. For fish with presporogonic stages, histopathological examination to identify these stages and *M. cerebralis* specific PCR would be adequate to confirm the diagnosis, although mature spores may not be readily observed.

Multiple fish should be submitted. Up to five fish can be pooled for screening with pepsin–trypsin digest but histopathological examination requires individual fish.

Whole heads should be submitted if fish are not too large. These should be from freshly killed fish and shipped on ice. If the fish is very large a wedge shaped sample or core sample can be submitted in lieu of a fresh head. For fish > 15 centimetres, a triangle-shaped wedge is cut posterior to the orbit at the dorsal surface almost to the ventral edge of the opercula. The top (dorsal) portion of the wedge should measure 1.5 centimetres. Additionally, fresh tissue samples from the head should be submitted preserved in 10% neutral buffered formalin.

Equipment for collecting samples, reagents for sample preparation and facilities for chilled or frozen storage and transport of samples will be required. Sampling equipment may be available on-site, or may be obtained from state or territory authorities. See AQUAVETPLAN Enterprise Manual for contact details (DA 2015b).

Microscopy

The parasite initially has an affinity for the skin and subsequently for the nerves and skeletal cartilage in the salmonid host. The granulomatous response seen in cartilage is the most prominent sign of infection.

In acute stages:

- there is little cellular response in the first few days
- macrophages attacking residual epithelial stages may be seen in sub-cutis
- nervous tissue containing parasite appears normal with no tissue reaction.

In later stages:

- lysis and phagocytosis of the cartilage by trophozoites initiate an intense inflammatory response in susceptible species (El-Matbouli et al. 1995; Feist & Longshaw 2006)
- lesions typically contain remnant cartilage, developmental and sporogonic stages of the parasite, and focal to diffuse granulomatous inflammation. Granulomas consist predominantly of epithelioid and mononuclear cells, fibroblasts and multinucleate giant cells.

A grading system has been developed to assess the histological lesions seen in whirling disease (Baldwin et al. 2000).

Histopathological examination

Presumptive diagnosis depends on visualising characteristic pre-spore or sporogonic stages of the parasite in cartilage surrounded by bone, and confirmatory diagnosis requires amplification of *M. cerebralis* DNA using a nested PCR assay (Andree et al.

1998). Characteristic pre-spore or sporogonic stages of the parasite are visualised with histopathological examination.

Fish heads (wedges or core samples for large heads) are placed in fixative (either 10% neutral buffered formalin or Davidson's fixative). Small samples (fish < 15 centimetres) fixed in Davidson's fixative can be transferred to 70% ethanol after 24–48 hours; larger samples require 48 hours and may require additional decalcification. Samples fixed in 10% formalin should be decalcified after 24–48 hours. Decalcified samples are then transferred to ethanol.

Tissues are embedded and sectioned using standard methods. They are then stained with haematoxylin and eosin or Giemsa and examined to confirm that the myxospores or developmental stages of the parasite are present in cartilage (MacConnell & Bartholomew 2012). This is essential for diagnosis, as there are other *Myxobolus* myxospores that can be found associated with other head tissues of fish (Andree et al. 1998).

Failure to detect spores of the correct morphology in any tissue is not sufficient to determine that the sample is negative for *M. cerebralis*. Detection of characteristic spores in tissues other than cartilage can be reported as negative for *M. cerebralis* (MacConnell & Bartholomew 2012).

A 'Slide of the Quarter' (Case# 03-3011: rainbow trout *(Oncorhynchus mykiss),* whirling disease caused by the myxosporean protozoan, *Myxobolus cerebralis*) was circulated around government laboratories in July–September 2007. Material was provided by the Fish Diseases Laboratory, CSIRO-AAHL.

Preparation for parasite identification

To further aid identification of *M. cerebralis* spores in tissue, purification of myxospores (e.g. with pepsin–trypsin digestion) can be undertaken. Further staining methods (e.g. silver nitrate or direct fluorescent antibody [Wolf & Markiw 1979]) can be used to aid identification. An experienced parasitologist will be required as identification of *M. cerebralis* myxospores can be difficult.

Molecular techniques

Amplification of *M. cerebralis* DNA allows a confirmatory diagnosis of infection with *M. cerebralis*.

Tissue samples for PCR analysis should be frozen or preserved in 80–95% analytical-grade ethanol. Several PCR tests have been researched and compared with traditional methods (Andree et al. 1998; Cavender et al. 2004; Kelley et al. 2006; Kelley et al. 2004b; Thompson 2007). PCR methods generally perform well, are highly sensitive, and allow diagnosis earlier than histopathological diagnosis and at low concentrations of the parasite. The qPCR is arguably the most useful test but challenges remain with using qPCR in the regulatory framework of aquatic animal health (Purcell et al. 2011). A validated and standardised qPCR is used in New Zealand. A nested PCR is currently the test recommended by the Fish Health Section of the American Fisheries Society (Andree et al. 1998; MacConnell & Bartholomew 2012).

1.4.6   Confirmation of infection

For the purposes of this manual, confirmatory diagnosis of whirling disease or infection depends principally on amplification and sequencing of *M. cerebralis* DNA, for example using a nested PCR assay (Andree et al. 1998).

1.4.7   Differential diagnosis

Acute whirling disease should be on the differential diagnosis list whenever young salmonids in Australia show neurological signs such as tail-chasing, spinning or spiralling in the water column.

The differential diagnoses that should be considered when the clinical signs associated with whirling disease are seen in Australian salmonids include:

- septicaemic conditions that cause inflammatory responses in the brain (e.g. *Yersinia ruckeri*); the aetiological agent causing the associated inflammation may be bacterial, viral or protozoal
- nutritional disorders; some, e.g. vitamin C deficiency, have been associated with skeletal deformities in salmonids
- early infection (e.g. salmonids < 5 grams) with *Flexibacter* species resulting in shortened operculae in fish that survive
- high incubation temperatures (e.g. in Atlantic salmon, > 8°C until first feeding) and fluctuating temperatures during incubation causing skeletal deformities, ranging from minor lesions in single vertebrae, 'short tails' and 'humpbacks', to short body dwarfism, in which the vertebral column is compressed and ankylosed (Grete Baeverfjord, Research Scientist, Akvaforsk, pers. comm.)
- electroshock injuries causing skeletal deformities and/or melanosis (Margolis et al. 1996; Wolf et al. 1981)
- injection damage causing caudal melanosis, where the caudal vein has been used as the injection site.

Sound judgment of fish health must be used to distinguish between the many conditions on the list of differential diagnoses.

1.5   Resistance and immunity

1.5.1   Resistance to infection

Interspecific differences in susceptibility to infection with *M. cerebralis* are well documented in fish. Only salmonids are affected, and there is also wide variability between salmonid species in susceptibility to both subclinical infection and whirling disease. See Section 1.2 (especially Table 1).

There is also good evidence of intraspecific variation in resistance to infection. Considerable variation in susceptibility to clinical disease has been shown within susceptible species, depending on several factors. Older and larger rainbow trout are more resistant to clinical disease, although the level of skeletal ossification was not important in the development of clinical disease (Ryce et al. 2005).

Within highly susceptible species such as rainbow trout, some German hatchery strains (and their crosses) are resistant to clinical disease, but other strains from the United States are not (Fetherman et al. 2014). There is a genetic basis for this (Baerwald 2013); a single QTL genomic region explains much of the phenotypic variation in resistance to *M. cerebralis* (Baerwald et al. 2011). Natural selection for resistant fish is also possible within a few generations (Granath & Vincent 2010; Miller & Vincent 2008).

1.5.2   Innate immunity

The initial portals of entry for the waterborne infective stage of the parasite include the epidermis, respiratory epithelium and buccal cavity. Non-specific, innate defence mechanisms such as lysozymes are located in these areas (Gomez et al. 2014) but little is

known about interactions between myxozoan parasites and fish hosts (Kallert et al. 2009).

Skin penetration by the sporoplasm is facilitated by the enzymatic role of proteases (Kelley et al. 2003; Kelley et al. 2004a). While *M. cerebralis* exhibits non-specific invasion behaviour (Kallert et al. 2011; Kallert et al. 2009), non-salmonid species (e.g. carp) can either prevent initial skin penetration of the sporoplasm (Kallert et al. 2009), or prevent subsequent developmental phases that would otherwise result in the formation of spores in tissues (El-Matbouli et al. 1999a; Kallert et al. 2009).

Myxobolus cerebralis targets immune-privileged host tissues such as nervous tissue (avoiding the host immune system), depresses the immune system of infected fish, and modifies its antigen expression at different life-cycle stages (avoiding host immune responses) (Densmore et al. 2004; El-Matbouli et al. 1995; Knaus & El-Matbouli 2005; Sitja-Bobadilla 2008a; 2008b).

1.5.3   Adaptive immunity

Myxobolus cerebralis infection in fish may be characterised by a relatively short exposure to the host immune system (approximately four days), during the parasite's migration through the epidermal layers and into the nervous tissue (El-Matbouli et al. 1992).

Circulating antibodies to the parasite have been detected in rainbow trout (Griffin & Davis 1978) and there is evidence that serum with anti-*M. cerebralis* spore antibodies collected from infected trout (at both early and late stages of infection) may offer incomplete passive protection when transferred to young rainbow trout (Hedrick et al. 1998).

Cell-mediated immunity may also be important in this disease (Hedrick et al. 1998). There is little cellular response seen in the first few days after infection, but macrophages are seen soon after, attacking residual epithelial stages of the parasite (El-Matbouli et al. 1999a). This response occurs during the active feeding developmental phase of the parasite, and during cartilage destruction, but does not occur once myxospores are formed (Halliday 1974).

Presporogonic and sporogonic stages associated with cartilage induce inflammation and a granulomatous response. This response may eliminate some, but not all, of the parasites, and varies with species susceptibility. In later stages, a key characteristic of the disease is the formation of granulomas. This has also been shown to vary between species, from an extensive, diffuse granulomatous response, to a well-defined, encapsulated granuloma containing few intact parasites.

1.5.4   Vaccination

While fish do have the physiological elements to mount an adaptive immune response and acquire immunity, and this has been observed for some myxozoan parasites, there are currently no vaccines for any myxozoan parasite, including *M. cerebralis*. UV-irradiated *M. cerebralis* may induce immunity in rainbow trout while not causing disease (Hedrick et al. 2012). In the absence of treatment and immunoprophylactic agents, avoidance of infection is the primary means of controlling myxozoan disease. See Gomez et al. (2014) for an extensive review of fish immunological responses to myxozoan infections.

1.6   Epidemiology

The major species affected by *M. cerebralis* include the salmonids, many of which can be infected and some of which are more susceptible to clinical disease (e.g. rainbow trout

and cutthroat trout, see Section 1.2 for details). Transmission occurs from *T. tubifex* worms to salmonids via TAMs and from salmonids back to *T. tubifex* via myxozoan spores. Temperature and ecological factors affect the distribution and prevalence of *T. tubifex* in freshwater aquatic environments, including the amount of available sediment. Resistance in some strains of susceptible fish can emerge and some lineages of *T. tubifex* are also resistant to infection. Susceptible salmonid species are more susceptible at young and less-developed stages and when higher infectious doses are received.

1.6.1   Incubation period

Salmonids can be infected without showing signs of clinical disease. When clinical disease occurs, clinical signs are generally due to the parasite causing inflammation and damage to cartilage. The parasite can localise in cartilage 20 days after exposure, with mature spores forming 52–120 days after exposure at 7–17 °C (Halliday 1973b). The first clinical signs can be seen at three to eight weeks after exposure (MacConnell & Vincent 2002).

1.6.2   Persistence of the pathogen

The two infective stages of *M. cerebralis*, myxospores and triactinospores, can each survive for the lifespan of their host (salmonid fish and *T. tubifex*, respectively) (Gilbert & Granath 2001; Hedrick et al. 2008). Once outside the host, spores survive for a variable time and are dispersed in fresh water.

After release from *T. tubifex*, TAMs remain infective for susceptible fish for six to 15 days or more at 7–15 °C (El-Matbouli et al. 1999b; Markiw 1992).

The myxospores can survive for considerable periods of time after release from an infected fish — usually a carcass. Early research examined myxospore survivability using vital staining and without knowledge of the two-host life-cycle. More recent research has examined survivability of the myxospore based on its ability to infect *T. tubifex* (Hedrick et al. 2008). Myxospores were able to infect *T. tubifex* for at least two months at 4, 10 and 20 °C, although little transmission occurred at 20 °C (Hedrick et al. 2008). El-Matbouli & Hoffmann (1991) showed that spores can retain infectivity for up to five months in mud at 13 °C. Experimental studies suggest that myxospores remain infective to susceptible lines of *T. tubifex* for between six and 12 months at low temperatures (5–15 °C) (Nehring et al. 2015).

Please see Section 2.2.6 and the [AQUAVETPLAN decontamination manual](#) (DAFF 2008) for information on how to reduce the persistence of spores using disinfection.

Vectors for myxospores include predatory fish and bird species (El-Matbouli & Hoffmann 1991; Hallett & Bartholomew 2012; Koel et al. 2010).

1.6.3   Modes of transmission

Transmission is indirect and horizontal with no known vertical transmission in *T. tubifex* worms or salmonids.

Transmission from salmonids to *T. tubifex* worms is via myxospores in water (see Section 1.1).

Infected salmonids are the predominant means of dispersal of myxospores (Hallett & Bartholomew 2012). Therefore live salmonids (e.g. stray migrating anadromous fish or fish from restocking programs), salmonid products, and by-products that contain raw cartilage, can transmit myxospores to clean water where uninfected worm populations can become infected. The movement of water or sediment containing sinking myxospores can disperse infection. Fomites such as fishing or aquaculture equipment, waders, boots (Gates et al. 2008) and other objects can transmit myxospores.

Piscivorous birds (El-Matbouli & Hoffmann 1991; Koel et al. 2010) and fish (El-Matbouli & Hoffmann 1991) can also transmit myxospores, as myxospores can retain their infectivity after transition through the gut.

Of most concern in an outbreak are movements of fish, fish products and fomites by people (Gates et al. 2008), and dispersal by predatory birds, as these movements may transmit infectious organisms most easily to new catchments (Koel et al. 2010).

Transmission from *T. tubifex* worms to salmonids is via TAMs (see Section 1.1). Transport of *T. tubifex* worms or water containing TAMs can lead to the release of TAMs into clean waterways with the subsequent transmission of *M. cerebralis* to salmonids. *Tubifex tubifex* worms may live longer than three years so any fallow period for fresh waters that have been depopulated of salmonids would need to remain so for at least three years (Nehring et al. 2015). *Tubifex tubifex* worms, both live and imported freeze-dried product, are traded widely in the aquarium industry as fish food.

Salmonid eggs from *M. cerebralis*-infected brood stock have been shown to be free of whirling disease (O'Grodnick 1975). However, there is the potential for mechanical transmission of myxospores and TAMs in packing material and water used for egg transport.

1.6.4   Factors influencing transmission and expression of disease

Several risk factors have been identified that influence clinical disease expression, including temperature, age and size of susceptible species, strains of susceptible fish and husbandry.

Individual fish of susceptible strains that are smaller and younger are more likely to show clinical disease than older fish (Ryce et al. 2004). For example, fingerlings and yearlings are most susceptible (MacConnell & Bartholomew 2012). The development of resistance to clinical disease in rainbow trout is associated with both the age and size of the fish at time of exposure. Rainbow trout must be both nine weeks of age or older and at least 40mm in fork length at time of exposure to exhibit increased resistance to whirling disease (Ryce et al. 2005).

The primary determinant of clinical disease is temperature. The optimal temperature for development and multiplication of *M. cerebralis* within its hosts is 10–15 °C (Blaylock & Bullard 2014). Temperatures above 20 °C are refractory to development of *M. cerebralis* in either host (in Feist & Longshaw 2006).

Susceptibility of both species and strain is also important for development of clinical or subclinical infection (see Section 1.2).

Certain husbandry practices facilitate infection. For example, earthen ponds, raceways or tanks provide habitat for *T. tubifex* and the completion of the life-cycle of the parasite. The use of concrete or plastic-lined pens will reduce the availability of suitable habitat, although *T. tubifex* can live in accumulated sediments. Therefore, facilities must be kept clean to reduce the incidence of infection.

Likewise, parasite transmission is facilitated by using untreated water from infected waterways.

1.7   Impact

Whirling disease caused large economic losses in the formative years of rainbow trout aquaculture in Europe. Control measures led to increased costs, mortality led to reduced

production, and some facilities required de-population to prevent contamination of nearby waterways.

Ecological impacts have been observed in North America with large and sometimes persistent population crashes in endemic salmonids (especially cut-throat and rainbow trout). Salmonid community and population structures have changed as a result. Despite this, recreational fishing has been relatively unharmed as a shift to resistant species occurred. See Hallett & Bartholomew (2012) for a discussion of the impacts.

There is no evidence that Australian native fish are susceptible to whirling disease (see Section 1.2). Australian impacts will likely be limited to fresh-water salmonid aquaculture facilities, and recreational fisheries that rely on salmonid species. Large population impacts would be possible in infected areas in these circumstances. Some rural areas benefit greatly from recreational fishing, so this disease may have direct economic impacts on the immediately affected townships and neighboring rural communities.

2   Principles of control and eradication

2.1   Introduction

The presence of *M. cerebralis* in Australia would be likely to cause severe impacts in infected freshwater salmonid aquaculture facilities and recreational fisheries relying on salmonid species. This section provides background information to enable the most appropriate response to detection of *M. cerebralis* in Australia.

There are three broad response options available. These options are:

1. ***Eradication***

Eradication of *M. cerebralis* from Australia would involve the highest level of control and would have the highest cost.

2. ***Containment and control via zoning and/or compartmentalisation***

This would involve containment of the parasite to areas with endemic infection, prevention of further spread and protection of uninfected areas.

3. ***Control and mitigation***

Implementation of management practices that decrease the incidence and severity of clinical outbreaks would involve the lowest level of control and cost.

The basic principles of eradication and other response options are described in the AQUAVETPLAN Enterprise Manual (DA 2015b) and the AQUAVETPLAN Control Centres Management Manual (DAFF 2001). The AQUAVETPLAN Enterprise Manual, Appendix 1, lists state and territory legislation relating to disease control and eradication.

The nature of an outbreak will affect which response option is considered most appropriate. Outbreaks which are widely disseminated or in both wild and aquaculture populations may make eradication more difficult, or impossible. For example, infection may have already been transmitted to wild populations of *T. tubifex* worms, or there may have been a release of myxospores across a wide area of natural habitat. In contrast, an outbreak at a single aquaculture facility may be relatively easy to eradicate.

The general strategies that should be used in the control of this disease include:

- Rapidly delineating the infection according to:

    - time (e.g. by ageing of lesions to estimate introduction date)
    - fish (e.g. aquaculture versus wild salmonids or species)
    - place (geographical extent of epizootic).

- Rapidly preventing further dissemination of the infection by implementing:

    - immediate zoning and movement restrictions (between and within farms) of stock, water, fomites and vectors
    - enhanced biosecurity (reduce fomite spread)
    - education of relevant groups.

- Reducing or eradicating infection by:

- depopulation, disinfection and disposal of infected aquaculture populations (consider practicality for wild populations)
- breaking life-cycle of parasite
- using water with low TAMs risk (filtered, treated or ground water)
- moving fish to saltwater
- eliminating *T. tubifex* worm habitat (using concrete raceways or by habitat restoration)
- implementing excellent management practices and hygiene standards.

There are limitations in our ability to satisfy these principles in the control and/or the eradication of *M. cerebralis*. For example, if the outbreak has spread to wild populations, it may be difficult to accurately survey distribution and manage infection.

Acknowledging such limitations, the most appropriate control option will depend on the probability of successful eradication and resources available to attempt management. These probabilities may be assessed by examining:

- the scale and size of an outbreak
- whether infection is confined to aquaculture facilities or has spread to wild reservoirs (salmonids or *T. tubifex* worms)
- the nature of wild reservoirs:

    - susceptibility (e.g. strain of worm or salmonid)
    - ability to de-stock localised wild populations (e.g. viability without restocking, fishing pressure etc.)
    - ecology
    - accessibility and tools to depopulate.

- the ability to identify wild reservoirs (distribution and species)
- the availability of alternative stock for restocking of aquaculture facilities
- expertise and capabilities of fish health management and response personnel
- the level of risk accepted for future spread of infection (e.g. associated with grow-out of infected populations)
- cost–benefit analyses.

Susceptible salmonid populations will generally be found in two production phases, a hatchery phase and a grow-out phase. In the hatchery, eggs can be hatched in tanks with re-circulating or flow-through water systems, and these can therefore be considered closed or semi-closed production systems, respectively. In the grow-out phase in Australia, salmonids are reared in cages, generally in sea water but sometimes in fresh water. There are also a few raceway-type systems in freshwater. These can be considered semi-open systems. See the AQUAVETPLAN Enterprise Manual for further information (DA 2015b). Wild populations are considered to meet the definition of an open system.

In closed systems (i.e. tanks that use re-circulating water), it may be possible to treat inflow and outflow water to reduce the risk of spore entry or exit from the tanks, meaning control of spores and prevention of transmission is possible. Sourcing uncontaminated water (e.g. ground water) is best for general disease management.

In semi-closed systems (i.e. hatcheries with flow-through water), the ability to control the movement of spores in water is reduced (myxospores in effluent, TAMs in influent). This makes control of transmission much harder (either spread of *M. cerebralis* to or from captive populations). Although control and treatment of input water is theoretically possible for the removal of viable TAMs (e.g. sand filtration, ozonation and

UV light (Arndt & Wagner 2004; Arndt et al. 2006; Wagner et al. 2003)), under the current management practices of many farms such treatment would not be feasible. A better option would be eliminating potential habitat for *T. tubifex* worms (e.g. lining earthen ponds with concrete or synthetic liners and keeping them clean).

Treatment to ensure inactivation of myxospores in effluent water is also considered less feasible. This could possibly be done with UV light treatment (Hedrick et al. 2008; Hedrick et al. 2007) but may not be practical in many facilities. Daily removal of dead fish will reduce myxospore release from the decomposing carcasses. Daily 'mort' (mortality) removal is possible in semi-closed systems, and should be standard practice in any aquaculture system as far as is practical.

Semi-open systems for grow-out have no control of water movement into or out of pens, but most grow-out occurs in salt or estuary water with high salinity. Fish initially reared in fresh water and exposed to TAMs could remain infected when transferred to the marine environment, but myxospores released directly from fish into the marine environment are unlikely to encounter a susceptible worm host, as *Tubifex* spp. do not tolerate salinities above 10 parts per thousand (Pinder & Brinkhurst 2000). Therefore, grow-out in semi-open sea pens is a viable option to control transmission risk and allow production to continue. There is a low theoretical risk that fish contained in cages moored in estuaries or sheltered areas of the sea could escape into the wild and migrate to fresh water. If fish are grown out in semi-open cages or raceways in fresh water there is a high risk of transmission.

Control of infection in open systems (i.e. wild populations) will be very difficult (e.g. depopulation and disinfection may be impossible). Despite this, eradication may be possible in some small isolated catchments of wild salmonids if infection was limited to these areas and salmonid populations were non-viable without restocking. Sustained heavy fishing pressure may reduce the abundance of fish to a very low level, and prevention of restocking may further reduce populations to negligible numbers. Habitat modification to remove *T. tubifex* (in sediments) may reduce the other host reservoir. Sentinel fish could then be used after several years to gauge success of eradication. Caution is required because this is a theoretical discussion and eradication from wild populations has not been achieved overseas (where salmonid populations are endemic and hence widely dispersed and abundant). In Australia, it may be a viable option in some circumstances, particularly where affected populations are entirely dependent on stocking. A fallow period of three years before restocking is recommend to allow infected *T. tubifex* worms to die out naturally (Nehring et al. 2015).

2.2   Methods to prevent spread and eliminate pathogens

2.2.1   Quarantine and movement controls

The following quarantine and movement restrictions should be implemented immediately on suspicion of *M. cerebralis*.

Establishment of quarantine areas

Establishment of specified areas (Figure 2) (see AQUAVETPLAN Enterprise Manual Section A for more details) (DA 2015b), including:

- declared area—infected, restricted and control areas:

    - infected area or premises—the premises (e.g. farm) or area where the infection is present, and the immediate vicinity

- ▪ restricted area—area around and containing infected premises or area
- ▪ control area—a buffer between the restricted area and free areas.

- free area—uninfected area; not considered a declared area and may include large areas of Australia in which the presence of *M. cerebralis* has not been detected through surveillance activities.

Figure 2 Establishment of specified areas to control *M. cerebralis*
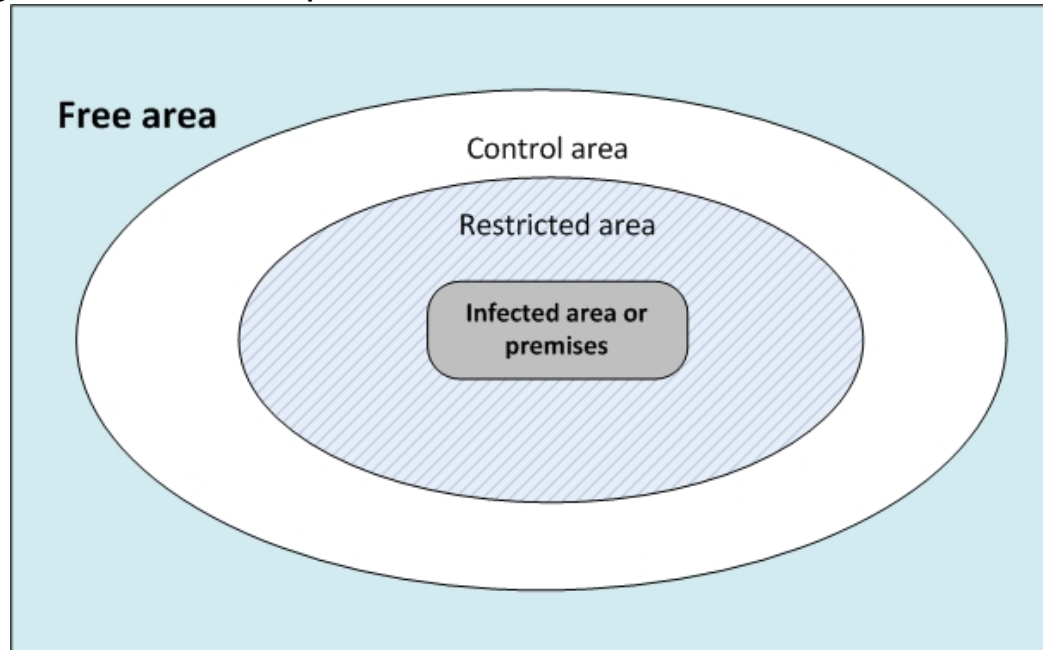
In the declaration of quarantine areas, the following factors need to be taken into account:

Epidemiological and surveillance information

Epidemiological and surveillance data on infection distribution and susceptible populations is the best means of establishing zones, but this is unlikely to be comprehensively available, especially early in an outbreak. Consideration of the following information may allow prediction of likely transmission and dispersal and early establishment of zones in the absence of good surveillance data.

Factors affecting transmission

Natural factors that could facilitate or hinder transmission include:

- contiguous distribution of naturalised salmonid populations
- vector movements (e.g. movements of piscivorous species)
- movements of salmonid carriers (e.g. adults or less susceptible salmonid species)
- water movements that can disperse spores
- natural catchment divisions that may control infection because water or wildlife does not move between catchments.

Anthropogenic factors (from the aquaculture industry) that could facilitate or hinder transmission include:

- connectivity between aquaculture facilities (e.g. company structure and movement of stock, equipment or fomites)

- proximity of other aquaculture facilities with susceptible species
- facilitation of business continuity (e.g. when possible, declared areas should facilitate movement of essential equipment, personnel and product between farms or to processing plants).

Anthropogenic factors (from recreational fishing) that could facilitate or hinder transmission include:

- establishment of areas based on likely historical movement of fishers that may have transmitted infection
- structuring of areas to minimise disruption to recreational fishing (where possible)
- structuring of areas so that movement bans can be legally and practically enforced.

The following practices must be considered when implementing response options:

- transport of fish, fomites and products
- transport of stock and equipment between farms
- transport to and between harvesting and processing
- transport of consumer products
- movements of recreational fishers
- movements of other river users
- discharge of effluent from processors and farms
- recreational fishing
- movement of potential vectors
- disposal of dead fish and products
- decontamination.

Movement controls

Movement controls include:

- banning movements within and out of restricted areas, including infected areas:

    – bans on the movement of live and dead salmonids, *T. tubifex* worms (or oligochaete worms if identification is difficult), water, aquatic plants (especially if mud and potentially *T. tubifex* worms are attached) and equipment should be considered, including movements within restricted (including infected areas) areas, and movements from restricted areas to control or disease-free areas
    – due to the freshwater life-cycle of the worm host, movement of fish from a restricted area into saltwater may be permitted, and it may be permissible to move fish directly to slaughter facilities if fish are carefully processed and the resultant waste is treated to kill myxospores (or otherwise disposed of safely)
    – some movements could be permitted for low-risk fomites after decontamination

- permitting movement from control areas to free areas:

    – permits required before movement of live and dead salmonids, *T. tubifex* (or oligochaete worms generally if identification is difficult), water, decontaminated equipment or other fomites and plants from control areas to free areas

- permitting movement from the control area to a restricted (including infected) area:

    – permits required before movement of live and dead salmonids, *T. tubifex* (or oligochaete worms generally if identification difficult), water, any equipment or other fomites and plants from control areas to restricted (including infected) areas

- unrestricted movements in the free area:

    – implementation of movement restrictions can assist significantly in the early stages of a disease response by preventing further dissemination of infection, and can also buy time while the extent of the infection is assessed
    – implementation of movement bans and restrictions will be a dynamic process, determined by the location and extent of the disease outbreak and whether the aim is to eradicate the disease agent or to control its spread; some restrictions may be impractical or unnecessary but others will be critically important to eradication or control
    – the feasibility of movement restrictions and bans and the extent to which they can be enforced will depend on the location of infection, the location and type of enterprises affected and the control response option chosen.

2.2.2   Zoning and compartmentalisation

It is sometimes possible to maintain a sub-population of salmonids with distinct aquatic animal health status (e.g. infected or free of whirling disease). This can be done on a geographical basis (referred to as zoning) or on the basis of common biosecurity factors, e.g. management practices (referred to as compartmentalisation). These processes are discussed below.

Zoning

If *M. cerebralis* were to become endemic in specific regions of Australia, a zoning policy specific for *M. cerebralis* may be necessary to protect non-infected areas and to prevent further spread of infection. Zones would be based on the distribution of *M. cerebralis* and of any vector species present (if appropriate), the geographical and hydrological characteristics of water bodies and landforms, and predictions of the most likely method of spread of infection. Zoning may rely on the identification of biogeographical barriers. A corresponding surveillance and monitoring program for *M. cerebralis* would be required to support the zoning policy. Principles of zoning for infected and non-infected zones in Australia are outlined in the AQUAPLAN Zoning Policy Guidelines (DAFF 2000) and in the OIE Aquatic Animal Health Code (OIE 2015a).

Establishing definitive infected and uninfected zones for whirling disease may be challenging in Australia. Difficulties in identifying both *T. tubifex* and infected fish, and excluding infection from disease-free areas (e.g. fomites such as fishing waders can transmit spores) may make it difficult to maintain or confirm zones. The presence of geographical barriers would assist zoning, for example Tasmanian salmonids may be protected from an outbreak in mainland Australia.

Compartmentalisation

A compartment in this context is defined as one or more aquaculture establishments under a common biosecurity management system, containing an aquatic animal population with a distinct health status relating to a specific disease or diseases, for which required surveillance and control measures are applied and basic biosecurity

conditions are met. Such compartments must be clearly documented by the 'competent authority' (the veterinary authority of the jurisdiction) if used for international trade.

A compartment does not have to include contiguous facilities—it can apply to a series of farms over a large area, including over several jurisdictions. The key is that it must have in place a biosecurity management system that meets the guidelines detailed in Chapters 4.1 and 4.2 of the OIE Aquatic Animal Health Code, and that these systems have been documented by the competent authority.

2.2.3   Disease management in aquatic environments

The establishment of Disease Management Area (DMA) boundaries during an outbreak of whirling disease presents particular difficulties requiring detailed consideration beyond that normally required for terrestrial animal disease control. Water movement through and around farms and within streams or rivers represents a substantial risk for spread of *M. cerebralis* through transfer of spores in the water column, movement of infected material (particularly dead fish parts and sediment containing spores) and any infected wild salmonids.

For example, although an infected area may be established around an individual land-based hatchery or farm, water bodies adjacent to the infected area and in the same catchment should be considered for monitoring and control measures. The establishment of DMA boundaries around wild salmonid fisheries may need to be comparatively large, and take into consideration local flows, natural barriers and the range of susceptible wild salmonids.

Establishment of the relevant DMA boundaries must also take into account the dispersal of spores in water discharged from any infected semi-closed aquaculture systems (e.g. hatcheries) or potentially infected processing facilities, and how this enters adjacent water. Potential spread of infection by other species also needs to be considered, as piscivorous fish and birds can act as vectors, with viable spores passed in faeces that can infect *T. tubifex* (El-Matbouli & Hoffmann 1991; Koel et al. 2010).

Therefore, rather than property boundaries, the geography, water flow, distribution of susceptible species, distance between farming areas and the range of susceptible species will define where DMA boundaries are placed.

Establishment of DMA boundaries and their classification must also take into account potential mechanisms by which *M. cerebralis* may move beyond these boundaries (e.g. recreational fishers, movement of live fish associated with restocking, vector movements or aquaculture activities). In most circumstances, it is advisable to overestimate the size of DMAs and reduce their area as the response takes effect. In most cases, in the initial response, the DMA boundaries will need to include the whole catchment area in freshwater systems.

2.2.4   Tracing

Tracing a disease outbreak is the process of retrospectively determining the method and pattern of disease spread. Tracing investigations are crucial in determining all confirmed and potential locations of the disease, as well as defining restricted and control areas. The information gathered from tracing will help determine the most appropriate response action. The immediate steps required are to trace back all contacts with infected salmonids and *T. tubifex*, premises and sites (to establish the origin of the outbreak), and to trace forward all contacts with infected salmonids and *T. tubifex*, premises and sites (to establish the current location and potential spread of infection). Aquatic surveillance information is available from the Department of Agriculture and Water Resources aquatic disease surveillance guidelines (Cameron 2004).

The following items must be traced:

- fish movements (including live fish, dead fish and fish products)
- effluent and water from the facility (potentially including hydrological modelling)
- personnel
- vehicles
- equipment
- natural movements of vectors, wild salmonids and water (including modelling theoretical movements when tracing actual movements of these entities is impractical)
- distribution of *T. tubifex* assemblages.

Fish farms on the same watercourse or in the same watershed may already be infected. Some freshwater operations slaughter and process fish on site. Any waste from such processing could be a source of myxospores if it reaches fresh water. Infection could be established downstream if susceptible *T. tubifex* worms are present. Maps with the location of neighbouring fish farms, waterways and hydrographic data are necessary to monitor the potential spread of the pathogen. The location of susceptible fish species should also be noted both upstream and downstream of the infected site. Further sources of infection may be identified if a number of facilities share a common water source. Both myxospores and TAMs (which are neutrally buoyant) can travel considerable distances in water.

Predators such as birds and fish can also carry infected fish to neighbouring waterways. Myxospores can survive passage through the gut of some birds and fish.

For information on the location of farming establishments and wild fish populations at risk of infection, contact the relevant state or territory authority (see AQUAVETPLAN Enterprise Manual for contact details) (DA 2015b).

2.2.5   Surveillance

Surveillance, by screening for clinical signs and by laboratory testing of samples for subclinical infection, is necessary to:

- define the extent of the infection and establish restricted and control areas to which quarantine and movement restrictions are applied
- detect new outbreaks
- establish infected and non-infected areas for an *M. cerebralis* zoning and/or compartmentalisation program
- monitor the progress and success of an eradication strategy
- demonstrate freedom from infection after an outbreak.

A surveillance program needs to be designed to meet the requirements of the investigation—whether it is for delimitation of infected area for control purposes, or for longer term management including certification for translocation or export purposes.

During surveillance at a premises, laboratory samples required for submission include fish heads from several fish preserved in chilled and 10% neutral buffered formalin or Davidson's fixative (see Section 1.4). These should be from susceptible salmonid species of appropriate ages (e.g. young rainbow trout), preferably showing clinical signs. Other salmonid species or older rainbow trout can be sampled to detect subclinical carriers. These samples will be used for PCR tests, microscopic and histopathological examination, and possibly for spore extraction and visualisation.

Subclinically infected stock must be identified before eradication can occur. Diagnosis of subclinical infection is more complex than diagnosis of clinical disease, because there will be little evidence of which individual fish to sample and fewer spores to visualise on histopathological examination. To diagnose subclinically infected stock requires epidemiological investigation (e.g. age, species and contacts) and, importantly, application of diagnostic tests, especially a well validated and correctly interpreted PCR test. Given the reduced sensitivity of histopathological examination in the diagnosis of subclinical infection, PCR is the recommended test for surveillance purposes.

Conversely, the presence of a known outbreak of whirling disease in a region (i.e. where an index case has already been diagnosed) and clinical signs of disease in salmonids of known susceptibility (e.g. young rainbow trout) will be enough evidence to presumptively diagnose suspect whirling disease cases without using diagnostic tests. Despite this, application of a PCR test or histopathological examination is required for confirmatory diagnosis.

2.2.6   Treatment of host products and by-products

There is no treatment available to eliminate the parasite from live infected fish, although some treatments have reduced both the prevalence of disease and the number of generated spores. For a more complete discussion of the literature relating to this, see Hallett & Bartholomew (2012).

Trade regulations, market requirements, food safety standards and potential spread of the pathogen must be considered when determining the treatment, processing and destination of fish products and by-products.

Untreated product from infected fish can transmit infection as myxospores will be present in cartilage. *Myxobolus cerebralis* can survive well in dead fish, even when frozen at –20 °C for up to three months. Brined fish also retain viable spores, although hot-smoking at 66 °C for 40 minutes inactivates spores (Wolf & Markiw 1982).

The species of salmonid affected will influence the potential number of myxospores per fish. Infected rainbow trout can have high numbers of myxospores in cartilage, and affected fish products and by-products will potentially have higher myxospore burdens compared to less susceptible species, such as brown trout.

Eggs

Myxobolus cerebralis is not transmitted vertically. Myxospores and TAMs could be mechanically transmitted during transport in packing material and fluids surrounding fish eggs if contaminated water has been used in spawning. The OIE Aquatic Animal Health Code has a chapter on disinfection techniques for salmonid eggs. Those methods were specifically developed to limit virus transmission, but they would reduce risk of transmission of all diseases (see http://www.oie.int/en/international-standard-setting/aquatic-code/access-online/).

Human health

Myxobolus cerebralis is not known to infect or cause clinical disease in humans. Infected fish are likely to be safe to eat, but clinically diseased fish may not be marketable due to the gross pathological changes associated with disease.

2.2.7   Destruction of hosts

Destruction of fish

Slaughter must be both hygienic and humane. It is unlikely that the chosen destruction method will influence myxospore shedding at slaughter, as most myxospores are released from cartilage on death and decomposition of fish.

There are many different methods to anaesthetise and/or slaughter fish, all of which have limitations. A common procedure involves lowering the water level in a tank of fish and using a water-soluble anaesthetic agent to sedate the animals before slaughter (e.g. isoeugenol [Aqui-S®], which has no withholding period). The dose of anaesthetic can be varied depending on whether the fish are to be subsequently harvested or immediately euthanased.

Stunning (often followed by slaughter and bleeding) may be mechanised (percussive stunning) or manual (e.g. with a club).

Pithing (e.g. using the *ike jime* method) is rapid and effective, although it is labour-intensive.

Fish may also be killed by removing water which asphyxiates and crushes fish.

The most appropriate method of slaughter depends on the following factors:

- size and number of fish
- deadline for slaughter (which depends on the mortality rates and the ability to contain the disease)
- destination (for human consumption or disposal)
- slaughter facilities (site, equipment and methods available)
- welfare considerations.

The National Aquaculture Council's Aquatic Animal Welfare Guidelines (Johnston & Jungalwalla n.d.), OIE Aquatic Animal Health Code (chapter 7.3 and 7.4) (OIE 2015a) and the AQUAVETPLAN Destruction Manual (DAFF 2009a) provide useful guidelines and information relating to salmonid welfare during destruction. In general, fish should be killed rapidly (for example with pithing); or should be stunned (e.g. using a mechanical percussion stunning device) or sedated (e.g. with Aqui-S) before being killed (Johnston & Jungalwalla n.d.). Other methods can achieve a similar effect. In some instances during an emergency, less humane methods may be necessary to maximise biosecurity outcomes.

Experience and availability of personnel

Experienced staff should conduct destruction to ensure it is done appropriately and safely. The availability of staff may influence which method of destruction is best, as some are more intensive and require more experienced staff.

Any chemicals used must be approved for that use by the Australian Pesticide and Veterinary Medicines Authority (APVMA) (see Appendix 2).

In addition, any chemical that is used directly or indirectly for the control of an animal disease is governed in its use by relevant 'control of use' legislation in each state and territory. The relevant state or territory authority should be consulted for advice before use of the chemical.

See the AQUAVETPLAN Destruction Manual for further information (DAFF 2009a).

Elimination of oligochaete worms

Physical alteration of natural habitats (generally habitat alteration to remove sediments) has been attempted in other countries to reduce the density of *T. tubifex*

with the aim to reduce the prevalence of whirling disease in natural settings. Significant engineering and costs can be associated with such measures. These studies have produced variable results; several demonstrated reduced whirling disease prevalence after the habitat modification (Granath 2014; Hansen & Budy 2011) but others showed no significant change in whirling disease prevalence with habitat modification (Pierce et al. 2014; Thompson 2011). It is likely that the success of habitat alteration is context-specific and prediction of the outcome is complex. Significant habitat alteration is unlikely to be socially acceptable even if it were practical or cost-effective.

In aquaculture facilities, the best means of removing the worm host is to eliminate worm habitat, either through the use of concrete or lined raceways, or with regular cleaning of ponds to remove sediments. If it is not viable to replace earthen facilities, destocking and drying raceways is an effective means of reducing worm numbers and disinfecting sediment of viable myxospores (Bartholomew et al. 2007).

Generally, disinfection of ponds with calcium cyanide, calcium canamide or chlorine will render spore stages non-viable and kill the invertebrate host. A molluscicide, Bayluscide (5,2'-dichloro-4'-nitrosalicylanilide), reduced worm densities by 73–82% (Kowalski & Bergersen 2003). This compound is toxic to fish at the doses used to kill worms. Any use of unregistered products would require an emergency-use permit from the APVMA (see Appendix 2), and appropriate permissions from local environmental authorities for disposal of waste to safeguard the environment.

2.2.8   Disposal of hosts

Correct disposal of carcasses is critical because of the highly resistant nature of myxospores. Rapid removal of carcasses ('morts') from ponds or raceways is also essential to both minimise myxospore release and prevent exposure of susceptible *T. tubifex* worms. Disposal sites must be carefully chosen to ensure there is no contact with waterways or birds and other animals that could transmit the myxospores to *T. tubifex* habitats.

Myxospores are *not* directly infectious to the fish, so infected fish are not a direct source of infection to uninfected fish.

See the AQUAVETPLAN Operational Procedures Manual (Disposal) for details of destruction and disposal of fish carcasses (DAFF 2009b).

2.2.9   Decontamination

Due to differences in farming enterprises, disinfection protocols may need to be determined on an individual basis and involve the farm manager, the state or territory Chief Veterinary Officer (CVO) and/or the Director of Fisheries. The protocol should take into consideration the factors outlined in Section 2 of the AQUAVETPLAN Decontamination Manual, in particular:

- the source and location of infection
- the type of enterprise (e.g. farm, processing plant, hatchery, grow-out ponds, water source etc.)
- the construction materials of the buildings and structures on the site
- the design of the site and its proximity to other waterways or buildings
- current disinfection protocols
- workplace safety concerns
- environmental impact of the disinfectant protocol
- legislative requirements (occupational health and safety, environmental protection, chemical use)
- availability of approved, appropriate and effective disinfectants.

See the AQUAVETPLAN Operational Procedures Manual – Decontamination for details of decontamination methods and their indicators (DAFF 2008), in which, as well as the general information detailed above, there are some specific details for *M. cerebralis*.

Myxospores

Due to the resistant nature of myxospores, effective decontamination of equipment, materials, personnel, tanks and buildings must inactivate this stage of the parasite, or ensure that viable myxospores do not enter freshwater waterways containing susceptible *T. tubifex* worms. Decontamination requires thorough cleaning *before* disinfection. The water used for the cleaning which then enters a freshwater habitat may contain viable myxospores if it is not disinfected before release.

Practices and compounds effective in killing myxospores (reviewed in Wagner [2002] unless otherwise stated) include:

- calcium hydroxide at > 0.5% for 24 hours
- calcium oxide (quicklime) or potassium hydroxide at > 0.25% for 24 hours
- chlorine at 1600 parts per thousand for 24 hours, or at 5000 parts per thousand for 10 minutes; 500 parts per thousand for 15 minutes disinfected most myxospores (Hedrick et al. 2008)
- heating for 10 minutes at 90 °C, and 20 °C for two months (Hedrick et al. 2008)
- drying of ponds may be effective; contaminated mud dried for 13–19 months did not induce infection when the pond was restocked
- UV irradiation at ≥ 40 MJ/cm^2 (Hedrick et al. 2008)
- freezing at –20 ° and –80 °C inactivates myxospores after seven and 56 days, respectively (Hedrick et al. 2008)
- alkyl dimethyl benzyl ammonium chloride (ADBAC) at 1500 mg/L for 10 minutes
- chitinase inactivates myxospores from other species of myxozoans (Liu et al. 2011) but is not currently a viable product for commercial use.

TAMs

TAMs are less resistant than myxospores, e.g. drying or freezing for at least an hour will inactivate TAMs (Wagner et al. 2003). Other methods of TAM inactivation include:

- temperatures > 75 °C for at least five minutes (at 7 °C, TAMs can survive seven to eight days) (Wagner et al. 2003)
- chlorine at 130 parts per million for 10 minutes is effective at temperatures ranging from ice-water to room temperature, and total hardness ranging from 10 to 500 mg/L
- hydrogen peroxide at 10% for 10 minutes (Wagner et al. 2003)
- povidone-iodine at 5000 parts per million active iodine, for 60 minutes (Wagner et al. 2003)
- UV irradiation of ≥ 40 MJ/cm^2 will inactivate TAMs (Hedrick et al. 2007)
- sand filters are successful at removing most TAMS from hatchery water, thereby markedly reducing the infection prevalence in fish (Arndt & Wagner 2004; Arndt et al. 2006).

Stringent decontamination of saltwater facilities from which fish need to be removed (i.e. fish infected in freshwater, then transferred to saltwater) will not be required, as it is unlikely that myxospores released into a saltwater environment would infect a freshwater *T. tubifex* worm. Likewise, processing plants discharging into saltwater are unlikely to need stringent decontamination protocols unless there is potential traffic

between the facility and freshwater, or the potential for birds or other animals to carry carcasses or offal to freshwater habitats.

2.2.10 Vaccination

Generally, fish can mount an adaptive immune response and acquire immunity. Immunological memory specific for some myxozoan parasites has been observed. Additionally, UV-irradiated *M. cerebralis* have induced immunity in rainbow trout while not causing disease (Hedrick et al. 2012). Currently, however, there are no vaccines for any myxozoan parasite, including *M. cerebralis*. See Gomez et al. (2014) for an extensive review of current research.

2.2.11 Vector control

Myxospores can survive passage through the gut of piscivorous birds or fish (El-Matbouli & Hoffmann 1991; Koel et al. 2010). Of the nine bird species tested by Koel et al. (2010), all species had *M. cerebralis* DNA detected in the faecal material of birds fed infected rainbow trout. However, only one species (the great blue heron) was shown to be a definitive vector, as measured by the production of viable TAMs in laboratory *T. tubifex* worms exposed to myxospores of infected bird faeces. The ability of Australian birds and fish to act as vectors is unknown.

Open air tanks, ponds and especially processing facilities and areas where carcasses are disposed of may attract birds and must be covered (e.g. using nets or tank roofs). Predatory or scavenging fish that may act as vectors (or become infected) must be excluded from aquaculture and processing facilities.

Water rats could also act as mechanical vectors of myxospores and will easily move between natural and aquaculture habitats unless adequate fencing is in place.

2.3   Environmental considerations

Environmental considerations in the control of whirling disease include:

- Discharge of infected or potentially infected effluent into freshwater catchment areas or natural waterways may lead to further spread of infection, and could lead to the establishment of reservoirs of infection in *T. tubifex*, wild fish populations and waterways.
- The use of disinfectants could have an impact on the environment, especially if used in larger quantities or concentrations than usual. The local environmental protection agency will need to be consulted (see the AQUAVETPLAN Enterprise Manual [DA 2015b]).
- Environmental impacts related to the destruction and disposal of infected carcasses and material must be minimised while ensuring that there is no dissemination of infection.

2.4   Sentinel animals and restocking measures

Removing all salmonids from an area is the only way to ensure there are no susceptible fish hosts in which the parasite can complete its life-cycle. If *T. tubifex* is removed from a facility, and there is no possibility of TAMs entering the facility, then restocking can start immediately.

For eradication in farming facilities, restocked fish must be free of subclinical infection or clinical disease. If areas are declared free of *M. cerebralis*, fish introduced into those areas must also be free from infection. Declaring an area free of the parasite may be very difficult.

Rainbow trout are considered the most susceptible fish to infection. Young rainbow trout may be stocked as sentinel fish to determine the presence or absence of the parasite. For examples, see Bartholomew et al. (2007) and Kelley et al. (2006).

Atlantic salmon and brown trout can be infected with the parasite and myxospores can form in their cartilage, but clinical disease is rare and morbidity low. Hence restocking with less susceptible species (and when fish are older e.g. at > nine weeks) may be an option in some areas to maintain production if eradication is not feasible. Restocking with fish strains that are resistant to clinical disease may also be an option.

2.5   Public awareness

A community engagement program emphasising education, surveillance and cooperation from industry and the community is essential. The public should be informed that:

- whirling disease does not infect people
- the two-host life-cycle is complex
- good biosecurity is required to prevent further spread
- surveillance is essential to determine the scale of the epizootic
- cooperation is essential.

2.6   Feasibility of control or eradication of whirling disease in Australia

The feasibility of controlling an outbreak of whirling disease (or infection with *M. cerebralis*) depends on the nature and location of the outbreak. As outlined in Section 2.1, there are three response options: eradication; containment and control via zoning and/or compartmentalisation; and control and mitigation.

2.6.1   Response Option 1: Eradication

Eradication relies on rapidly imposed movement restrictions, surveillance and tracing, destruction and disposal of infected fish, and decontamination. If the technical capabilities to implement each of these steps, or resources required are limited, attempted eradication is less likely to succeed.

If wild populations are affected, it is generally accepted that *M. cerebralis* cannot be eliminated once it is established. Failed eradication efforts from areas where the parasite is now endemic (e.g. many states in the United States) have been documented in the literature, including drastic measures such as chlorinating an entire stream. As Wagner (2002) states, 'The best management is to avoid infecting negative waters'. These failed attempts, however, have been in high-density, widely dispersed and well-established native salmonid populations. In contrast, there may be the potential for eradication in some areas of Australia where an epizootic is limited to small isolated populations of non-endemic salmonids that are not sustainable without restocking. This is a theoretical possibility and has not been rigorously investigated.

Eradication is unlikely to be successful or feasible if epidemiological investigations determine that infection is widespread, has no detectable point source, is unable to be contained and is present or potentially widely established in wild fish species, or natural water bodies. This is due to:

- the ability of *M. cerebralis* to spread rapidly and establish reservoirs of infection in wild fish populations that may be impossible to eradicate
- the ability of *M. cerebralis* to produce subclinical infections that are difficult to detect

- the ability of infected wild fish and vectors to transmit and establish infection in rivers and other freshwater habitats
- close contact between, and relative lack of control over, farmed and wild fish populations, and water in Australian salmonid farming operations (both semi-open and semi-closed systems)
- experience in affected countries where eradication was unsuccessful once reservoirs of infection became established in wild fish populations and the natural environment.

In certain circumstances, it may be possible to eradicate *M. cerebralis* from a fish farming facility. This has occurred overseas (Anderson 1993). The principles are to ensure that:

- source water is either free of TAMs or there is the potential to adequately treat incoming water
- there are no potential habitats for *T. tubifex* worms within the facility.

The option chosen must ensure that there is no further:

- exposure of unexposed fish populations to TAMs
- spread of infection via the release of myxospores into the environment.

Unexposed salmonids

In an eradication response, unexposed salmonids in disease-free areas would not be subject to destruction notices, and commercial farms could continue normal operations, provided that future exposure to infection can be prevented. Young (pre-market sized) unexposed fish in declared control areas may be allowed to grow-out, also under the proviso that future exposure to infection can be prevented, while older unexposed fish may be emergency-harvested and slaughtered for human consumption. Alternatively, fish may be transferred to salt water to negate the possibility of exposure to TAMs. This is only a viable option when:

- fish will tolerate such salinity changes
- saltwater facilities are available and suitable.

Emergency destocking of unexposed fish populations located within declared control areas could be considered as part of an eradication response (with the aim being to increase the size of the buffer zone between infected and uninfected areas, and to increase confidence in their putative disease status). However, given the nature of the parasite and the likely high commercial value of the affected fish, this action may be considered excessive, even within the conservative approach typical of a disease eradication response. *Myxobolus cerebralis* cannot be transmitted directly from fish to fish, and there is a significant temporal lag for development of the parasite within the *T. tubifex* worm host before infective TAMs are released (> 90 days, depending on temperature).

A more measured approach, involving concurrent and rigorous surveillance of unexposed fish populations in control and disease-free areas, may therefore be preferable to emergency destocking in control areas. Detection of *M. cerebralis* infection in control or disease-free areas would indicate that containment of the outbreak had failed. A decision would need to be made about whether disease eradication remained feasible, or whether the response would need to transition to a containment and control phase (Section 2.6.2).

Exposed or potentially exposed fish

All live salmonid fish within an infected or restricted area are assumed to be exposed. Immediate destruction and disposal of these fish could be an option during an eradication response. However, this will not eradicate the parasite if there is the possibility that effluent water or escaped infected fish from the affected facility have spread myxospores to downstream worm habitats. There is no need for immediate destruction if the fish are located in saltwater.

Normal or controlled grow-out of exposed, or potentially exposed, clinically normal farmed salmonid populations could also be considered, provided any myxospores potentially released during the grow-out period are prevented from coming in contact with *T. tubifex* worms. Grow-out in saltwater may achieve this where fish can tolerate such salinities (e.g. smolted Atlantic salmon), and where the facilities exist. Other grow-out options include facilities that have the ability to send effluent water to ground, or where there is no possibility of contaminated water entering areas with suitable worm habitat.

Treatment of exposed or potentially exposed fish is not an option during an eradication response, as there are no effective treatments that destroy all myxospores in the fish host. Emergency harvesting of exposed fish can be considered, and is unlikely to result in further transfer of infection providing there is no possibility of untreated processing waste, especially skeletal elements, being released into freshwater habitats. Infected fish are safe for human consumption.

The strict control measures necessary to prevent further spread of infection include:

- disinfection of all equipment/personnel involved in harvesting, slaughter and processing to eliminate the risk of transferring myxospores off site.
- quarantine restrictions and procedures applying to the infected site, including personnel, equipment and vehicles; quarantine is aimed at myxospores, as it is unlikely that TAMs would be transferred off site by this route
- processing, possibly onsite or offsite, provided waste and fish carcasses cannot come in contact with freshwater *T. tubifex* habitats
- holding, treatment and safe disposal of slaughter and processing effluent (including holding water and waste offal)
- ensuring that the final product will not result in the spread of infection
- disinfection of effluent water.

Infected fish

If clinically diseased and dead fish are located in fresh water, and there is no possibility of disinfecting or redirecting effluent water from the facility, their removal, destruction and disposal is essential to prevent myxospores transmitting infection. If clinically infected or dead fish are to be removed, burial sites should be chosen carefully to ensure there is no contact with waterways, birds or animals.

Tubifex tubifex worms may also be infected, and will remain infected for the life of the worm, which can be up to three years.

2.6.2   Response Option 2: Containment and control via zoning and/or compartmentalisation

The purpose of this response option is to contain the parasite to areas with endemic infection by preventing further spread and protecting uninfected areas. It may be feasible where an epizootic is limited to a well circumscribed geographical area (e.g. an

island such as Tasmania or a remote wild population in a well circumscribed catchment) or where the epizootic is limited to a single industry compartment.

There are challenges to implementing this response. For example, it may be difficult to effectively contain the infection; fishers and wild vectors may transmit infection between catchments; and spores (both TAMs and myxospores) can be transmitted long distances in water. Additionally, the subclinical nature of the parasite in some salmonid species and older fish, the long incubation period and difficulty in identifying *T. tubifex* may make surveillance and delineating infected areas difficult.

In summary, if an epizootic was widely dispersed either across wide geographic areas or across several sectors of commercial industry, and if significant resources were not available for surveillance and enforcement of biosecurity measures, containment and control would be very difficult. In contrast, if the outbreak was detected early, before wide dissemination, was confined to well delineated geographic or industry compartments, and public and industry cooperation was high, then containment and control may be possible.

Unexposed fish

Management options for unexposed fish are the same as those outlined for eradication. The implementation of a zoning and/or compartmentalisation program, and associated control measures, to maintain uninfected areas would be necessary. For zoning, see Section 2.2.2.

Exposed or potentially exposed fish

Exposed or potentially exposed fish within an infected zone or compartment are assumed to be infected. Immediate destruction of these fish is an option for a containment and control response, as it can help decrease the infectious load on a site and minimise the spread of infection. If susceptible *T. tubifex* hosts are also infected, then destruction of fish must be carefully considered against the overall benefit of removing one host but not the other. Removing both hosts by destruction and elimination of *T. tubifex* habitat, if this is possible (e.g. cleaning and drying ponds, or conversion to concrete raceways), will be a more effective option.

In an infected zone or compartment, normal or controlled grow-out and slaughter may be feasible without further spread of infection. To prevent the spread of infection, however, final products must be processed to prevent spread to uninfected areas.

There are no effective treatments that destroy all myxospores in the fish host, and treatment is not considered a viable option as part of a containment and control response in Australia.

A successful containment and control response will rely heavily on the implementation of movement restrictions for exposed or potentially exposed fish to prevent spread of *M. cerebralis* to uninfected zones or compartments. The zoning and/or compartmentalisation program must also take into account water movement and possible spread of the parasite's two spore stages by this means. The feasibility of these restrictions will depend on farm management practices, the extent to which infection has already spread and the location of reservoirs of infection. Feasibility can only be assessed at the time of the outbreak, taking into account required movement restrictions on fish, people, vehicles and watercraft, and also market access for the fish products and by-products. Even then, assessment may be limited.

Infected fish

Clinically diseased and dead fish, along with infectious wastes, are considered to be the main source of myxospores in the environment. Unmanaged, they constitute an unacceptable risk for spreading the infection to uninfected zones due to the resistant nature of myxospores.

The only real option for clinically diseased fish, if effluent water cannot be contained or disinfected, is immediate destruction or transport to saltwater for grow-out. It is unlikely that myxospores would contact a susceptible *T. tubifex* host in this environment. Escaped, infected fish may be a risk if they return to freshwater.

2.6.3   Response Option 3: Control and mitigation

In a control and mitigation program, the aim may simply be to reduce the prevalence and severity of clinical disease to biologically and/or economically acceptable levels. Critically, there may be a level of disease in the population below which the cost of further expenditure on control would be greater than the likely benefits.

The principles of control and mitigation are to reduce the impact of disease. Therefore, the use of any options (or parts of options) listed in Section 2.2 could be considered. In general, the resources used to implement available control and mitigation measures, and the extent of their imposition, will often be less than if eradication or containment and control were pursued.

Considerable research into control and mitigation options in wild salmonid populations has occurred in recent years, and habitat restoration is one means that is sometimes successful (see Section 2.2.6). Restocking programs that concentrate on releasing older fish that are less susceptible to clinical disease can reduce the impacts of whirling disease. In recent years, restocking with resistant strains of rainbow trout has been practised in the United States with evidence that resistance spreads rapidly in the wild population (Fetherman et al. 2014).

2.7   Trade and industry considerations

In countries where whirling disease is endemic, affected industries include salmonid farming and recreational fishing. It is unlikely that other aquatic farming industries in Australia would be affected by this disease.

Trade regulations, market requirements and food safety standards must be considered as part of a control strategy. Permits may be required from the relevant authorities to allow products derived from disease control programs to be released and sold for human consumption.

2.7.1   Export markets

Whirling disease is endemic throughout many parts of the United States, Europe, North and South Africa, North East Asia and the east coast of the south island of New Zealand. It is not listed as a notifiable disease by the OIE. Despite this, there are some countries that require imports to be certified free from whirling disease. Some countries also have regional requirements that differ within the country; for example some states of the United States.

The Department of Agriculture and Water Resources is responsible for the health certification of all exports and should be contacted for further information (export@agriculture.gov.au).

2.7.2   Domestic markets

A cautious approach is required for the salvage of exposed or potentially exposed product for the domestic market. The myxospore is highly resistant and can survive for long periods. Decisions on the release of salmonids or salmonid products to the domestic market will depend on the control strategy implemented.

Eradication

If eradication is considered, decisions relating to the release of product for the domestic market must ensure there is no potential for the spread of *M. cerebralis*.

Containment and control via zoning and/or compartmentalisation

The release of exposed or potentially exposed salmonid product to the domestic market must be carefully controlled to ensure there is no potential spread of viable *M. cerebralis* to uninfected areas.

Control and mitigation

Requirements for the release of exposed or potentially exposed salmonid product to the domestic market would be less stringent if whirling disease became endemic in Australia.

3   Preferred Australian response options

3.1   Overall policy for whirling disease

Whirling disease has the potential to cause significant mortality and morbidity in farmed and wild salmonid populations in Australia. Rainbow trout are particularly susceptible to clinical disease, especially if infected when less than seven weeks old.

It takes three to eight weeks after infection for clinical signs to develop in susceptible fish; many infected fish may never show clinical signs. Fish can only be infected with spores released from the *T. tubifex* worm host.

There are three potential response options:

- Option 1: Eradication of the parasite
- Option 2: Containment and control via zoning and/or compartmentalisation (restriction of the parasite to areas with endemic infection, prevention of further spread and protection of uninfected areas)
- Option 3: Control and mitigation by implementing management practices that decrease the incidence and severity of the disease.

The disease response option chosen will depend on the nature of the outbreak and/or isolation of the parasite. The Director of Fisheries and/or the CVO of the state or territory in which the parasite is isolated will decide the control option(s).

It is important that the selected response is dynamic, to allow evolution of the strategy with the changing situation, e.g. choosing containment and control via zoning and/or compartmentalisation in the short term does not preclude adopting eradication as a long-term policy.

Strategies which may be used under these options include:

- quarantine and movement controls on fish, fish products, *T. tubifex* worms and fomites in declared areas to prevent spread of infection
- destruction and disposal of clinically diseased and dead fish to prevent further myxospore release into the environment
- decontamination of facilities to inactivate the resistant myxospore stage of the parasite on infected premises and to prevent spread to *T. tubifex* worms
- surveillance and tracing to determine the extent of possible infected worm and fish hosts, and to provide proof of freedom from the parasite
- zoning and/or compartmentalisation to define and maintain infected and parasite-free zones
- restocking with older, less susceptible fish, less susceptible species or resistant strains of fish unlikely to develop clinical disease
- alteration of the habitat to eliminate the *T. tubifex* worm host, e.g. changing earthen ponds to concrete raceways or natural habitat restoration
- prevention of predators (e.g. birds and fish) gaining access to infected fish
- education of the public, aquaculturists and government.

The Director of Fisheries and/or the CVO in the state or territory in which the outbreak occurs will be responsible for developing an emergency animal disease (EAD) response plan. This plan will be submitted to the Aquatic Consultative Committee on Emergency

Animal Diseases (AqCCEAD), who will provide advice on the technical soundness of the plan and its consistency with AQUAVETPLAN.

Directors of Fisheries and/or CVOs in the affected states or territories will implement the disease control measures as agreed in the EAD response plan and in accordance with relevant legislation. They will make ongoing decisions on follow-up disease-response measures in consultation with AqCCEAD. The detailed response measures adopted will be determined using the principles of control and eradication (see Section 2), epidemiological information about the outbreak, and the financial feasibility of the option.

For information on the responsibilities of the other state or territory disease control headquarters and local disease control centres, see the AQUAVETPLAN Control Centres Management Manual (DAFF 2001).

3.2   Response options

The circumstances surrounding an outbreak of whirling disease (or infection with *M. cerebralis*) will greatly influence selection of the most suitable response option. Figure 3 details the actions that should occur on initial suspicion of whirling disease. Figure 4 is a flow chart to assist selection of the appropriate response option.

Figure 3 Actions to be undertaken by key response agencies during the initial phase of a suspected outbreak of whirling disease
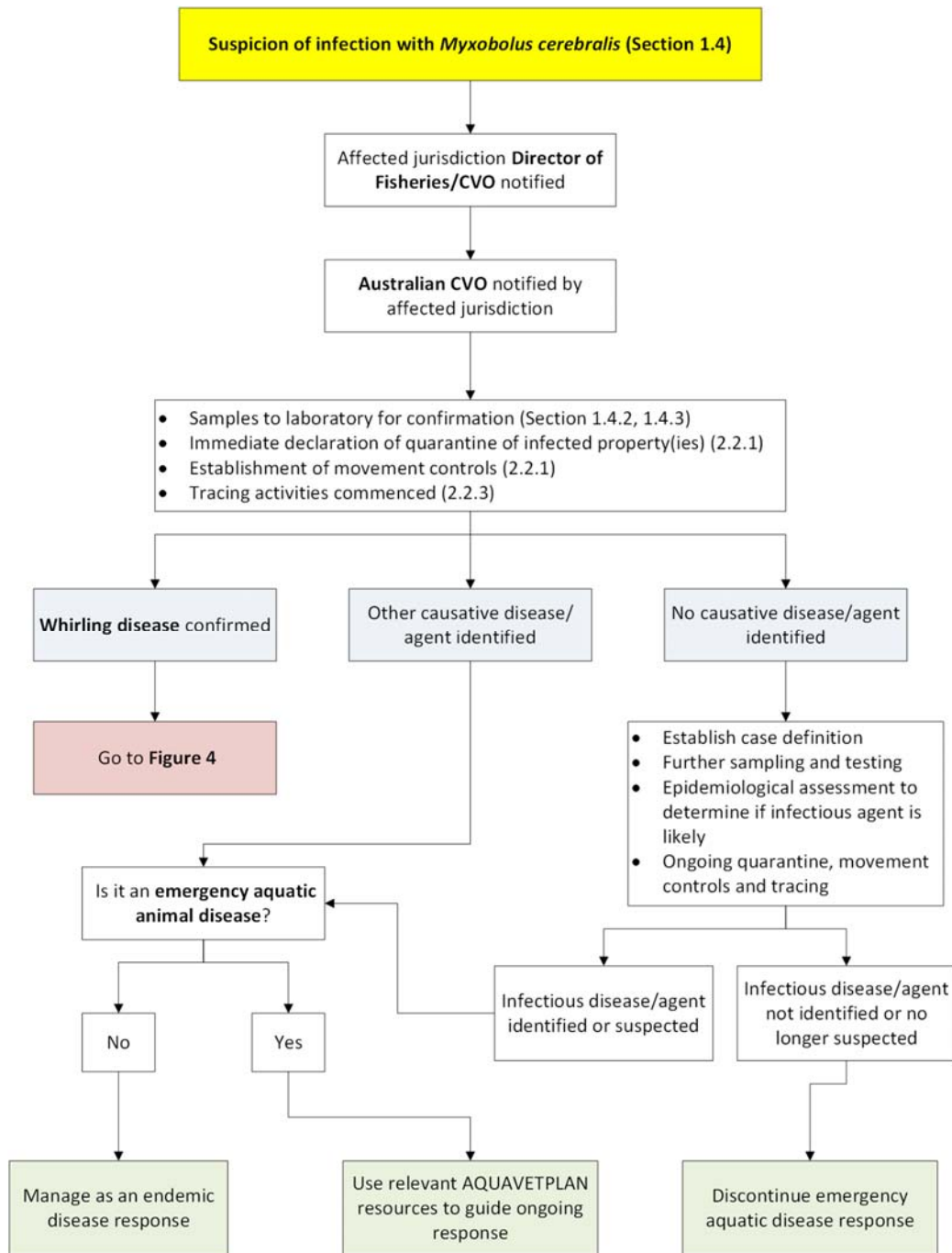
Figure 4 Selection of most appropriate response to a whirling disease outbreak
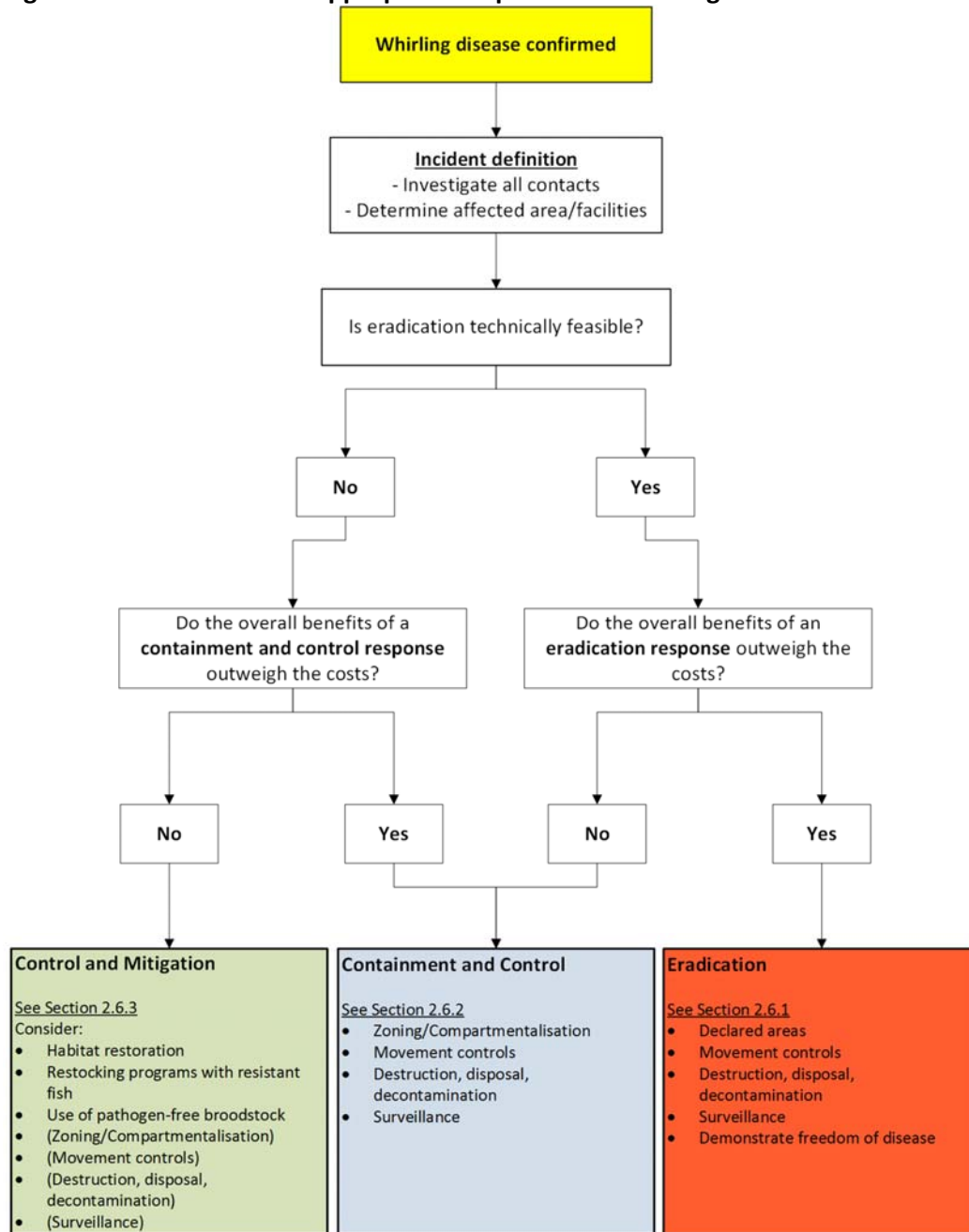
3.2.1   Option 1: Eradication

Eradication of whirling disease has the highest short-term economic costs, but if successful, the long-term economic benefits will likely outweigh these short-term costs.

Eradication is likely to be successful in a closed system or where the outbreak is an obvious point source epizootic with minimal spread. In open, semi-open and possibly semi-closed systems where myxospores may have widely contaminated the surrounding area, success of an eradication strategy would depend on whether the:

- infection had spread to wild salmonid populations

- resources for surveying and destocking wild salmonids in the immediate area were sufficient
- infection was widely dispersed across multiple aquaculture facilities.

An eradication plan must include the following activities:

- Quarantine and movement controls must be declared immediately and stringently enforced on salmonids, salmonid products, water, *T. tubifex* and any vectors or fomites located in declared areas. Restrictions must apply to movement out of the infected area of anything capable of transmitting *M. cerebralis* from infected to uninfected salmonids, and to aquaculture facilities or processing plants. Movement controls should be maintained until the agent is either eradicated or declared endemic. However, movement to processing plants with good biosecurity procedures and adequate effluent treatment could be permitted, providing that adequate biosecurity is observed on route to the plant. Given the resistant nature of spores, any effluent would need treatment sufficient to kill spores, safe disposal (e.g. seepage pits away from natural waters), or disposal in areas where *T. tubifex* cannot live (e.g. sea water). Proposals to move potentially infected fish for processing would need individual consideration.
- Surveillance and tracing should be rapidly implemented to allow detection of all infected premises and populations so that containment, implementation of responses and appropriate decision-making can occur.
- Destruction or depopulation of all exposed fish and worms should occur. In some instances, flexibility can be allowed in how this is achieved (e.g. fish can be grown out in salt water first).
- All exposed dead and culled fish and products should be disposed of appropriately to prevent escape of myxospores into fresh water.
- All contaminated areas, equipment and material should be decontaminated and effluent treated.
- Restocking of susceptible sentinel fish (e.g. young rainbow trout) can occur after destocking, thorough decontamination and removal of the source of TAMs. This will allow detection of residual infection (e.g. remnant infected worm populations).
- Australian native fish are likely to be resistant and can be restocked, as can less susceptible salmonid species (e.g. brown trout).

3.2.2   Option 2: Containment and control via zoning and/or compartmentalisation

If eradication is discounted, then containment and control is the preferred response option in order to protect and maintain uninfected areas.

Measures to be taken under this response option include:

- *Zoning and/or compartmentalisation program*: care must be exercised in the development of such a program for whirling disease. Factors such as laboratory test performance, case definitions and bias in sampling procedures must be considered before implementation to ensure such a program would achieve the desired objectives. Principles of zoning and compartmentalisation for infected and non-infected zones in Australia are outlined in the AQUAPLAN Zoning Policy Guidelines (DAFF 2000).
- *Management options to reduce the severity and incidence of* infection: managers of farms in the affected areas would need to consider options to reduce exposure to *M. cerebralis* (see Chapter 2).

- *Restocking with less susceptible fish*: farms in control areas may elect to restock with less susceptible fish species (e.g. Australian natives or hybrid salmonids) if this is deemed economically viable. Alternatively, preventing exposure of young salmonids (< seven weeks) to TAMs will significantly reduce the incidence of clinical disease, although such fish may still be infected.

3.2.3   Option 3: Control and mitigation of disease

If this control option is chosen, measures taken will be aimed at managing the disease in affected areas. Such measures are detailed in Section 2, and which measures to use are context-specific.

3.3   Criteria for proof of freedom

Due to the effectively global distribution of whirling disease, export of Australian salmonid fish products is not likely to be severely affected. The OIE does not list whirling disease and has no guidelines for proof of freedom, but individual countries may still have restrictions on the import of fish products.

Despite this, proof of freedom tools are available and can be used to design an appropriate surveillance program in Australia. Tools include suitable diagnostic tests and surveillance techniques. There are suitable diagnostic tests for identifying both *T. tubifex* (Eszterbauer et al. 2006; Hallett et al. 2005; Lodh et al. 2012; Lodh et al. 2011) and *M. cerebralis* (Kelley et al. 2006; Kelley et al. 2004b; MacConnell & Bartholomew 2012); and suitable surveillance techniques include passive and active surveillance.

Passive surveillance uses existing systems and processes for disease identification and notification. If a suitable passive surveillance system exists, the absence of disease reports from putatively disease-free areas (e.g. zones or eradicated areas) can be used as evidence that such areas are indeed disease-free.

Active surveillance relates to collection of new information (e.g. samples of fish or worms) to detect disease if it is present. There are practical active sampling strategies to demonstrate freedom from disease (Cameron 1999; 2002; 2004; Cameron & Baldock 1998a; 1998b). For example, a surveillance program can be undertaken using a sample size that is sufficient to detect infection if it was present at a specified design prevalence value (often set at 2% for the purposes of demonstrating disease freedom; see OIE 2015a). The absence of infection from the surveillance program can then be used to show that whirling disease is effectively absent.

Approaches have also been developed that allow a holistic analysis of disparate surveillance sources (e.g. active and passive sources) to determine the probability that disease freedom has been achieved in a farm, region or country (Cameron 2012; Martin et al. 2007).

These techniques are widely accepted internationally and are often considered required information during international trade negotiations. They could be applied to whirling disease to show freedom from disease.

3.4   Funding and compensation

There are currently no national cost-sharing agreements in place for emergency responses to aquatic animal diseases, including whirling disease. It is the responsibility of the users of this publication to seek advice in relation to any relevant funding or compensation arrangements within the relevant jurisdictions.

3.5   Export markets

Some countries may have import conditions in place related to whirling disease, such as requiring imports to be certified free of whirling disease. The Department of Agriculture and Water Resources is responsible for the health certification of all exports and should be contacted for further information (export@agriculture.gov.au).

4   Appendix 1 OIE Aquatic Animal Health Code and Manual of Diagnostic Tests for Aquatic Animals

Note: current versions of the Aquatic Code and Aquatic Manual are available on the OIE website (http://www.oie.int/en/international-standard-setting/overview/). These standards are updated annually.

OIE Aquatic Code

The objective of the OIE *Aquatic Animal Health Code* (OIE 2015a) is to prevent the spread of aquatic animal diseases, while facilitating international trade in aquatic animals and aquatic animal products. This annually updated volume is for use by veterinary departments, import and export services, epidemiologists and people involved in the international trade of aquatic animals and their products.

The current edition of the OIE Aquatic Code (18th edition) was published in 2015 and is available on the OIE website (OIE 2015a).

OIE Aquatic Manual

The purpose of the OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE 2015b) is to contribute to the international harmonisation of methods for the surveillance and control of the most important aquatic animal diseases. Standards are described for laboratory diagnostic tests and the production and control of biological products (principally vaccines) for veterinary use globally.

The current edition of the OIE Aquatic Manual (7th Edition) was published in 2015 and is available on the OIE website (OIE 2015b).

Further information

Further information about the OIE Aquatic Code and Aquatic Manual is available on the OIE website at:

http://www.oie.int/international-standard-setting/overview/
(Accessed 22/07/2016)

5   Appendix 2   Approval of chemicals for use in Australia

The Australian Pesticides and Veterinary Medicines Authority (APVMA) evaluates, registers and regulates agricultural and veterinary chemicals. Before a veterinary chemical (e.g. an antibiotic or vaccine) can enter the Australian market, it must go through the APVMA's rigorous assessment process to ensure that it meets high standards of safety and effectiveness. In addition, an import permit is required from the Department of Agriculture and Water Resources if a product containing biological material is to be sourced from overseas.

Detailed data about the product and its proposed use pattern must be submitted to the APVMA with the application for registration or permits. Because the assessment process is so detailed, the evaluation may take some time to complete.

Registration

Registration is the default method for the APVMA to allow the use of a veterinary chemical in Australia. Registration is time consuming and expensive and it may be necessary to apply for a minor or emergency use permit during an emergency.

Minor use permit

The minor use permit (MUP) system is a temporary approval system for the use of drugs and chemicals. The system was devised by the APVMA for Australia, and allows the restricted use of a limited amount of a drug or chemical in a specified species when inadequate data are available to satisfy APVMA requirements for registration. Conditions are applied to the permit, which often include the collection of data related to the use of the product. The MUP system aims to enable restricted use of a drug or chemical until sufficient data are available to enable full registration.

For example, the APVMA may set a temporary withholding period with a wide margin of safety for a MUP. This withholding period may have been extrapolated from data relating to the use of the product in other species. In such cases, a condition of the MUP will be the collection of residue data for testing. Results from the data are assessed by the APVMA (usually after 12 months, which is the duration of most permits) and used to more accurately set a withholding period for the product.

Emergency use permit

The APVMA has a permit system for the emergency use of a product that is either unregistered in Australia or registered for use in a different species or for a different use. The APVMA will verify with the appropriate state and territory coordinators that the emergency is genuine.

For further details or permit application forms, visit the APVMA website (APVMA 2015).

Appendix 3 Extraction and PCR method for whirling disease (*Myxobolus cerebralis*) — New Zealand Ministry for Primary Industries

Supplied courtesy of the MPI, Wallaceville Laboratories, New Zealand, with some minor modifications (including the removal of references to NZ-specific forms). The test, current as at July 2015, is accredited to ISO 17025 by International Accreditation New Zealand. Assistance with sourcing positive control material can be obtained from:

National Centre for Biosecurity and Infectious Disease
Ministry for Primary Industries, Manatū Ahu Matua, Investigation and Diagnostic Centre
66 Ward St, PO Box 40742, Upper Hutt 5018, New Zealand

Whirling disease (*Myxobolus cerebralis*) — extraction and PCR method   TM-111

Safety statement

Care should be taken when handling samples that may contain zoonotic agents; all biological samples should be treated as potentially harmful. Use the assigned protective clothing and equipment at all times. Check that the work area is safe by locating fire extinguishers, first aid kit and any safety equipment that may be required. Check MSD sheets for any chemicals or reagents that may be used and have a clear understanding of the dangers associated with them.

Whirling disease (*Myxobolus cerebralis)* — extraction and PCR method

1.   Purpose

To extract DNA from the cartilage of juvenile salmon heads for use with a PCR assay to test for *Myxobolus cerebralis*.

2.   Scope

Examination of farmed or wild salmonid populations for spores of the chondrophilic protozoan parasite *M. cerebralis*.

3.   Limitations of Method

This method is used for fish longer than 120 mm (i.e. heads longer than 26 mm, nose to caudal margin of operculum).

The rate of *M. cerebralis* spore development is dependent on temperature. Salmonids can be tested by this method if the period between exposure to *M. cerebralis* and sampling is greater than four months.

M. cerebralis infested cartilage is not routinely available for control purposes. *Escherichia coli* transformants with *M. cerebralis* DNA inserts are stored on beads in –70°C freezer:

DNA is extracted from this and used as a positive control with each PCR.

4.   Sample Requirements

Sixty fish are sampled from each 'lot'* at the location, giving a statistical confidence level of 95% that a 100% sensitive test will detect at least one individual in a population with a 5% or greater prevalence of *M. cerebralis*. Any fish showing clinical signs of whirling disease are sampled first and the sample number made up with randomly selected individuals.

Whole fish or heads are kept chilled during transport to the laboratory.

*A 'lot' is a group of fish of the same species and age which have shared the same holding facilities and water supply.

5.   Quality Control Material

Positive control DNA:

*   Cloned *Myxobolus cerebralis* used at a concentration of 0.1 pg/μL
*   **or**
*   DNA from infected tissue of *Myxobolus cerebralis* used at a concentration of 1 ng/μL

This DNA is run with each assay.

6.   Quality Control Procedures

As per laboratory requirements.

7.   Equipment

*   Water bath: 45°C (± 5°C), refer SOP EQ-46
*   Scissors
*   Sterile forceps
*   Large beaker
*   Sterile blades size 22
*   Plastic bottles
*   Sterile petri dishes
*   Ultra-turrax homogeniser: type T25, IKA-Labortechnik
*   Heating blocks - 56°C and 80°C, 1.5 mL microfuge tubes
*   Thermocyclers
*   Pipettors,
*   Bench centrifuge,
*   Freezer –20°C

8.   Reagents and Solutions

*   96% Ethanol
*   Distilled water
*   Instagene matrix
*   10% buffered formalin
*   Instagene lysis buffer
*   Zymo Genomic DNA Clean and Concentrator™ kit

- PCR reagents

9.   Procedure

9.1   Processing of salmon heads

- Fish heads should arrived at the laboratory in pools of 5 — if not, pool into lots of 5 and label.
- Sagittally halve the heads, and place half in 10% neutral buffered formalin. Keep these formalin fixed tissues for 3 months for confirmation if needed.
- Soften remaining heads, as pools in separate bags, by warming the heads in a large beaker of warm tap water, which is immersed in a water-bath at 45°C. The heads are ready to be processed when the eyes have turned opaque (5–10 minutes for small heads less than 40 mm long, and 20–30 minutes for large heads greater than 40 mm). Occasionally stir the heads to maintain an even temperature. Remove the heads from the beaker.
- Using forceps remove and discard the eyes, brain, lower jaw, skin and other soft tissue, retaining all the cartilage/bone from each pool in separate petri dishes.
- Fish showing clinical signs are to be pooled together.
- Process each batch separately as follows:

    – cut remaining cranial elements with scissors or scalpel blade and place into a small plastic container. Further disperse this chopped material for 30 seconds in up to 30 mL of distilled water (5–10 mL water is preferable) in an Ultra-turrax homogeniser. Rinse all solid material off the probe into a 1% solution of sodium hypochlorite rinse, then sterilise the probe by flaming with 96% ethanol between each pool.

- Use homogenate for the DNA extraction, Store the remaining homogenate at –20°C for 3 months.
- Brush clean and sterilize all equipment in a 1% solution of sodium hypochlorite solution for 4 hours. Do not leave metallic equipment in sodium hypochlorite for longer than this.
- Flesh scrapings from the fish heads and disposable pipettes should be placed in the biohazard bins for disposal.
- Clean the water bath by raising the water temperature to 90°C for a minimum of one hour. After cooling the contents can be disposed of down the sink and the bath washed with detergent.

9.2   DNA extraction — Modified Instagene matrix method

- Add ~750 µL homogenate to a microfuge tube using a transfer pipette.
- Pellet this homogenate by centrifuging at 10,000 x g for 1 minute. Remove supernatant.
- Weigh pellet and repeat if necessary to obtain between 150 mg and 250 mg of material.
- Add 200 µL lysis buffer to pellet. Vortex and incubate at 56°C for at least 30 minutes.
- Centrifuge at 10,000 x g for 1 minute. Remove supernatant.
- Add 200 µL Instagene matrix to the pellet and incubate at 80°C for at least 30 minutes. Vortex.
- Incubate at 100°C for 8 minutes.
- Centrifuge at 10,000 x g for 3 minutes. Remove supernatant and place into a microfuge tube for clean up

- Clean up extracted DNA using the Zymo Genomic DNA Clean and Concentrator™ kit — follow manufacturers protocol with the inclusion of the following steps:

  – extend the final wash spin by 2 minutes to ensure complete removal of the wash buffer
  – elute in 15 μL using DNA elution buffer warmed at 60–70°C.

9.3   Conventional PCR

- Interpretation — quantify the extracted cleaned DNA and verify the DNA is amplifiable by running through an internal control PCR e.g. 18S rRNA.
- Run samples through the whirling disease conventional PCR.
- Note the DNA concentration on the tubes, and store in the storage boxes in the sample prep laboratory –20°C freezer.
- Note storage location in the fish path DNA storage workbook located in the sample prep lab.
- All samples will be stored for three months before being discarded.
- Record all information on the appropriate molecular worksheets

10.   Interpretation and Recording of Results

Expected product size is 507 bp.

Positive control:

- No product = run not valid, repeat
- 507 bp product = valid run

Samples:

- No product or non-specific banding = negative
- 507 bp product = not negative

Not negative samples:

- PCR to be repeated
- if a not negative result is produced, the product should be sequenced to confirm the identification of *M. cerebralis*. The PCR primers (Tr5-16/Tr3-17) are used to sequence part of the 18*s* rRNA gene.
- Sagitally sectioned heads fixed in formalin should be processed histologically, stained with haematoxylin and eosin, and examined microscopically for evidence of *Myxosporean* organisms in the cartilage.

11.   Calculations

Not applicable.

12.   Reporting of Results

A typed report is entered in LIMS or appropriate lab recording system which details the particulars of the fish sampled, the test method and result. This report is sent to the person/s who requested the test and copies are held both electronically and with the case papers.

13.   Reference Documents

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IANZ accredited test

Whirling Disease (*Myxobolus cerebralis***) Conventional PCR**

Nucleic acid extraction–DNA

Primers	Name	Sequence (5'-3')	Size	Position	Target*
Forward	Tr 5-16	GCA TTG GTT TAC GCT GAT GTA GCG A	25	211	18s rRNA EF370480
Reverse	Tr 3-17	GGC ACA CTA CTC CAA CAC TGA ATT TG	26	693	18s rRNA EF370480

* Please include a Genbank accession number for the target wherever possible.
PCR kit: Kapa 2G Fast Hotstart Readymix
Reaction volume: 25 µL

Reagent mix	Volume (µL)
Kapa 2G ready mix	12.5
Nuclease free water	10.7
F primer (10 µM)	0.4
R primer (10 µM)	0.4
DNA template (1–100 ng)	1*

* DNA volume may vary depending on concentration of DNA.

PCR controls	Description
Positive	DNA extracted from *Myxobolus cerebralis* clone used at 0.1 pg/uL or DNA extracted from infected tissue used at 1 ng/uL
Reagent	Nuclease-free water

Cycling parameters	Temp (°C)	Time (sec)	No. cycles
Hold	95	120	1
Denature	95	15	35

Anneal	67	15	35
Extension	72	1	35

Electrophoresis	Description	Size of amplicon (bp)
Agarose gel	1.5%	507 bp
MW marker	100 bp	

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