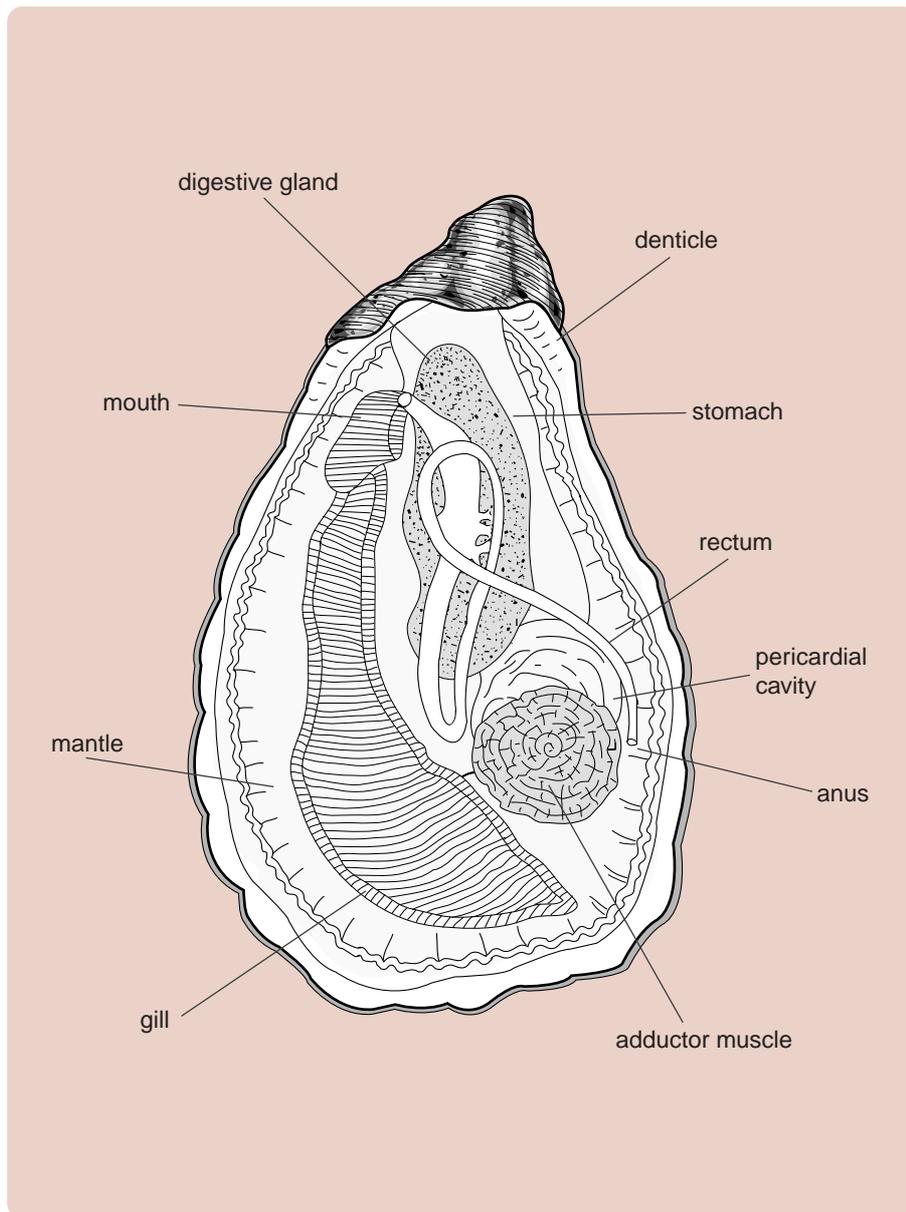


Basic Anatomy of an Oyster



Basic anatomy of an oyster.

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SECTION 3 - MOLLUSCAN DISEASES

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M.1 GENERAL TECHNIQUES

(SE McGladdery)



Fig.M.1.1.1. Gaping hard shell clam, *Mercenaria mercenaria*, despite air exposure and mechanical tapping.

(MG Bondad- Reantaso)



Fig.M.1.1.2c,d. *Pteria penguin* shell with dense multi-taxa fouling, Guian Pearl Farm, Eastern Philippines (1996).

(MG Bondad-Reantaso)



Fig.M.1.1.2a. Mollusc encrustment (arrows) of winged oyster, *Pteria penguin*, Guian Pearl Farm, Eastern Samar, Philippines (1996).

(SE McGladdery)



Fig.M.1.1.2e. *Polydora* sp. tunnels and shell damage at hinge of American oyster, *Crassostrea virginica*, plus barnacle encrusting of other shell surfaces.

(D Ladra)



Fig.M.1.1.2b. *Pteria penguin* cultured at Guian Pearl Farm, Eastern Samar, Philippines with extensive shell damage due to clionid (boring) sponge (1992).

(MG Bondad-Reantaso)



Fig.M.1.1.2f. Winged oyster, *Pteria penguin*, shell with clionid sponge damage. Guian Pearl Farm, Eastern, Philippines (1996).

M.1 General Techniques

General molluscan health advice and other valuable information are available from the OIE Reference Laboratories, Regional Resource Experts in the Asia-Pacific, FAO and NACA. A list is provided in Annexes M.A1 and M.AII, and up-to-date contact information may be obtained from the NACA Secretariat in Bangkok (e-mail: naca@enaca.org). Other useful guides to diagnostic procedures which provide valuable references for molluscan diseases are listed in Annex M.AIII.

M.1.1 Gross Observations

M.1.1.1 Behaviour (Level I)

It is difficult to observe behavioural changes in molluscs in open-water, however, close attention can be made of behaviour of both broodstock and larvae in hatcheries. Since disease situations can erupt very quickly under hatchery conditions, regular and close monitoring is worth Level I efforts (see Iridovirus - M.8).

Feeding behaviour of larval molluscs is also a good indicator of general health. Food accumulation in larval tanks should be noted and samples of larvae examined, live, under a dissecting microscope for saprobiotic fungi and protists (e.g. ciliates) and/or bacterial swarms. Pre-settlement stages may settle to the bottom prematurely or show passive circulation with the water flow currents in the holding tanks.

Juvenile and adult molluscs may also cease feeding, and this should be cause for concern under normal holding conditions. If feeding does not resume and molluscs show signs of weakening (days to weeks depending on water temperature) samples should be collected for laboratory examination. Signs of weakening include gaping (*i.e.* bivalve shells do not close when the mollusc is touched or removed from the water) (Fig. M.1.1.1), accumulation of sand and debris in the mantle and on the gills, mantle retraction away from the edge of the shell, and decreased movement in mobile species (*e.g.* scallop swimming, clam burrowing, abalone grazing, *etc.*).

Open-water mortalities that assume levels of concern to the grower should be monitored to determine if there are any patterns to the losses. Sporadic mortalities following periods of intense handling should be monitored with minimal additional handling if at all possible. If the mortalities persist, or increase, samples should be collected for laboratory analysis. Mortalities that appear to have a uniform distribution should be examined

immediately and environmental factors pre- and post-mortality recorded. Mortalities that appear to spread from one area to another suggest the presence of an infectious disease agent and should be sampled immediately. Affected animals should be kept as far away as possible from unaffected animals until the cause of the mortalities can be determined.

M.1.1.2 Shell Surface Observations (Level I)

Fouling organisms (barnacles, limpets, sponges, polychaete worms, bivalve larvae, tunicates, bryozoans, *etc.*) are common colonists of mollusc shell surfaces and do not normally present a threat to the health of the mollusc (Fig. M.1.1.2a,b). Suspension and shallow water culture, however, can increase exposure to fouling and shells may become covered by other animals and plants (Fig. M.1.1.2c,d). This can affect health directly by impeding shell opening and closing (smothering) or indirectly through competition for food resources. Both circumstances can weaken the mollusc so cleaning may be required. Such defouling should be undertaken as rapidly as possible, to minimise the period of removal from the water, during cooler periods of the day. Rapid cleaning is usually achieved using high pressure water or mechanical scrapers. Defouled molluscs should be returned to clean water. Fouling organisms should not be discarded in the same area as the molluscs, since this will accelerate recolonisation. Signs of weakening that persist or increase after cleaning, should be investigated further by laboratory examination.

Shell damage by boring organisms, such as sponges and polychaete worms (Fig. M.1.1.2e, f) is normal in open-water growing conditions. Although usually benign, under certain conditions (especially in older molluscs) shells may be rendered brittle or even become perforated. Such damage can weaken the mollusc and render it susceptible to pathogen infections.

Shell deformities (shape, holes in the surface), fragility, breakage or repair should be noted, but are not usually indicative of a disease condition (Fig.M.1.1.2g, h). Abnormal colouration and smell, however, may indicate a possible soft-tissue infection which may require laboratory examination.

M.1.1.3 Inner Shell Observations (Level I)

The presence of fouling organisms (barnacles, sponges, polychaete worms, *etc.*) on the inner shell surface is a clear indication of a weak/



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(MG Bondad-Reantaso)



Fig.M.1.1.2g,h. *Pinctada maxima*, shell with clionid sponge damage due to excavation of tunnels exhalant-inhalant openings (holes) to the surface (arrows). Other holes (small arrows) are also present that may have been caused by polychaetes, gastropod molluscs or other fouling organisms. Guian Pearl Farm, Eastern Philippines (1996).

(MG Bondad-Reantaso)



Fig.M.1.1.3a. Winged oyster, *Pteria penguin*, shell showing clionid sponge damage through to the inner shell surfaces, Guian Pearl Farm, Eastern Philippines (1996).

(B Jones)



Fig.M.1.1.3a1. Abalone (*Haliotis roei*) from a batch killed by polydoriid worms.

(D Ladra)



Fig.M.1.1.3b,c. b. Shells of *Pinctada maxima* showing a erosion of the nacreous inner surfaces (arrows), probably related to chronic mantle retraction; c. Inner surface of shell showing complete penetration by boring sponges (thin arrows).

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(MG Bondad-Reantaso)

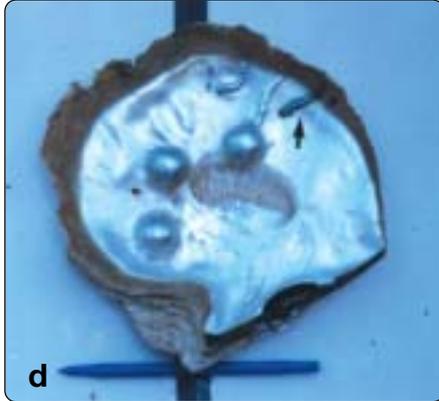


Fig.M.1.1.3d,e,f. *Pinctada maxima* (d), *Pteria penguin* (e) and edible oyster (*Crassostrea* sp.) (f) shells showing *Polydora*-related tunnel damage that has led to the formation of mud-filled blisters.

(MG Bondad-Reantaso)



Fig.M.1.1.3g. Inner shell of winged pearl oyster showing: tunnels at edge of the shell (straight thick arrow); light sponge tunnel excavation (transparent arrow); and blisters (small thick arrow) at the adductor muscle attachment site. Guian Pearl Farm, Eastern Philippines (1996).

(SE McGladdery)



Fig.M.1.1.3.h. Extensive shell penetration by polychaetes and sponges causing weakening and retraction of soft-tissues away from the shell margin of an American oyster *Crassostrea virginica*.

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sick mollusc (Fig.M.1.1.3a and Fig.M.1.1.3a1). The inner surfaces are usually kept clean through mantle and gill action. Perforation of the inner surface can be sealed off by deposition of additional conchiolin and nacre (Fig.M.1.1.3b,c). This may result in the formation of a mud- or water-filled “blister” (Fig.M.1.1.3d,e,f). Shell coverage can also occur over irritants attached to, or lying up against the inner shell, a process that may result in a “blister pearl” (Fig.M.1.1.3g).

Where perforation or other irritants exceed repair, the health of the mollusc is jeopardised and it becomes susceptible to opportunistic infections (Fig.M.1.1.3.h). The degree of shell perforation can be determined by holding the shell up to a strong light.

Where abnormalities occurring within the matrix of the shell warrant further investigation, freshly collected specimens can be sent intact to the laboratory or fixed for subsequent decalcification, as required.

M.1.1.4 Soft-Tissue Surfaces (Level I)

The appearance of the soft-tissues is frequently indicative of the physiological condition of the animal. Gross features which should be recorded include:

- condition of the animal as listed below:
 - fat** - the soft-tissues fill the shell, are turgid and opaque
 - medium** - the tissues are more flaccid, opaque and may not fill the shell cavity
 - watery** - the soft-tissues are watery/translucent and may not fill the shell cavity (Fig.M.1.1.4a, Fig.M.1.1.4b)
- colour of the digestive gland – e.g., pale, mottled, dark olive
- any abnormal enlargement of the heart or pericardial cavity – e.g., cardiac vibriosis, tumours
- presence of focal lesions such as:
 - abnormal coloration (e.g., patches of green, pink, red, black, etc.)
 - abscesses (Fig. M.1.1.4c)
 - tumour-like lesions (Fig. M.1.1.4d)
 - tissue (e.g. gill) erosion
- presence of water blisters in the viscera, palp, or mantle (Fig.M.1.1.4e)
- presence of pearls or other calcareous deposits (Fig.M.1.1.4f) within the soft tissues
- presence of parasites or commensals such as:

pea crabs in mantle cavity
parasitic copepods attached to gills
polychaetes, nematodes and turbellarians in mantle cavity or on surrounding surfaces (Fig. M.1.1.4g)
redworm (*Mytilicola* spp.) usually exposed only on dissection of the digestive tract
ciliates (sessile or free-swimming) and other protists (for larvae only)
bacteria (for larvae only)

- any mechanical (e.g., knife) damage to the soft-tissues during the opening of the shell.

Abscess lesions, pustules, tissue discolouration, pearls, oedema (water blisters), overall transparency or wateriness, gill deformities, etc., can be present in healthy molluscs, but, if associated with weak or dying animals, should be cause for concern. Record the levels of tissue damage and collect samples of both affected and unaffected animals for laboratory examination. Moribund animals, or those with foul-smelling tissues may be of little use for subsequent examination (especially from warm water conditions), however, numbers affected should be recorded.

Worms or other organisms (e.g. pea crabs, copepods, turbellarians) on the soft-tissues are also common and not generally associated with disease. If present in high numbers on weak molluscs, however, numbers should be noted and samples of intact specimens collected for laboratory examination and identification. Fixation in 10% buffered formalin is usually adequate for preserving the features necessary for subsequent identification.

M.1.2 Environmental Parameters (Level I)

Environmental conditions have a significant effect on molluscan health, both directly (within the ranges of physiological tolerances) and indirectly (enhancing susceptibility to infections). This is especially important for species grown under conditions which differ significantly from the wild (e.g. oysters grown in suspension). Important environmental factors for molluscan health include water temperature, salinity, turbidity, fouling and plankton blooms. Extremes and/or rapid fluctuations in these can seriously compromise molluscan health. Anthropogenic factors include a wide range of biologic and chemical pollutants. Since molluscs are, essentially, sessile species (especially under culture conditions) this renders them particularly susceptible to pollution. In addition, molluscs have

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(SE McGladdery)



Fig.M.1.1.4a. Normal oyster (*Crassostrea virginica*) tissues.

(SE McGladdery)



Fig.M.1.1.4e. Water blister (oedema/edema) in the soft-tissues of the mantle margin of an American oyster (*Crassostrea virginica*).

(SE McGladdery)



Fig.M.1.1.4b. Watery oyster (*Crassostrea virginica*) tissues – compare with M.1.1.4a.

(SE McGladdery)

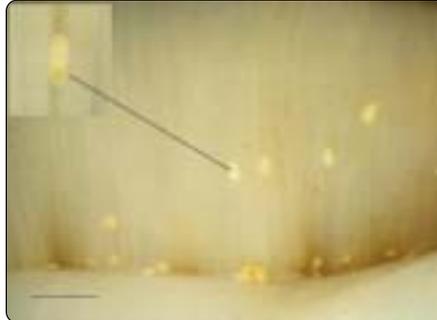


Fig.M.1.1.4f. Calcareous deposits (“pearls”) in the mantle tissues of mussels in response to irritants such as mud or digenean flatworm cysts.

(SE McGladdery)



Fig.M.1.1.4c. Abscess lesions (creamy-yellow spots, see arrows) in the mantle tissue of a Pacific oyster (*Crassostrea gigas*).

(SE McGladdery and M Stephenson)



Fig.M.1.1.4g. Polydoriid tunnels underlying the nacre at the inner edge of an American oyster (*Crassostrea virginica*) shell, plus another free-living polychaete, *Nereis diversicolor* on the inner shell surface.

(MS Park and DL Choi)



Fig.M.1.1.4d. Gross surface lesions in Pacific oyster (*Crassostrea gigas*) due to *Marteiliodes chungmuensis*.

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low tolerance of some other water-uses/abuses (e.g., dynamite and cyanide fishing; dragging; creosote and other anti-fouling chemical compounds; agricultural run-off).

Maintaining records of temperature, salinity (in estuarine or coastal areas), turbidity and man-made disturbances provide valuable background data, essential for accurate interpretation of mortality observations and results from laboratory analyses.

M.1.3 General Procedures

M.1.3.1 Pre-Collection Preparation

Wherever possible, check the number of specimens required for laboratory examination with laboratory personnel *before* collecting the sample. Ensure that each specimen is intact, i.e., no empty or mud-filled shells. Larger sample numbers are generally needed for screening purposes compared with numbers required for disease diagnosis.

M.1.3.2 Background Information (Level I)

All samples being submitted for laboratory examination should include as much background information as possible, such as:

- reason(s) for submitting the sample (mortalities, abnormal growth/spawning, health screening, etc.);
- gross observations and environmental parameters (as described under M.1.1 and M.1.2);
- where samples are submitted due to mortalities, approximate prevalences and patterns of mortality (acute or chronic/sporadic cumulative losses), and
- whether or not the molluscs are from local populations or from another site. If the stock is not local, the source and date of transfer should also be noted.

The above information will help identify if handling stress, change of environment or infectious agents may be a factor in mortalities. It will also help speed up accurate diagnosis of a disease problem or disease-risk analysis.

M.1.3.3 Sample Collection for Health Surveillance

The most important factors associated with collection of specimens for surveillance are:

- sample numbers that are high enough (see Table M.1.3.3 below)

- susceptible species are sampled
- samples include age- or size-groups that are most likely to manifest detectable infections. Such information is given under specific disease sections.

The standard sample sizes for screening healthy aquatic animals, including molluscs, is given in Table M.1.3.3 below.

M.1.3.4 Sample Collection for Disease Diagnosis

All samples submitted for disease diagnosis should include as much supporting information as possible including:

- reason(s) for submitting the sample (mortalities, abnormal growth, etc.)
- handling activities (de-fouling, size sorting/grading, site changes, new species/stock introduction, etc.)
- history and origin(s) of the affected population(s);
- environmental changes

M.1.3.5 Live Specimen Collection for Shipping (Level I)

Once the required number of specimens has been determined, and the laboratory has provided a date or time for receipt of the sample, the molluscs should be collected from the water. This should take place as close to shipping as possible to reduce air-storage changes in tissues and possible mortalities during transportation. This is especially important for moribund or diseased mollusc samples.

The laboratory should be informed of the estimated time of arrival to ensure they have the materials required to process the sample prepared before the sample arrives. This helps reduce the time between removal from the water and preservation of the specimens for examination.

The molluscs should be wrapped in paper soaked with ambient seawater. For small seed (<10 mm), these can be packed in paper or styrofoam cups along with damp paper towel to prevent movement during transportation. Larger molluscs should be shipped in insulated and sealable (leakproof) coolers (styrofoam or plastic). Where more than one sample is included in the same cooler, each should be placed in a separate and *clearly* labeled plastic bag (tied or ziploc). Use of plastic bags is required to prevent exposure of marine species

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Population Size	Prevalence (%)						
	0.5	1.0	2.0	3.0	4.0	5.0	10.0
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	71	57	27
10000	579	292	146	96	72	29	27
100000	594	296	147	97	72	57	27
1000000	596	297	147	97	72	57	27
>1000000	600	300	150	100	75	60	30

Table M.1.3.3¹, Sample sizes needed to detect at least one infected host in a population of a given size, at a given prevalence of infection. Assumptions of 2% and 5% prevalences are most commonly used for surveillance of presumed exotic pathogens, with a 95% confidence limit.

to freshwater ice (gel-paks or plastic bottles containing frozen water are recommended over loose ice to keep specimens cool) and to reduce loss of mantle fluids.

Label containers clearly:

“Live Specimens, Store at _____°C to _____°C
DO NOT FREEZE”

If being shipped by air also indicate:

“HOLD AT AIRPORT AND CALL FOR PICK-UP”

Clearly indicate the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the laboratory.

Ship **early in the week** to avoid arrival during the weekend with possible loss of samples due to improper storage. Inform the contact person as soon as the shipment has been sent and, where appropriate, give them the name of the carrier and waybill number.

M.1.3.6 Preservation (Fixation) of Tissue Samples (Level I - with basic training)

For samples that cannot be delivered live to a diagnostic laboratory, due to distance or slow transportation, specimens should be fixed (preserved) on site. This is suitable for subsequent histology examination, but means that routine bacteriology, mycology or media culture (e.g., Fluid Thioglycollate Medium culture of *Perkinsus* spp.) cannot be performed. Diagnostic needs should, therefore, be discussed with laboratory personnel prior to collecting the sample.

The following fixatives can be used for preservation of samples:

i) 1G4F solution (1% Glutaraldehyde : 4% Formaldehyde)

*Stock 1G4F solution - may be held at 4°C for up to 3 months:

120 ml	37-40% buffered formalin solution**
20 ml	50% glutaraldehyde
360 ml	tap water

**Buffered formalin solution:

1 litre	37-40% formaldehyde
15 gm	disodium phosphate (Na ₂ HPO ₄)

¹ Ossiander, F.J. and G. Wedermeyer. 1973. Journal of Fisheries Research Board of Canada 30:1383-1384.

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0.06 gm sodium hydroxide (NaOH)
0.03 gm phenol red (pH indicator)

Working solution – should be prepared immediately prior to use:

500 ml filtered ambient seawater or Instant Ocean
500 ml Stock 1G4F solution*

The required tissue thickness is about 2-3 mm. Tissues can tolerate long storage in this fixative at room temperature. (N.B. Thicker tissues, or whole animals, may be fixed using the 10% buffered formalin solution as described below).

ii) 10% Buffered formalin in filtered ambient seawater (This is the easiest solution to prepare and store).

10 ml 37-40% buffered formalin solution**
90 ml filtered ambient seawater

N.B. Whole specimens less than 10 mm thick can be fixed with this solution. If the specimens are larger, cut them into two or more pieces before fixing (ensure that pieces from different specimens do not get mixed up).

iii) Davidson's Fixative

Tissue up to 10 mm in thickness can be fixed in Davidson's fixative. Prior to embedding, tissues need to be transferred to either 50% ethanol for 2 hours (minimum) and then to 70% ethanol, or directly to 70% isopropanol. Best results are obtained if fixative is made up in the following order of ingredients.

Stock Solution:

400 ml glycerin
800 ml formalin(37-40% formaldehyde)
1200 ml 95% ethanol (or 99% iso propanol)
1200 ml filtered natural or artificial seawater

Working Solution: dilute 9 parts stock solution with 1 part glacial acetic acid

Important Notes:

- All fixatives should be kept away from open water and used with caution against contact with skin and eyes.
- If the molluscs cannot be fixed intact, contact the diagnostic laboratory to get guidance for cracking shell hinges or removing the required tissues.

M.1.3.7 Shipping Preserved Samples (Level I)

Many transport companies (especially air carriers) have strict regulations regarding shipping any chemicals, including fixed samples for diagnostic examination. Check with the carrier *before* collecting the sample to prevent loss of time and/or specimens due to inappropriate packaging, labeling, etc.. If the tissues have been adequately fixed (as described in M.1.3.4), most fixative or storage solution can be drained from the sample for shipping purposes. As long as sufficient solution is left to keep the tissues from drying out, this will minimise the quantity of chemical solution being shipped. Pack fixed samples in a durable, leak-proof container.

Label containers clearly with the information as described for live specimens (M.1.3.3.). Clearly indicate the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the laboratory. Ship **early in the week** to avoid arrival during the weekend with possible loss of samples due to improper storage. Inform the contact person as soon as the shipment has been sent and, where appropriate, give them the name of the carrier and waybill number.

If being shipped by air also indicate:

“HOLD AT AIRPORT AND CALL FOR PICK-UP”

M.1.4 Record-Keeping (Level I)

Record keeping is essential for effective disease management. For molluscs, many of the factors that should be recorded are outlined in sections M.1.4.1, M.1.4.2, and M.1.4.3.

M.1.4.1 Gross Observations (Level I)

Gross observations can be included with routine monitoring of mollusc growth, either by sub-sampling from suspension cages, lines or stakes, or by guess estimates from surface observations.

For hatchery operations, the minimum essential information which should be recorded/ logged are:

- feeding activity
- growth
- mortalities

These observations should be recorded on a daily basis for larval and juvenile molluscs, including date, time, tank, broodstock (where

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there are more than one) and food-source (algal culture batch or other food-source). Dates and times for tank and water changes should also be noted, as well as dates and times for pipe flushing and/or disinfection. Ideally, these logs should be checked regularly by the person responsible for the site/animals.

For open-water mollusc sites, the minimum essential observations which need to be recorded/ logged include:

- growth
- fouling
- mortalities

These should be recorded with date, site location and any action if taken (e.g., defouling or sample collection for laboratory examination). Ideally, these logs should be checked regularly by the person responsible for the site/animals.

M.1.4.2 Environmental Observations (Level I)

This is most applicable to open water sites, but should also be included in land-based systems with flow-through or well-based water sources. The minimum essential data which should be recorded are:

- temperature
- salinity
- turbidity (qualitative evaluation or secchi disc)
- algal blooms
- human activity

The frequency of these observations will vary with site. Where salinity or turbidity rarely vary, records may only be required during rainy seasons or exceptional weather conditions. Temperate climates will require more frequent water temperature monitoring than tropical climates. Human activity should be logged on an “as it happens” basis for reference if no infections or natural environmental changes can be attributed to a disease situation.

M.1.4.3 Stocking Records (Level I)

Information on movements of molluscs into and out of a hatchery should be recorded. This should include:

- exact source of the broodstock/seed
- condition on arrival
- date, time and person responsible for receiving delivery of the stock
- date, time and destination of stock shipped out of the hatchery

Where possible, animals from different sources should not be mixed.

All movements of molluscs onto and off an open-water site should also be recorded, including:

- exact source of the molluscs
- condition on arrival
- date, time and person responsible for receiving delivery of the stock
- date, time and destination of stock shipped off site

In addition, all movements of stocks within a hatchery, nursery or grow out site should be logged with the date for tracking purposes if a disease situation arises.

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MOLLUSCAN DISEASES

M.2 BONAMIOSIS

(*BONAMIA* SP., *B. OSTREAE*)

M.2.1 Background Information

M.2.1.1 Causative Agents

Bonamiosis (*a.k.a.* Microcell Disease; haemocyte disease of flat or dredge oysters) is caused by two Protistan (= Protozoan = single-celled) species belonging to the Haplosporidia: *Bonamia ostreae* and *Bonamia* sp.. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.2.1.2 Host Range

Bonamia ostreae occurs naturally in *Ostrea edulis* (European oyster) and *O. conchaphila* (*O. lurida*) (Olympia oyster). Other ostreiid species can become infected if transferred to enzootic areas, namely *O. puelchana*, *O. angasi* and *Ostrea lutaria* (*Tiostrea lutaria*) (New Zealand oyster), *Tiostrea chilensis* (*Ostrea chilensis*) (South American oyster), thus, all species of *Ostrea*, *Tiostrea* and some *Crassostrea* (*C. ariakensis*) should be considered susceptible. To date, *Crassostrea gigas* (Pacific oyster), *Mytilus edulis* and *M. galloprovincialis* (edible mussels) and *Ruditapes decussatus* and *R. philippinarum* (European and Manila clams) have been found to be resistant to infection. These species have also been shown to be incapable of acting as reservoirs or sub-clinical carriers of infection.

M.2.1.3 Geographic Distribution

Bonamia ostreae: The Netherlands, France, Spain, Italy, Ireland, the United Kingdom (excluding Scotland) and the United States of America (States of California, Maine and Washington). Denmark, although stocked with infected oysters in the early 1980's, has shown no sign of persistence of the infection and their European oysters are now considered to be free of *B. ostreae*.

Bonamia sp.: Australia (Western Australia, Victoria and Tasmania) and New Zealand (South Island and southern North Island).

M.2.1.3 Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999 to 2000)

For the reporting year 1999, *Bonamia* sp. was positively reported in Australia in April, July and October in Tasmania; July and October in Western Australia. For the year 2000, *Bonamia* sp. was reported in March and April in Western Australia. In New Zealand, *Bonamia* sp. was

reported every month for 1999 and 2000 reporting periods (OIE 1999, OIE 2000b).

M.2.2 Clinical Aspects

Most infections show no clinical signs until the parasites have proliferated to a level that elicits massive blood cell (haemocyte) infiltration and diapedesis (Fig.M.2.2a). The pathology of infection varies with the species of *Bonamia*, and with host species. *Bonamia ostreae* infects the haemocytes of European oysters (Fig.M.2.2b), where it divides until the haemocyte bursts, releasing the parasites into the haemolymph. Infections likely occur through the digestive tract, but gill infections suggest this may also be another infection route and macroscopic gill lesions are sometimes visible. The pathology of *Bonamia* sp. in Australian *Ostrea angasi* and New Zealand populations of *Tiostrea chilensis* is very different. In Australia's *O. angasi*, the first indication of infection is high mortality. Surviving oysters rapidly start to gape on removal from the water and may have "watery" tissues and a ragged appearance to the margin of the gill (unpublished data, B. Jones, Fisheries Western Australia). *Bonamia* sp. infects the walls of the gills, digestive ducts and tubules (Fig.M.2.2c), from which the parasites may be released into the gut or surrounding water. Infected haemocytes may contain up to 6 *Bonamia* parasites (Fig.M.2.2d). Infections induce massive abscess-like lesions (haemocytosis), even in the presence of only a few parasites. In *T. chilensis*, *Bonamia* sp. appears to enter via the gut wall (Fig.M.2.2e), and then infects the haemocytes, where up to 18 *Bonamia* per haemocyte can be found (Fig.M.2.2f). The resultant haemocytosis is less severe than in *O. angasi*. When infected haemocytes enter the gonad of *T. chilensis* to reabsorb unspawned gametes, the parasites proliferate and may be released via the gonoduct. Alternative release is also possible via tissue necrosis following the death of the host. Despite the differences in pathology, gene sequencing studies (unpublished data, R. Adlard, University of Queensland, Australia) have shown that the Australian and New Zealand *Bonamia* sp. are the same species.

M.2.3 Screening Methods

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.2 Bonamiosis (*Bonamia* sp., *B. ostreae*)

(SE McGladdery)



Fig.M.2.2a. Haemocyte infiltration and diapedesis across intestinal wall of a European oyster (*Ostrea edulis*) infected by *Bonamia ostreae*.

(PM Hine)

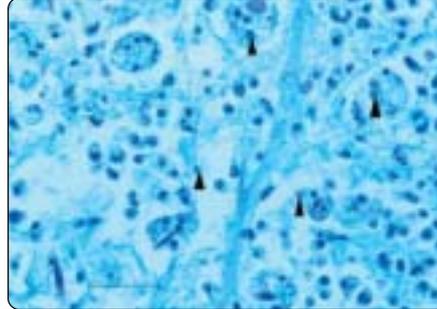


Fig.M.2.2d. Oil immersion of *Bonamia* sp. infecting blood cells and lying free (arrows) in the haemolymph of an infected Australian flat oyster, *Ostrea angasi*. Scale bar 20µm (H&E).

(SE McGladdery)

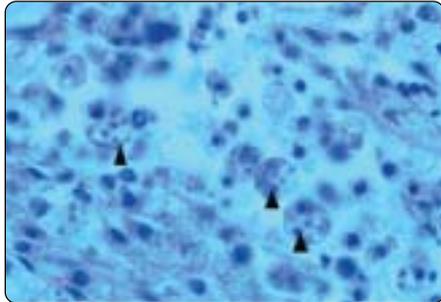


Fig.M.2.2b. Oil immersion of *Bonamia ostreae* inside European oyster (*Ostrea edulis*) haemocytes (arrows). Scale bar 20 µm.

(PM Hine)



Fig.M.2.2e. Focal infiltration of haemocytes around gut wall (star) of *Tiostraea lutaria* (New Zealand flat oyster) typical of infection by *Bonamia* sp. (H&E).

(PM Hine)



Fig.M.2.2c. Systemic blood cell infiltration in Australian flat oyster (*Ostrea angasi*) infected by *Bonamia* sp. Note vacuolised appearance of base of intestinal loop and duct walls (H&E).

(PM Hine)

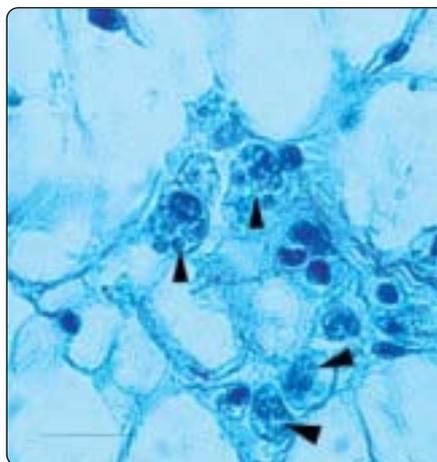


Fig.M.2.2f. Oil immersion of haemocytes packed with *Bonamia* sp. (arrows) in an infected *Tiostraea lutaria* (H&E). ➤

M.2 Bonamiosis (*Bonamia* sp., *B. ostreae*)

M.2.3.1 Presumptive

M.2.3.1.1 Gross Observations (Level I).

Slowed growth, presence of gill lesions (in some cases), gaping and mortalities of *Ostrea edulis* should be considered suspect for Bonamiosis. Gross signs are not disease specific and require Level II examination.

M.2.3.1.2 Cytological Examination (Level II)

Spat or heart (preferably ventricle) smears or impressions (dabs) can be made onto a clean microscope slide and air-dried. Once dry, the preparation is fixed in 70% methanol. Quick and effective staining can be achieved using commercially available blood-staining (cytological) kits, following the manufacturer's instructions. The stained slides are then rinsed (gently) in tapwater, allowed to dry and cover-slipped using a synthetic resin mounting medium. The parasite has basophilic (or colourless - *Bonamia* sp. in *O. angasi*) cytoplasm and an eosinophilic nucleus (depending on the stain used). An oil immersion observation time of 10 mins per oyster preparation is considered sufficient for screening cytology, tissue imprint and histology preparations (OIE 2000a).

M.2.3.2 Confirmatory

M.2.3.2.1 Histopathology (Level II)

It is recommended that at least two dorso-ventral sections through the cardiac cavity, gonad and gills of oysters over 18 months – 2 years (> 30 mm shell height) be examined for screening purposes. These sections should be fixed immediately in a fast fixative such as 1G4F. Fixatives such as Davidsons or 10% seawater buffered formalin may be used for whole oysters (see M.1.3.3.3), but these do not allow serial tissue sections to be collected for subsequent confirmatory Electron Microscopy (EM) diagnosis, if required. Davidson's fixative is recommended for subsequent PCR-based confirmation techniques.

Several standard stains (e.g., haematoxylin-eosin) enable detection of *Bonamia* spp.. The parasites measure 2-5 µm and occur within the haemocytes or epithelia (as described above) or, more rarely, loose within the haemolymph or gut/mantle lumens.

M.2.4 Diagnostic Methods

More detailed methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.2.4.1 Presumptive

M.2.4.1.1 Histopathology and Cytology (Level II)

Histology and cytology (Level II), as described under M.2.3.2.1, may be used. For first-time diagnoses, a back up tissue specimen fixed for EM is recommended (M.2.4.2.1).

M.2.4.2 Confirmatory

M.2.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

Tissue for TEM can be fixed in 1G4F (M.1.3.3.3), however, where it is likely that TEM will be required for confirmatory diagnosis (M.2.4.1.1), small (< 1 mm cubed) sub-samples of infected tissue should be fixed in 2-3% buffered glutaraldehyde prepared with ambient salinity filtered seawater. Fixation should not exceed 1 hr. Longer storage in glutaraldehyde fixative is possible, however membrane artifacts can be produced. Tissues should be rinsed in a suitable buffer prior to post-fixing in 1-2% osmium tetroxide (= osmic acid - *highly toxic*). This post-fixative must also be rinsed with buffered filtered (0.22 µm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues should be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides and stained with Toluidine Blue is one method of selecting the tissue specimens for optimum evidence of putative *Bonamia* spp. Ultrathin sections are then mounted on copper grids (with or without formvar coating) for staining with lead citrate + uranyl acetate or equivalent EM stain.

Ultrastructural differences between *B. ostreae* and *Bonamia* sp. include diameter (*B. ostreae* = 2.4 ± 0.5 µm; *Bonamia* sp. = 2.8 ± 0.4 µm in *O. angasi*, 3.0 ± 0.3 µm in *T. chilensis*); mean number of mitochondrial profiles/section (*B. ostreae* = 2 ± 1 ; *Bonamia* sp. = 4 ± 1 in *O. angasi*, 3 ± 1 in *T. chilensis*), mean number of haplosporosomes/section (*B. ostreae* = 7 ± 5 ;

M.2 Bonamiosis (*Bonamia* sp., *B. ostreae*)

Bonamia sp. = 10 ± 4 in *O. angasi*, 14 ± 6 in *T. chilensis*); percentage of sections containing large lipid globules (*B. ostreae* = 7%; *Bonamia* sp. in *O. angasi* = 30%; in *T. chilensis* = 49%), large lipid globules/section (*B. ostreae* = 0.3 ± 0.6 ; *Bonamia* sp. = 0.5 ± 0.8 in *O. angasi*, 0.8 ± 0.9 in *T. chilensis*). Both species are distinguished from *Mikrocytos* spp. by having a centrally-placed nucleus.

Plasmodial forms of *Bonamia* sp. in *T. chilensis* are distinguished from *Bonamia ostreae* by their size (4.0 -4.5 μm diameter), irregular cell and nucleus profile, amorphous cytoplasmic inclusions (multi-vesicular bodies) and arrays of Golgi-like smooth endoplasmic reticula. Other developmental stages are more electron dense and smaller in diameter (3.0 -3.5 μm).

M.2.5 Modes of Transmission

Prevalence and intensity of infection tends to increase during the warm water season with peaks in mortality in September/October in the northern hemisphere, and January to April, in the southern hemisphere. The parasite is difficult to detect prior to the proliferation stage of development or in survivors of an epizootic. Cohabitation and tissue homogenate/haemolymph inoculations can precipitate infections indicating that transmission is direct (no intermediate hosts are required). There is a pre-patent period of 3-5 months between exposure and appearance of clinical signs of *B. ostreae* infection. In New Zealand the pre-patent period for *Bonamia* sp. infection may be as little as 2.5 months and rarely exceeds 4 months.

M.2.6 Control Measures

None known. Reduced stocking densities and lower water temperatures appear to suppress clinical manifestation of the disease, however, no successful eradication procedures have worked to date. Prevention of introduction or transfer of oysters from *Bonamia* spp. enzootic waters into historically uninfected waters is recommended.

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M.3 MARTEILIOSIS (*MARTEILIA REFRINGENS*, *M. SYDNEYI*)

M.3.1 Background Information

M.3.1.1 Causative Agents

Marteiliosis is caused by two species of parasites, belonging to the Phylum Paramyxia. *Marteilia refringens* is responsible for Aber Disease (a.k.a Digestive Gland Disease) of European oysters (*Ostrea edulis*) and *Marteilia sydneyi* is responsible for QX Disease of *Saccostrea glomerata* (syn. *Crassostrea commercialis*, *Saccostrea commercialis*) and, possibly, *Saccostrea echinata*. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.3.1.2 Host Range

Ostrea edulis is infected by *Marteilia refringens*. Other host species include *Tiostrea chilensis*, *Ostrea angasi*, *O. puelchana*, *Cerastoderma* (= *Cardium*) *edule*, *Mytilus edulis*, *M. galloprovincialis*, *Crassostrea gigas* and *C. virginica*. *Marteilia sydneyi* infects *Saccostrea glomerata* and possibly *S. echinata*. Another marteliad, *Marteilia maurini*, infects mussels (*Mytilus edulis* and *M. galloprovincialis*) from France, Spain and Italy. This species is not readily distinguished morphologically from *M. refringens* and distinct species status is under investigation. An unidentified marteliad was responsible for mass mortalities of the Calico scallop (*Argopecten gibbus*) in Florida in the late 1980's, but has not re-appeared since. Another *Marteilia*-like species was reported from the giant clam, *Tridacna maxima*. Other species of *Marteilia* that have been described include *M. lenghi* from *Saccostrea* (*Crassostrea*) *cucullata* (Persian Gulf and north Western Australia) and *M. christenseni* in *Scrobicularia plana* (France). These are differentiated from *M. refringens* and *M. sydneyi* by the cytoplasmic contents of the sporangia and spore morphology.

M.3.1.3 Geographic Distribution

Marteilia refringens is found in *O. edulis* in southern England, France, Italy, Portugal, Spain, Morocco and Greece. *Marteilia sydneyi* is found in *S. glomerata* in Australia (New South Wales, Queensland and Western Australia).

M.3.1.4 Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)

No positive report of the disease in any country for the 2 year reporting periods. Most countries have no information about the occurrence of the disease (OIE 1999, OIE 2000b).

M.3.2 Clinical Aspects

Early stages of *Marteilia refringens* develop in the digestive ducts, intestinal and stomach epithelia and gills (Fig.M.3.2a). Later, spore-forming stages appear in the blind-ending digestive tubule epithelia (Fig.M.3.2b). Proliferation of the parasite is associated with emaciation and exhaustion of glycogen reserves, gross discoloration of the digestive gland, cessation of feeding and weakening. Mortalities appear to be associated with sporulation of the parasite and disruption of the digestive tubule epithelia.

M.3.3 Screening Methods

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.3.3.1 Presumptive

M.3.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Ostrea edulis* and other susceptible species should be considered suspect for Marteiliiosis. Gross signs are not specific for Bonamiosis or Marteiliiosis and require Level II examination.

M.3.3.1.2 Tissue Imprints (Level II)

Cut a cross-section through the digestive gland, blot away excess water with blotting paper and dab the cut section of the digestive gland onto a clean microscope slide. Fix tissue imprint for 2-3 min in 70% methanol. Quick and effective staining can be achieved with a commercially available blood-staining (cytological) kit, using the manufacturer's instructions. The stained slides are then rinsed (gently) under tap water, allowed to dry and cover-slipped using a synthetic resin mounting medium.

The parasite morphology is as described for histology (M.3.3.2.1), although colouration may vary with the stain chosen. Initial screening with a haematoxylin or trichrome stain, as used for his-

M.3 Marteiliosis (*Marteilia refringens*, *M. sydneyi*)

tology, may assist familiarisation with tissue imprint characteristics prior to using a dip-quick method. An observation time of 10 mins at 10-25x magnification is considered sufficient for screening purposes.

M.3.3.2 Confirmatory

M.3.3.2.1 Histopathology (Level II)

Two dorso-ventral tissue section (2-3 mm thick) are recommended for screening purposes. These can be removed from oysters over 18-24 months old (or >30 mm shell height) for immediate fixation in a fast fixative, such as 1G4F. Davidson's or 10% buffered formalin may be used for larger samples or whole oysters (see M.1.3.3.3) but these provide less satisfactory results if subsequent processing for Transmission Electron Microscopy (TEM) (M.3.4.2.1) is required (e.g., for species identification). Several standard stains (e.g., haematoxylin-eosin) enable detection of *Marteilia* spp..

The early stages of development occur in the stomach, intestine and digestive duct epithelia (usually in the apical portion of the cell) and appear as basophilic, granular, spherical inclusions (Fig.M.3.2a). Later stages occur in the digestive tubules, where sporulation may induce hypertrophy of the infected cell. *Marteilia* spp. spores contain eosinophilic, "refringent", bodies which are easily detected at 10-25 x magnification under light microscopy (Fig.M.3.2b).

(SE McGladdery)

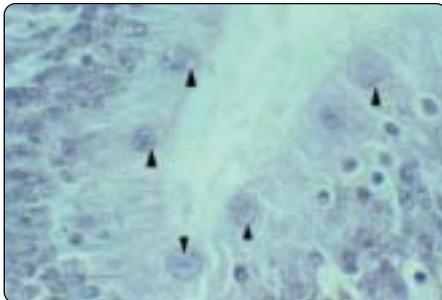


Fig.M.3.2a. Digestive duct of a European oyster, *Ostrea edulis*, showing infection of distal portion of the epithelial cells by plasmodia (arrows) of *Marteilia refringens*. Scale bar 15 µm (H&E).

(SE McGladdery)

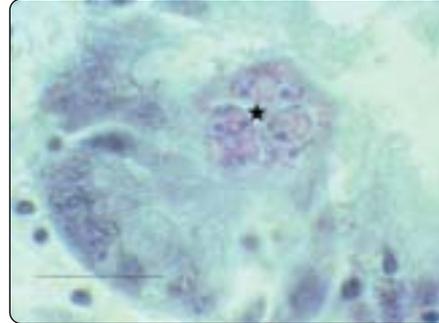


Fig.M.3.2b. Digestive tubule of a European oyster, *Ostrea edulis*, showing refringent spore stage of *Marteilia refringens* (star). Scale bar 50 µm (H&E).

(RD Adlard)

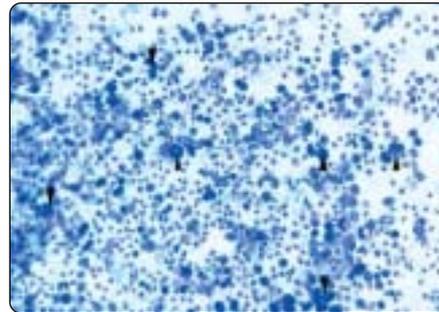


Fig.M.3.4.1.a. Tissue imprint from *Saccostrea commercialis* (Sydney rock oyster) heavily infected by *Marteilia sydneyi* (arrows) (QX disease). Scale bar 250 µm (H&E).

(RD Adlard)

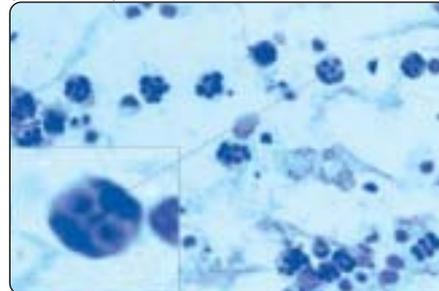


Fig.M.3.4.1.b. Oil immersion of tissue squash preparation of spore stages of *Marteilia sydneyi* from Sydney rock oyster (*Saccostrea commercialis*) with magnified inset showing two spores within the sporangium. Scale bar 50 µm (H&E).

M.3 Marteiliosis (*Marteilia refringens*, *M. sydneyi*)

M.3.4 Diagnostic Methods

More detailed methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.3.4.1 Presumptive

M.3.4.1.1 Tissue Imprints (Level II)

As described under M.3.3.1.2, tissue imprints may also be used for presumptive diagnoses (Fig.M.3.4.1.1a,b). For first-time diagnoses back up tissues should be fixed for histology and EM confirmatory diagnosis.

M.3.4.1.2 Histopathology (Level II)

Histology techniques as described under M.3.3.2.1, may be used. For first-time diagnoses a back up tissue specimen fixed for EM is recommended, as described below.

M.3.4.2 Confirmatory

M.3.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

TEM tissue preparation involves fixing tissues either in 1G4F (M.1.3.3.3) or small (< 1 mm cubed) sub-samples of infected tissue in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Ideally, fixation in 2-3% glutaraldehyde should not exceed 1 hr, since longer storage may induce membranous artifacts. Tissues should be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse tissues in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - highly toxic). Secondary fixation should be complete within 1 hr. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 μm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 μm sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of possible *Marteilia* spp. Ultrathin sections are then mounted on copper grids (with or without formvar coating), and stained with lead citrate + uranyl acetate (or equivalent EM stain).

Marteilia refringens plasmodia contain striated inclusions, eight sporangial primordia, with up to four spores to each mature sporangium. *Marteilia sydneyi* has a thick layer of concentric membranes surrounding the mature spore, lacks striated inclusions in the plasmodia, forms eight to sixteen sporangial primordia in each plasmodium and each sporangium contains two (rarely three) spores.

M.3.4.2.2 In situ Hybridization (Level III)

In situ hybridization (Level III) techniques are under development but not yet available commercially. Information on the current status of these and related molecular probe techniques may be obtained from IFREMER Laboratory at La Tremblade, France (OIE 2000a, Annex MAI).

M.3.5 Modes of Transmission

Marteilia refringens transmission appears to be restricted to periods when water temperatures exceed 17°C. High salinities may impede *Marteilia* spp. multiplication within the host tissues. *Marteilia sydneyi* also has a seasonal period of transmission with infections occurring generally from mid- to late-summer (January to March). Heavy mortalities and sporulation occur all year round. The route of infection and life-cycle outside the mollusc host are unknown. Since it has not been possible to transmit the infection experimentally in the laboratory, an intermediate host is suspected. This is reinforced by recent observations showing spores do not survive more than 7-10 days once isolated from the oyster. Cold temperatures prolong survival (35 days at 15°C). Spore survival within fish or birds was limited to 2 hrs, suggesting they are an unlikely mode of dispersal or transmission.

M.3.6 Control Measures

None known. High salinities appear to suppress clinical manifestation of the disease, however, no eradication attempts have been successful, to date. Prevention of introduction or transfer of oysters or mussels from *Marteilia* spp. enzootic waters into historically uninfected waters is recommended.

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M.4 MIKROCYTOSIS (MIKROCYTOS MACKINI, M. ROUGHLEYI)

M.4.1 Background Information

M.4.1.1 Causative Agents

Mikrocytosis is caused by two species of parasites of uncertain taxonomic affinity. *Mikrocytos mackini* is responsible for Denman Island Disease (Microcell disease) of Pacific oysters (*Crassostrea gigas*), and *Mikrocytos roughleyi* is responsible for Australian Winter Disease (Winter Disease, Microcell Disease) of Sydney rock oysters, *Saccostrea glomerata*. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.4.1.2 Host Range

Mikrocytos mackini naturally infects *Crassostrea gigas* (Pacific oysters), *Ostrea edulis* (European oysters), *O. conchaphila* (= *O. lurida*) (Olympia oyster) and *Crassostrea virginica* (American oysters) growing in enzootic waters are also susceptible to infection. *Saccostrea glomerata* (*Crassostrea commercialis*, *Saccostrea commercialis*) (Sydney rock oyster) is the only known host for *Mikrocytos roughleyi*.

M.4.1.3 Geographic Distribution

Mikrocytos mackini is restricted to specific localities around Vancouver Island and southwest coast of the Pacific coast of Canada. The parasite is limited to waters with temperatures below 12°C. *Mikrocytos roughleyi* occurs in central to southern New South Wales, and at Albany and Carnarvon, Western Australia.

M.4.1.4 Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)

No positive report during the reporting periods for 1999 and 2000. In Australia, last year of occurrence was 1996 (in New South Wales and Western Australia). Most countries have no information about occurrence of the disease (OIE 1999, OIE 2000b).

M.4.2 Clinical Aspects

Mikrocytos mackini initiates focal infections of the vesicular connective tissue cells. This elicits haemocyte infiltration and abscess formation. Grossly visible pustules (Fig.M.4.2a), abscess lesions and tissue ulcers, mainly in the mantle, may correspond to brown scar formation on the

adjacent surface of the inner shell. However, such lesions are not always present. Small cells, 1-3 µm in diameter, are found (rarely) around the periphery of advanced lesions, or in connective tissue cells in earlier stages of disease development. Severe infections appear to be restricted to oysters over 2 years old.

Mikrocytos roughleyi induces a systemic intracellular infection of the haemocytes (never the connective tissue cells) which may result in focal lesions in the gills, connective, gonadal and digestive tract.

M.4.3 Screening Methods

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.4.3.1 Presumptive

M.4.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Crassostrea gigas* and *Saccostrea glomerata* should be considered suspect for Mikrocytosis. Gross signs are not pathogen specific and require Level II examination, at least for first-time observations.

M.4.3.1.2 Cytological Examination and Tissue Imprints (Level II)

Heart impressions (dabs) can be made onto a clean microscope slide and air-dried. Once dry, the slide is fixed in 70% methanol. Quick and effective staining can be achieved with commercially available blood-staining (cytological) kits, using the manufacturer's instructions. The stained slides are then rinsed (gently) under tap water, allowed to dry and cover-slipped using a synthetic resin mounting medium. Intracytoplasmic parasites in the haemocytes will match the descriptions given above for histology. This technique is more applicable to *M. roughleyi* than *M. mackini*.

Tissue sections through mantle tissues (especially abscess/ulcer lesions, where present) are cut and excess water removed with blotting paper. The cut section is dabbed onto a clean microscope slide, fixed for 2-3 minutes in 70% methanol and stained. Quick and effective staining can be achieved using a commercially available blood-staining (cytological) kits, using the manufacturer's instructions. The stained slides

M.4 MIKROCYTOSIS

(*Mikrocytos mackini*, *M. roughleyi*)

(SM Bower)

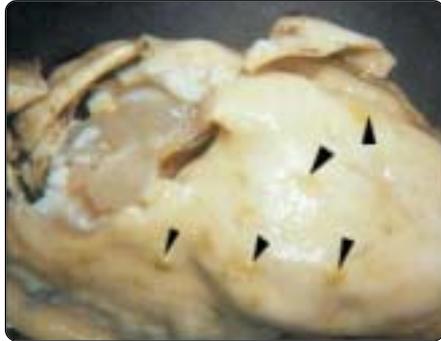


Fig.M.4.2a. Gross abscess lesions (arrows) in the mantle tissues of a Pacific oyster (*Crassostrea virginica*) severely infected by *Mikrocytos mackini* (Denman Island Disease).

(SM Bower)



Fig.M.4.3.2.1a. Histological section through mantle tissue abscess corresponding to the gross lesions pictured in Fig.M.4.2a, in a Pacific oyster (*Crassostrea gigas*) infected by *Mikrocytos mackini* (H&E).

(SM Bower)

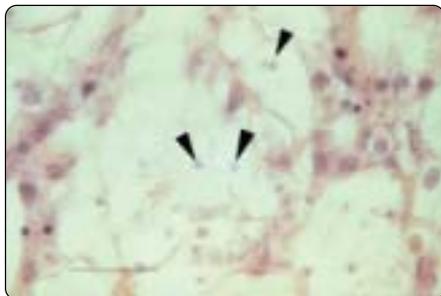


Fig.M.4.3.2.1b. Oil immersion of *Mikrocytos mackini* (arrows) in the connective tissue surrounding the abscess lesion pictured in Fig.M.4.3.2.1a. Scale bar 20 μ m (H&E).

are then rinsed (gently) under tap water, allowed to dry and cover-slipped using a synthetic resin mounting medium.

The parasite morphology is as described for histology (M.4.3.2.1), although colouration may vary with the stain chosen. Initial screening with a haematoxylin or trichrome stain, as used for histology, may assist familiarisation with tissue imprint characteristics prior to using a dip-quick method. An observation time of 10 mins under oil immersion is considered sufficient for screening purposes.

M.4.3.2 Confirmatory

M.4.3.2.1 Histopathology (Level II)

It is recommended that at least two dorso-ventral sections (2-3 mm) through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F. Davidsons or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.4.4.2.1), or species identification, if required. Also smaller oysters are not the recommended size-group for *Mikrocytos* screening. Sections through pustules, abscess or ulcer lesions should be selected where present. Several standard stains (e.g., haematoxylin-eosin) enable detection of *Mikrocytos* spp.

Mikrocytos mackini appears as 2-3 μ m intracellular inclusions in the cytoplasm of the vesicular connective tissues immediately adjacent to the abscess-like lesions (Fig. M.4.3.2.1a,b). It may also be observed in muscle cells and, occasionally, in haemocytes or free, within the lesions. It is distinguished from *Bonamia* by an eccentric nucleus and from *M. roughleyi* by the consistent absence of a cytoplasmic vacuole, and the presence of a mitochondrion in *M. roughleyi*. These features will not be clear under oil and require confirmation using 1 micron resin sections or TEM (described below). Neither of these techniques, however, are practical for screening purposes.

Mikrocytos roughleyi measures 1-3 μ m in diameter and occurs exclusively in the haemocytes. A cytoplasmic vacuole may or may not be present. When present, it displaces the nucleus peripherally. Nucleolar structures may or may not be visible under oil immersion resolution for this intracellular parasite.

M.4 Mikrocytosis (*Mikrocytos mackini*, *M. roughleyi*)

M.4.4 Diagnostic Methods

More detailed methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.4.4.1 Presumptive

M.4.4.1.1 Histopathology and Tissue Imprints (Level II)

Histology (M.4.3.2.1) may be used, however, for first-time diagnoses, EM confirmation is recommended (M.4.4.2.2). Tissue imprints may also be used for presumptive diagnoses, where they demonstrate the features described under M.4.3.1.2.

M.4.4.2 Confirmatory

M.4.4.2.1 Histopathology and Tissue Imprints (Level II)

Histology (M.4.3.2.1) and tissue imprints (M.4.3.1.2) may be used, however, for first-time diagnoses, EM confirmation is recommended (M.4.4.2.2).

M.4.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

Tissues should be fixed in 1G4F for 12-24 hours. Following primary fixation, rinse tissues in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO₄ = osmic acid - *highly toxic*). Secondary fixation should be complete within 1 hour. The OsO₄ fixative must also be rinsed with buffer/filtered (0.22 microns) seawater prior to dehydration and resin-embedding.

Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of putative *Mikrocytos* spp. Ultrathin sections are then mounted on copper grids (with or without formvar coating), and stained with lead citrate + uranyl acetate (or equivalent EM stain).

Mikrocytos mackini is distinguished ultrastructurally (as well as by tissue location and host species) from *Bonamia* spp. by the location of the nucleolus. In *M. mackini* it is in the centre of the nucleus, while in *B. ostreae* it is eccentric.

Mikrocytos mackini also lacks mitochondria. The ultrastructural characteristics of *Mikrocytos roughleyi* have not been published, however, it is distinguished from *M. mackini* by the presence of a cytoplasmic vacuole (along with completely different geographic, host and tissue locations!).

M.4.5 Modes of Transmission

Mikrocytos mackini transmission appears restricted to early spring (April-May) following periods of 3-4 months at water temperatures < 10°C. High salinities (30-35 ppt) appear to favour parasite proliferation and mortalities of approximately 40% occur in sub-tidal or low-tide populations of older oysters.

Mikrocytos roughleyi is also associated with low temperatures and high salinities killing up to 70% of mature Sydney rock oysters in their third winter before marketing. This usually follows a prepatent (sub-clinical) period of approximately 2.5 months.

Transmission of *M. mackini* has been achieved by exposure of susceptible oysters to homogenates from infected oysters as well as to proximal exposure, thus, it is believed that this species has a direct life-cycle. *M. roughleyi* is also thought to be transmitted directly from oyster to oyster.

M.4.6 Control Measures

Circumvention of mortalities has been achieved for *M. mackini* at enzootic sites by relaying oysters to high tide levels during the peak transmission period in April-May to reduce exposure to the water-borne infectious stages. No control measures are known for *M. roughleyi*.

M.4.7 Selected References

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M.4 Mikrocytosis (*Mikrocytos mackini*, *M. roughleyi*)

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M.5 PERKINSOSIS (*PERKINSUS MARINUS*, *P. OLSENI*)

M.5.1 Background Information

M.5.1.1 Causative Agents

Perkinsosis is caused by two species of protistan parasite belonging to the phylum Apicomplexa (although recent nucleic acid investigations suggest a possible affiliation with the dinoflagellates). *Perkinsus marinus* is responsible for “Dermo” disease in *Crassostrea virginica* (American oysters) and *Perkinsus olseni* causes perkinsosis in many bivalve species in tropical and subtropical waters. Other perkinsiid species are known to infect clams in Europe (*Perkinsus atlanticus*) and the eastern USA (*Perkinsus* spp.), as well as Japanese (Yesso) scallops, *Patinopecten yessoensis* in Pacific Canada (*Perkinsus qugwadi*). The taxonomic relationship between these and the two species listed as ‘notifiable’ by OIE is currently under investigation. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.5.1.2 Host Range

Perkinsus marinus (formerly known as *Dermocystidium marinum* and *Labyrinthomyxa marinus*) infects *Crassostrea virginica* (American oysters). Experimental infection to *C. gigas* (Pacific oysters) is possible, but they appear more resistant than *C. virginica*. *Perkinsus olseni* shows a strong rDNA similarity to *Perkinsus atlanticus* of *Ruditapes decussatus* and the speciation within this genus, as mentioned under M.5.1.1, is currently under nucleic acid investigation. Recognised hosts of *P. olseni* are the abalone species: *Haliotis rubra*, *H. cyclobates*, *H. scalaris* and *H. laevigata*. More than 50 other molluscan species harbour *Perkinsus* spp., as well as other possibly related species, without apparent harmful effects (e.g., in *Arca* clams [Fig.M.5.1.2a] and *Pinctada* pearl oysters [Fig.M.5.1.2b]).

M.5.1.3 Geographic Distribution

Perkinsus marinus is found along the east coast of the United States from Massachusetts to Florida, along the Gulf of Mexico coast to Venezuela, and in Puerto Rico, Cuba and Brazil. It has also been introduced into Pearl Harbour, Hawaii. Range extension into Delaware Bay, New Jersey, Cape Cod and Maine are attributed to repeated oyster introductions and increased winter water temperatures. *Perkinsus olseni* occurs in South Australia. Other species occur in Atlantic and Pacific oceans and the Mediterranean Sea.

M.5.1.4 Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)

P. marinus was not reported in Australia during 1999 and 2000 reporting periods; *P. olseni* likewise not reported in 1999 and 2000 (last year of occurrence in South Australia in 1997, and in 1995 in New South Wales and Western Australia). Suspected in Korea RO for reporting period 1999 and 2000. In New Zealand, positively reported from April to December 2000. *Perkinsus olseni* occurs in wild populations of New Zealand cockles, *Austrovenus stutchburyi* (Family Veneridae) and two other bivalve species, *Macomona liliiana* (Family Tellinidae) and *Barbatia novae-zelandiae* (Family Arcidae). These species occur widely in the coast of New Zealand. Affected locations have been the Waitemata and Kaipara Harbours but the organism is probably enzootic in the warmer waters of northern New Zealand (OIE 1999, OIE 2000a).

M.5.2 Clinical Aspects

The effects of *Perkinsus marinus* on *Crassostrea virginica* range from pale appearance of the digestive gland, reduced condition indices, severe emaciation, gaping, mantle retraction, retarded gonadal development and growth and occasional abscess lesions. Mortalities of up to 95% have occurred in infected *C. virginica* stocks.

Proliferation of *Perkinsus olseni* results in disruption of connective and epithelial tissues and some host species show occasional abscess formation. Pustules up to 8 mm in diameter in affected *Haliotis* spp. reduce market value and have been associated with heavy losses in *H. laevis gata*.

M.5.3 Screening Methods

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.5.3.1 Presumptive

M.5.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Crassostrea virginica* and *Haliotis* spp., as well as other mollusc species in *Perkinsus*-enzootic waters should be considered suspect for Perkinsiosis. Gross signs are not pathogen-specific and require Level II examination, at least for first time observations.

M.5 Perkinsosis (*Perkinsus marinus*, *P. olseni*)

(PM Hine)

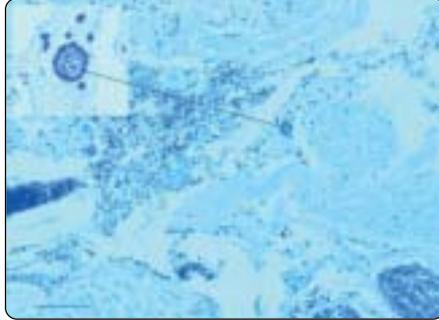


Fig.M.5.1.2a. *Arca* clam showing a *Perkinsus*-like parasite within the connective tissue. Magnified insert shows details of an advanced 'schizont' like stage with trophozoites showing vacuole-like inclusions. Scale bar 100 μ m. (H&E).

(SE McGladdery)

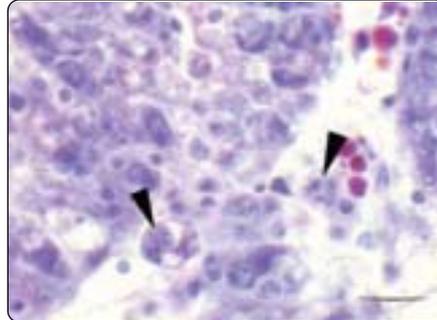


Fig.M.5.3.2.1b. Schizont ('rosette') stages of *Perkinsus marinus* (arrows), the cause of 'Dermo' disease in American oyster (*Crassostrea virginica*) digestive gland connective tissue. Scale bar 30 μ m (H&E).

(PM Hine)

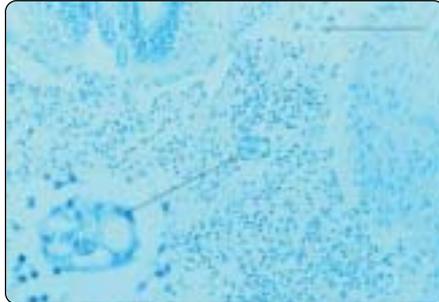


Fig.M.5.1.2b. *Pinctada albicans* pearl oyster showing a *Perkinsus*-like parasite. Magnified insert shows details of a 'schizont'-like stage containing 'trophozoites' with vacuole-like inclusions. Scale bar 250 μ m (H&E).

(SE McGladdery)

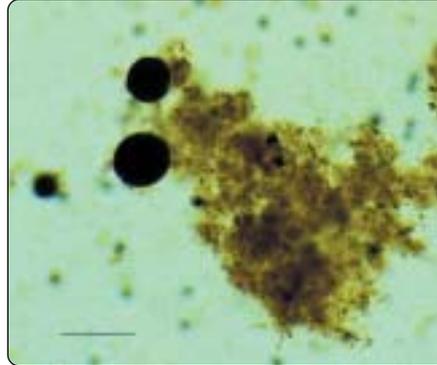


Fig.5.3.2.2. Enlarged hyphospores of *Perkinsus marinus* stained blue-black with Lugol's iodine following incubation in fluid thioglycollate medium. Scale bar 200 μ m.

(SM Bower)

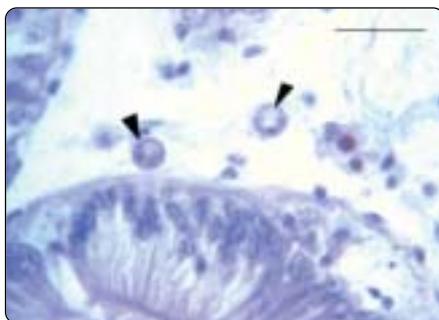


Fig.M.5.3.2.1a. Trophozoite ('signet-ring') stages of *Perkinsus marinus* (arrows), the cause of 'Dermo' disease in American oyster (*Crassostrea virginica*) connective tissue. Scale bar 20 μ m (H&E).

M.5.3.2 Confirmatory

M.5.3.2.1 Histopathology (Level II)

It is recommended that at least two dorso-ventral sections through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F. Davidson's or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.5.4.2.1), or species identification, if required. Sections through pustules, abscess or ulcer lesions should be selected, where present. Several standard stains (e.g.,

M.5 Perkinsosis (*Perkinsus marinus*, *P. olsenii*)

haematoxylin-eosin) enable detection of *Perkinsus* spp.

Perkinsus marinus infections are usually systemic, with trophozoites occurring in the connective tissue of all organs. Immature trophozoites (meronts, merozoites or aplanospores) measure 2-3 µm in diameter. “Signet-ring” stages are mature trophozoites, measuring 3-10 µm in diameter, each with a visible eccentric vacuole displacing the nucleus and cytoplasm peripherally (Fig.M.5.3.2.1a). The “rosette” stage (tomonts, sporangia or schizonts) measure 4-15 µm in diameter and can contain 2, 4, 8, 16 or 32 developing trophozoites (Fig.M.5.3.2.1b).

Perkinsus olsenii shows the same developmental stages although the trophozoite stages are larger ranging from 13-16 µm in diameter. Due to host and parasite diversity, however, morphological features cannot be considered specific.

M.5.3.2.2 Fluid Thioglycollate Culture (Level II)

Tissue samples measuring 5-10 mm are excised (select lesions, rectal and gill tissues) and placed in fluid thioglycollate medium containing antibiotics. Incubation temperature and time varies per host species and environment. The standard protocol for *P. marinus* is 22-25°C for 4-7 days in the dark. Warmer temperatures can be used for *P. olsenii*.

The cultured parasites expand in size to 70-250 µm in diameter. Following incubation, the tissues are placed in a solution of 1:5 Lugol's iodine:water for 10 minutes. The tissue is then teased apart on a microscope slide and examined, using low power on a light microscope, for enlarged hypnospores with walls that stain blue-black (Fig.M.5.3.2.2)

M.5.4 Diagnostic Methods

More detailed methods for diagnostics can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.5.4.1 Presumptive

M.5.4.1.1 Histopathology (Level II)

Histology (M.5.3.2.1), may be used. However, for first-time diagnoses a back up tissue specimen fixed for EM is recommended (M.5.4.2.1).

M.5.4.1.2 Fluid Thioglycollate Medium Culture (Level II)

Fluid thioglycollate medium culture (Level II) may also be used for presumptive diagnosis (M.5.3.2.2).

M.5.4.2 Confirmatory

M.5.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

TEM is required to detect the species-specific ultrastructure of the zoospore stage of development (collected from zoospore release from cultured aplanospores [prezoosporangia]). Tissue preparation involves fixing concentrated zoospores or zoosporangia (produced by placing the aplanospores into filtered ambient seawater, where they develop into zoosporangia and produce hundreds of motile zoospores) in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Oyster tissues can also be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse tissues in a suitable buffer and postfix in 1-2% osmium tetroxide (OsO_4 = osmic acid - highly toxic). Secondary fixation should be complete within 1 hr. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 µm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 µm-thick sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of putative *Perkinsus* spp. Such pre-screening should not be necessary for concentrated zoospore or zoosporangia preparations. Ultrathin sections are then mounted on copper grids (with or without formvar coating), and stained with lead citrate + uranyl acetate (or equivalent EM stain).

The anterior flagellum of *Perkinsus marinus* zoospores is ornamented with a row of hair- and spur-like structures. The posterior flagellum is smooth. An apical complex is present, consisting of a conoid, sub-pellicular microtubules, rhoptries, rectilinear micronemes and conoid-associated micronemes. Large vacuoles are also present at the anterior end of the zoospore.

M.5 Perkinsosis (*Perkinsus marinus*, *P. olsenii*)

M.5.5 Modes of Transmission

Proliferation of *Perkinsus* spp. is correlated with warm water temperatures (>20°C) and this coincides with increased clinical signs and mortalities. Effects appear cumulative with mortalities peaking at the end of the warm water season in each hemisphere. The infective stage is a biflagellate zoospore which transforms into the feeding trophozoite stage after entering the host's tissues. These multiply by binary fission within the host tissues. *Perkinsus marinus* shows a wide salinity tolerance range. *Perkinsus olsenii* is associated with full strength salinity environments.

Direct transmission of *Perkinsus* spp. has been demonstrated by exposure of susceptible hosts to infected hosts, including cross-species transmission for *P. olsenii*. There is currently no evidence of cross-genus transmission of *P. marinus*.

M.5.6 Control Measures

None known for *Perkinsus* spp. Most efforts against *P. marinus* have concentrated on development of resistant (tolerant) stocks of oysters. These currently show potential for surviving in enzootic areas, but are not recommended for use in non-enzootic areas due to their potential to act as sub-clinical carriers of the pathogen. Some success has been achieved, however, in preventing *P. marinus* infection of hatchery-reared larval and juvenile oysters using filtration and UV sterilization of influent water. The almost ubiquitous occurrence of *Perkinsus* in many bivalve species around mainland Australia makes control by restriction of movements impractical.

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M.5 Perkinsosis (*Perkinsus marinus*, *P. olsenii*)

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M.6 HAPLOSPORIDIOSIS (*HAPLOSPORIDIUM COSTALE*, *H. NELSONI*)

M.6.1 Background Information

M.6.1.1 Causative Agents

Haplosporidiosis is caused by two species of protistan parasite belonging to the phylum Haplosporidia. *Haplosporidium nelsoni* (syn. *Minchinia nelsoni*) is responsible for “MSX” (multi-nucleate sphere X) disease in *Crassostrea virginica* (American oysters) and *Haplosporidium costale* (*Minchinia costale*) causes “SSO” (sea-side organism) disease in the same species. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.6.1.2 Host Range

Both *Haplosporidium nelsoni* and *H. costale* cause disease in *Crassostrea virginica* (American oysters). Recently a *Haplosporidium* sp. from *Crassostrea gigas* (Pacific oyster) has been identified as *H. nelsoni* using DNA sequencing of small sub-unit ribosomal DNA.

M.6.1.3 Geographic Distribution

Haplosporidium nelsoni occurs in American oysters along the Atlantic coast of the United States from northern Florida to Maine. Endemic areas appear limited to Delaware Bay, Chesapeake Bay, Long Island Sound and Cape Cod. *Haplosporidium nelsoni* has been found in *C. gigas* from California and Washington on the Pacific coast of the USA, and Korea, Japan and France.

(PM Hine)

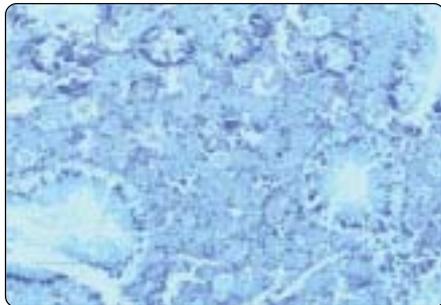


Fig.M.6.1.3a. Massive connective tissue and digestive tubule infection by an unidentified *Haplosporidium*-like parasite in the gold-lipped pearl oyster *Pinctada maxima* from north Western Australia. Scale bar 0.5 mm (H&E).

(PM Hine)

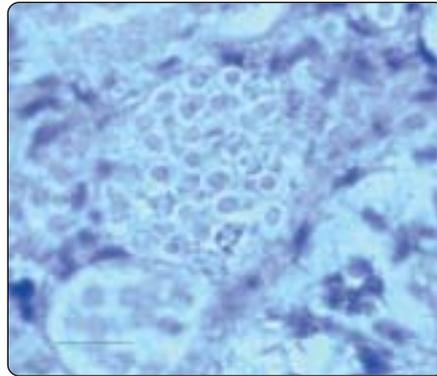


Fig.M.6.1.3b. Oil immersion magnification of the operculated spore stage of the *Haplosporidium*-like parasite in the gold-lipped pearl oyster *Pinctada maxima* from north Western Australia. Scale bar 10 µm. (H&E).

(PM Hine)



Fig.M.6.1.3c. Haemocyte infiltration activity in the connective tissue of a Sydney rock oyster (*Saccostrea cucullata*) containing spores of a *Haplosporidium*-like parasite (arrow). Scale bar 0.5 mm. (H&E).

(PM Hine)



Fig.M.6.1.3d. Oil immersion magnification of *Haplosporidium*-like spores (arrow) associated with heavy haemocyte infiltration in a Sydney rock oyster (*Saccostrea cucullata*). Scale bar 10 µm. (H&E).

M.6 Haplosporidiosis (*Haplosporidium costale*, *H. nelsoni*)

Haplosporidium costale has been reported solely from *C. virginica* from the Atlantic coast of the United States and has a small subunit rDNA distinct from that of *H. nelsoni*. *Haplosporidium costale* also has a narrower distribution, ranging from Long Island Sound, New York, to Cape Charles, Virginia.

Similar agents have been reported from hatchery-reared pearl oysters, *Pinctada maxima*, (Fig.M.6.1.3a,b) and the rock oyster, *Saccostrea cucullata* (Fig.M.6.1.3c,d), from north Western Australia.

M.6.1.4 Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)

No information or no positive report for this disease in any country for the reporting periods 1999 and 2000 (OIE 1999, OIE 2000b).

M.6.2 Clinical Aspects

Haplosporidium nelsoni occurs extracellularly in the connective tissue and digestive gland epithelia. It is often associated with a visible brown-red discolouration of gill and mantle tissues. Sporulation of *H. nelsoni* is prevalent in juvenile oysters (1-2 yrs) but sporadic in adults and occurs exclusively in the epithelial tissues of the digestive tubules. Sporulation of *H. costale* occurs throughout the connective tissues.

Haplosporidium nelsoni infections appear and continue throughout the summer (mid-May to the end of October). Gradual disruption of the digestive gland epithelia is associated with weakening and cumulative mortalities of oysters. A second wave of mortalities may occur in early spring from oysters too weak to survive over-wintering. Holding *in vivo* for up to 2 weeks in 10 ppt salinity seawater at 20°C kills the parasite but not the host. *H. nelsoni* does not cause disease at <15 ppt salinity.

Haplosporidium costale causes a pronounced seasonal mortality between May and June. Sporulation is more synchronous than with MSX infections, causing acute tissue disruption, weakening and death of heavily infected individuals. SSO disease is restricted to salinities of 25-33 ppt and infections appear to be lost at lower salinities.

M.6.3 Screening Methods

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic

Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.6.3.1 Presumptive

M.6.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Crassostrea virginica* and *C. gigas* should be considered suspect for Haplosporidiosis. Gross signs are not pathogen specific and require Level II examination, at least for first time observations.

M.6.3.1.2 Cytological Examination and Tissue Imprints (Level II)

As with histology (M.6.3.2.2), juvenile oysters are preferred for cytological or tissue imprint screening for *Haplosporidium nelsoni*. For *H. costale* adult oysters are preferred. Screening during May-June is recommended for both disease agents.

Heart smears or impressions (dabs) can be made onto a clean microscope slide and air-dried. Digestive gland and gill sections can also be used for smears by absorbing excess water from cut surfaces and dabbing the surface onto clean slides. Once dry, the slide is fixed in 70% methanol. Quick and effective staining can be achieved with commercially available blood-staining (cytological) kits, using the manufacturer's instructions. The stained slides are then rinsed (gently) under tap water, allowed to dry, and cover-slipped using a synthetic resin mounting medium.

The presence (especially between March and June in endemic areas) of multinucleate plasmodia measuring 2-15 µm in diameter is indicative of *H. costale* infection (Fig.6.3.1.2a). Plasmodia of *H. nelsoni* are detectable between mid-May and October throughout the tissues and measure 4-30 µm in diameter (Fig.M.6.3.1.2b).

Haemolymph suspensions can also be collected from live oysters, however, this is more time-consuming than heart/tissue imprints and is considered less useful for screening purposes.

M.6.3.1.3 Histopathology (Level II)

For *Haplosporidium nelsoni*, juvenile oysters are preferred for screening. For *H. costale* adult oysters are preferred. Screening during May-June is recommended for both disease agents. The

M.6 Haplosporidiosis (*Haplosporidium costale*, *H. nelsoni*)

(SE McGladdery)

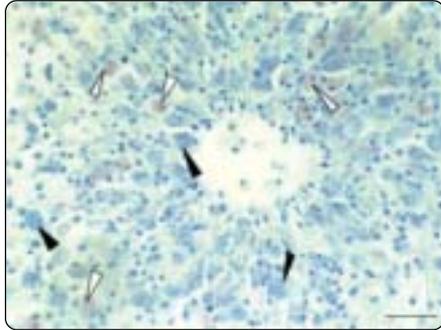


Fig.M.6.3.1.2a. Plasmodia (black arrows) and spores (white arrows) of *Haplosporidium costale*, the cause of SSO disease, throughout the connective tissue of an American oyster (*Crassostrea virginica*). Scale bar 50 μ m.

(SE McGladdery)

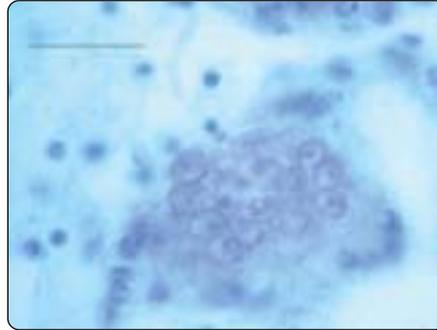


Fig.M.6.4.2.2b. Oil immersion magnification of MSX spores in the digestive tubule epithelium of an American oyster *Crassostrea virginica*. Scale bar 25 μ m. (H&E).

(SE McGladdery)

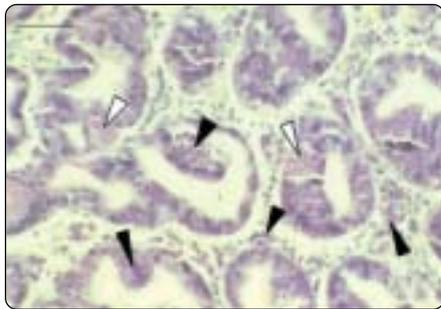


Fig.M.6.3.1.2b. Plasmodia (black arrows) and spores (white arrows) of *Haplosporidium nelsoni*, the cause of MSX disease, throughout the connective tissue and digestive tubules of an American oyster (*Crassostrea virginica*). Scale bar 100 μ m.

(SE McGladdery)

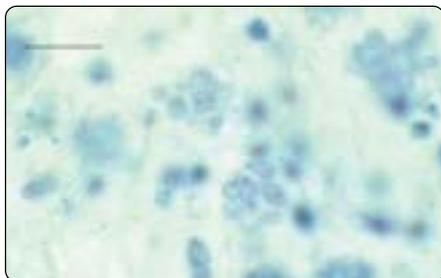


Fig.M.6.4.2.2a. Oil immersion magnification of SSO spores in the connective tissue of an American oyster *Crassostrea virginica*. Scale bar 15 μ m.

techniques used are the same as described for confirmatory diagnosis (M.6.4.2.2).

M.6.4 Diagnostic Methods

More detailed methods for diagnostics can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at <http://www.oie.int> or selected references.

M.6.4.1 Presumptive

M.6.4.1.1 Gross Observations (Level I)

The only presumptive diagnosis would be gross observations of cumulative mortalities of American oysters in early spring and late summer in areas (12-25 ppt salinity) with an established history of MSX epizootics. Such presumptive diagnosis requires confirmation via another diagnostic technique (histology). Likewise, summer mortalities of the same oyster species in waters with a history of SSO disease may be presumed to be due to SSO. Both must be confirmed, however, since infection distributions for both species of *Haplosporidium* may overlap.

M.6.4.2 Confirmatory

M.6.4.2.1 Cytological Examination and Tissue Imprints (Level II)

Positive cytological or tissue imprints (M.6.4.1.1) can be considered confirmatory where collected from susceptible oyster species and areas with a historic record of the presence of *Haplosporidium* spp. infections.

M.6 Haplosporidiosis (*Haplosporidium costale*, *H. nelsoni*)

M.6.4.2.2 Histopathology (Level II)

Positive histological sections can be considered confirmatory where collected from susceptible oyster species and areas with a historic record of the presence of *Haplosporidium* spp. infections.

It is recommended that at least two dorso-ventral sections through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F. Davidson's or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.6.4.2.3), or species identification, if required. Several standard stains (e.g., haematoxylin-eosin) enable detection of *Haplosporidium* spp..

Haplosporidium spp. infections are usually systemic and characterised by massive infiltration by hyalinocyte haemocytes (agranular haemocytes with a low cytoplasm: nucleoplasm ratio). The sporoplasm of spores of *H. costale* which are smaller than those of MSX and often masked by the intense haemocyte infiltration response, can be differentially stained using a modified Ziehl-Nielsen stain. Sporocysts of *H. costale* occur in the connective tissues (Fig.M.6.3.1.2a), measure approximately 10-25 µm in diameter and contain oval, operculate, spores approximately 3 µm in size (Fig.M.6.4.2.2a). Sporocysts of *H. nelsoni* occur in the digestive tubule epithelia and measure 20-50 µm in diameter. The operculate spores of MSX measure 4-6 x 5-8 µm (Fig.M.6.4.2.2b). In *C. gigas* spores may also occur in other tissues. Older foci of infection in both oyster species may be surrounded by haemocytes and necrotic tissue debris. A similar infectious agent occurs in the pearl oyster *Pinctada maxima* in north Western Australia (Fig.M.6.1.3a,b). The spore size of this *Haplosporidium* resembles *H. nelsoni* but differs from MSX infections in both *C. virginica* and *C. gigas* by being found exclusively in the connective tissue.

The plasmodial stages of both *H. costale* and *H. nelsoni* are as described under M.6.3.1.2.

M.6.4.2.3 Transmission Electron Microscopy (TEM) (Level III)

TEM is required for confirmation of species-specific ultrastructure of the spores - especially in areas enzootic for both disease agents. Tissues are fixed in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Oyster tissues can also be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse tissues in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO₄ = osmic acid - highly toxic). Secondary fixation should be complete within 1 hr. The OsO₄ fixative must also be rinsed with buffer/filtered (0.22 µm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of *Haplosporidium* plasmodia or spores.

M.6.4.2.4 In situ Hybridization (Level III)

DNA-probes for both species of *Haplosporidium* have been produced at the Virginia Institute of Marine Science (VIMS), College of William and Mary, Gloucester, Virginia, USA. These are not yet commercially available, but labelled probes may be obtained for experienced users, or samples may be sent to VIMS¹ for *in situ* hybridization analysis.

M.6.5 Modes of Transmission

Neither parasite has been successfully transmitted under laboratory conditions and one (or more) intermediate host(s) is/are suspected.

M.6.6 Control Measures

None are known for *Haplosporidium* spp.. Most efforts have concentrated on development of resistant stocks of oysters. These currently show potential for survival in enzootic areas, but are not recommended for use in non-enzootic areas due to their potential as sub-clinical carriers of the pathogen. Some success has also been achieved in preventing infection of hatchery-reared larval and juvenile oysters through filtration and UV radiation of influent water.

¹ Attention Dr. N. Stokes, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062, USA. (E-mail: stokes@vims.edu).

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M.7 MARTEILIOIDOSIS (MARTEILIOIDES CHUNGMUENSIS, M. BRANCHIALIS)

M.7.1 Background Information

M.7.1.1 Causative Agents

Marteiloidosis is caused by two species of parasites, belonging to the protistan Phylum Paramyxea. *Marteiloides chungmuensis* is responsible for oocyte infections of Pacific oysters (*Crassostrea gigas*) and *Marteiloides branchialis* infects the gills of *Saccostrea glomerata* (syn. *Crassostrea commercialis*, *Saccostrea commercialis*).

M.7.1.2 Host Range

The Pacific oyster *Crassostrea gigas* is infected by *Marteiloides chungmuensis*. *Marteiloides branchialis* infects the Sydney rock oyster, *Saccostrea commercialis*.

M.7.1.3 Geographic Distribution

Marteiloides chungmuensis infects *C. gigas* in Japan and Korea. *Marteiloides branchialis* is found in Australia (New South Wales).

M.7.2 Clinical Aspects

Marteiloides chungmuensis infects the cytoplasm of mature oocytes and significant proportions of the reproductive output of a female oyster can be affected. The infected eggs are released or retained within the follicle, leading to visible distention of the mantle surface (Fig.M.7.2a, b). Prevalences of up to 8.3% have been reported from Korea. *Marteiloides branchialis* causes focal lesions on the gill lamellae and, in conjunction with infections by *Marteilia sydneyi* (M.3), is associated with significant mortalities of Sydney rock oysters being cultured in trays during the autumn.

M.7.3 Screening Methods

M.7.3.1 Presumptive

M.7.3.1.1 Gross Observations (Level I)

Marteiloides branchialis causes focal patches (1-2 mm in diameter) of discolouration and swelling on the gill lamellae. The presence of such lesions in Sydney rock oysters in the Austral autumn should be treated as presumptive Marteilioidosis.

M.7.3.2 Confirmatory

M.7.3.2.1 Histopathology (Level II)

The techniques used are the same as described for confirmatory disease diagnosis (M.7.4.2.1). Presence of histological inclusions, as described under M.7.4.2.1, can be considered confirmatory for *Marteiloides* spp., during screening.

(MS Park and DL Choi)

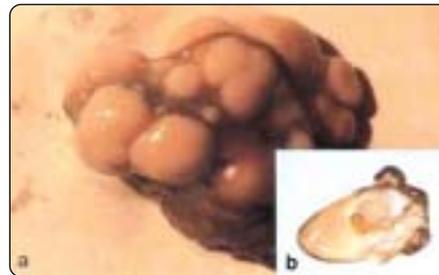


Fig.M.7.2a,b. a. Gross deformation of mantle tissues of Pacific oyster (*Crassostrea gigas*) from Korea, due to infection by the protistan parasite *Marteiloides chungmuensis* causing retention of the infected ova within the ovary and gonoducts; b. (insert) normal mantle tissues of a Pacific oyster.

(MS Park)

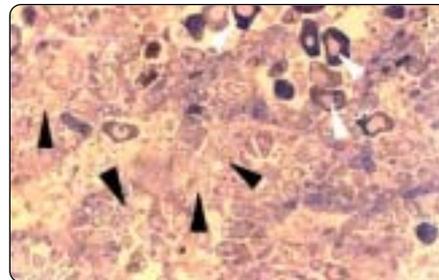


Fig.M.7.4.2.1. Histological section through the ovary of a Pacific oyster (*Crassostrea gigas*) with normal ova (white arrows) and ova severely infected by the protistan parasite *Marteiloides chungmuensis* (black arrows). Scale bar 100 μ m.

M.7.4 Diagnostic Methods

M.7.4.1 Presumptive

M.7.4.1.1 Gross Observations (Level I)

As for M.7.3.1.1, focal patches (1-2 mm in diameter) of discolouration and swelling on the gill lamellae of Sydney rock oysters in the Austral autumn can be treated as presumptive positives for *M. branchialis*.

M.7 Marteilioidosis (*Marteilioides chungmuensis*, *M. branchialis*)

M.7.4.1.2 Histopathology (Level II)

For first-time diagnoses a back up tissue specimen fixed for EM is recommended (M.7.4.2.3).

M.7.4.2 Confirmatory

M.7.4.2.1 Histopathology (Level II)

Positive histological sections can be considered confirmatory where collected from susceptible oyster species and areas with a historic record of the presence of *Marteilioides* spp. infections.

It is recommended that at least two dorso-ventral sections through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F, Davidsons or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.6.4.2.3), or species identification, if required. Several standard stains (e.g., haematoxylin-eosin) enable detection of *Marteilioides* spp..

Marteilioides chungmuensis is located in the cytoplasm of infected ova (Fig.M.7.4.2.1). Stem (primary) cells contain secondary cells. These may, in turn, contain developing sporonts, giving rise to a single tertiary cell by endogenous budding. Each tertiary cell forms a tricellular spore by internal cleavage.

Marteilioides branchialis causes epithelial hyperplasia and granulocyte infiltration at the site of infection. Uninucleate primary cells contain two to six secondary cells (some may contain up to 12) in the cytoplasm of epithelial cells, connective tissue cells and occasionally the infiltrating haemocytes within the lesion.

M.7.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

TEM is required for confirmation of species-specific ultrastructure of these parasites. Tissues are fixed in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Tissues can also be fixed in 1G4F for 12-24 hours. Following primary fixation, rinse in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO₄ = osmic acid - highly toxic). Secondary fixation should be complete within 1 hour. The OsO₄ fixative must also be rinsed with buffer/filtered (0.22 microns) seawater prior to dehydration and resin-embedding.

Post-fixed tissues should be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of putative *Marteilioides* spp. Ultrathin sections are then mounted on copper grids (with or without formvar reinforced support) for staining with lead citrate + uranyl acetate or equivalent EM stain.

Marteilioides branchialis is differentiated from the other *Marteilioides* spp. by the presence of two concentric cells (rather than three) within the spore. In addition *M. chungmuensis* in *C. gigas* contains only two to three sporonts per primary/stem cell compared with two-six (or up to 12) for *M. branchialis*. Multivesicular bodies resembling those of *Marteilia* spp. are present in *M. branchialis* stem cells, but absent from those of *M. chungmuensis*.

M.7.5 Modes of Transmission

Unknown.

M.7.6 Control Measures

None known.

M.7.7 Selected References

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- Anderson, T.J., T.F. McCaul, V. Boulo, J.A.F. Robledo, and R.J.G. Lester. 1994. Light and electron immunohistochemical assays on paramyxea parasites. *Aquat. Living Res.* 7(1): 47-52.
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- Comps, M., M.S. Park, and I. Desportes. 1986. Etude ultrastructurale de *Marteilioides chungmuensis* n.g. n.sp., parasite des ovocytes de l'huître *Crassostrea gigas* Th. *Protistol.* 22(3): 279-285.

M.7 Marteilioidosis (*Marteilioides chungmuensis*, *M. branchialis*)

Comps, M., M.S. Park, and I. Desportes. 1987. Fine structure of *Marteilioides chungmuensis* n.g. n.sp., parasite of the oocytes of the oyster *Crassostrea gigas*. *Aquac.* 67(1-2): 264-265.

Hine, P.M. and T. Thorne. 2000. A survey of some parasites and diseases of several species of bivalve mollusc in northern Western Australia. *Dis. Aquat. Org.* 40(1): 67-8.

M.8 IRIDOVIROSIS (OYSTER VELAR VIRUS DISEASE)

M.8.1 Background Information

M.8.1.1 Causative Agents

Oyster Velar Virus Disease (OVVD) (Iridovirus) is caused by an icosahedral DNA virus with morphological similarities to the Iridoviridae.

M.8.1.2 Host Range

Crassostrea gigas (Pacific oyster) larvae are the documented host species, although similar viral agents have been associated with gill disease ("Maladie des Branchies") and haemocyte infections in Portuguese oysters (*Crassostrea angulata*) and *C. gigas*.

M.8.1.3 Geographic Distribution

Infections have been reported solely from two hatcheries in Washington State, but are believed to have a ubiquitous distribution throughout juvenile *C. gigas* production, with clinical manifestation only under sub-optimal growing conditions.

M.8.2 Clinical Aspects

OVVD causes sloughing of the velar epithelium of larvae >150µm in length, and can cause up to 100% mortality under hatchery conditions. The larvae can not feed, weaken and die.

M.8.3 Screening Methods

M.8.3.1 Presumptive

Generally-speaking, since this is an opportunistic infection, only clinical infections will demonstrate detectable infections – as described under M.8.4.

M.8.3.1.1 Wet Mounts (Level I)

Wet mounts of veliger larvae which demonstrate sloughing of ciliated epithelial surfaces can be considered to be suspect for OVVD. As with gross observations, other opportunistic pathogens (bacteria and *Herpes*-like viruses) may be involved, so Level II/III diagnostics are required.

M.8.3.1.2 Histopathology (Level II)

Using the techniques described under M.8.4.2.1, detection of the features described in that section can be considered to be presumptively positive for OVVD. Such inclusions require TEM (Level III) (M.8.4.2.2) for confirmatory diagnosis, at least for first time observations.

M.8.3.2 Confirmatory

M.8.3.2.1 Transmission Electron Microscopy (Level III)

As described under M.8.4.2.2.

M.8.4 Diagnostic Methods

M.8.4.1 Presumptive

M.8.4.1.1 Gross Observations (Level I)

Slowed growth, cessation of feeding and swimming in larval *Crassostrea gigas* should be considered suspect for OVVD. Gross signs are not pathogen specific and require Level II examination (M.8.4.2), at least for first time observations.

M.8.4.1.2 Wet Mounts (Level I)

As described under M.8.3.1.1. For first-time diagnoses a back up tissue specimen fixed for TEM is recommended (M.8.4.2.2).

M.8.4.1.3 Histopathology (Level II)

As described under M.8.4.2.1.

M.8.4.1.4 Transmission Electron Microscopy (Level III)

As described under M.8.4.2.2.

M.8.4.2 Confirmatory

M.8.4.2.1 Histopathology (Level II)

Where larvae have a history of OVVD, detection of inclusions and ciliated epithelial pathology, as described below, can be considered confirmatory for the disease. However, it should be noted that other microbial infections can induce similar histopathology and electron microscopy is the ideal confirmatory technique (M.8.4.2.2).

Larvae must be concentrated by centrifugation or filtration into a pellet prior to embedding. This is best achieved post-fixation in Davidson's, 1G4F or other fixative. Although paraffin embedding is possible, resin embedding is recommended for optimal sectioning. Paraffin permits sectioning down to 3 µm using standard microtome. Resin embedded tissue can be sectioned down to 1 µm thick, but requires specialised microtomes and/or block holders and specialised staining.

M.8 Iridovirus (Oyster Velar Virus Disease)

Standard stains (e.g., haematoxylin-eosin) will detect intracytoplasmic inclusion bodies in ciliated velar epithelial cells. Early inclusion bodies are spherical, but become more irregular as the viruses proliferate. Inclusion bodies may also be detected in oesophageal and oral epithelia or, more rarely, in mantle epithelial cells.

M.8.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

TEM is required to visualise the causative viruses *in situ* in gill tissue sections of concentrated 'pellets' of larvae. Fixation in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater should not exceed 1 hour to reduce artifacts. Tissues can also be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - highly toxic). Secondary fixation should be complete within 1 hr. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 μm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues should be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the best specimens for ultrathin sectioning. Ultrathin sections are mounted on copper grids (with or without formvar reinforced support) for staining with lead citrate + uranyl acetate or equivalent EM stain.

Icosahedral viral particles (228 +/- 7 nm in diameter) with a bi-laminar membrane capsid should be evident to confirm Iridoviral involvement.

M.8.5 Modes of Transmission

The disease appeared in March-May at affected hatcheries. Direct transmission between moribund and uninfected larvae is suspected.

M.8.6 Control Measures

None known except for reduced stocking densities, improved water exchange and general hatchery sanitation methods (tank and line disinfection, etc.).

M.8.7 Selected References

Comps, M. and N. Cochenec. 1993. A Herpes-like virus from the European oyster *Ostrea edulis* L. *J. Inverteb. Pathol.* 62: 201-203.

Elston, R.A. 1979. Virus-like particles associated with lesions in larval Pacific oysters (*Crassostrea gigas*). *J. Inverteb. Pathol.* 33: 71-74.

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LeDeuff, R.M., T. Renault and A. G?rard. 1996. Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*. *Dis. Aquat. Org.* 24: 149-157.

Meyers, T.R. 1981. Endemic diseases of cultured shellfish of Long Island, New York: adult and juvenile American oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*). *Aquac.* 22: 305-330.

Nicolas, J.L., M. Comps and N. Cochenec. 1992. Herpes-like virus infecting Pacific oyster larvae, *Crassostrea gigas*. *Bull. Eur. Assoc. Fish Pathol.* 12: 11-13.

Renault, T., N. Cochenec, R.M. Le Deuff and B. Chollet. 1994. Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. *Bull. Eur. Assoc. Fish Pathol.* 14: 64 -66.

Annex M.AI. OIE Reference Laboratory for Molluscan Diseases

Disease	Expert/Laboratory
Mollusc pathogens	Dr. F. Berth IFREMER Laboratoire de Genetique Aquaculture et Pathologie BP 133, 17390 La Tremblade FRANCE Tel: 33(0)5 46.36.98.36 Fax: 33 (0)5 46.36.37.51 E-mail: fberthe@ifremer.fr

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	<p>Dr. Robert D. Adlard Queensland Museum PO Box 3300, South Brisbane, Queensland 4101 AUSTRALIA Tel: +61 7 38407723 Fax: +61 7 38461226 E-mail: RobertAd@qm.qld.gov.au; http://www.qmuseum.qld.gov.au</p>
Marteiliosis	<p>Dr. Sarah N. Kleeman Aquatic Animal Biosecurity Animal Biosecurity GPO Box 858 Canberra ACT 2601 AUSTRALIA Tel: +61 2 6272 3024 Fax: +61 2 6272 3399 E-mail: sarah.kleeman@affa.gov.au</p>
Molluscan Diseases	<p>Professor R.J.G. Lester Department of Microbiology and Parasitology The University of Queensland, Brisbane AUSTRALIA 4072. Tel: +61-7-3365-3305, Fax: +61-7-3365-4620 E-mail: R.Lester@mailbox.uq.edu.au http://www.biosci.uq.edu.au/micro/academic/lesterlester.htm</p>
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¹ The experts included in this list has previously been consulted and agreed to provide valuable information and health advice concerning their particular expertise.

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	<p>Dr. Sharon E. McGladdery Oceans and Aquaculture Science 200 Kent Street (8W160) Ottawa, Ontario, K1A 0E6 CANADA Tel: 613-991-6855 Fax: 613-954-0807 E-mail: mcgladderys@dfo-mpo.gc.ca</p>

² These experts outside the Asia-Pacific region has supported the regional programme on aquatic animal health and agreed to assist further in providing valuable information and health advise on molluscan diseases.

Annex M.AIII. List of Useful Diagnostic Manuals/ Guides/Keys to Molluscan Diseases

- **Australian Aquatic Animal Disease – Identification Field Guide (1999) by Alistair Herfort and Grant Rawlin**

Information: AFFA Shopfront – Agriculture, Fisheries and Forestry – Australia
GPO Box 858, Canberra, ACT 2601
Tel: (02) 6272 5550 or free call: 1800 020 157
Fax: (02) 6272 5771
E-mail: shopfront@affa.gov.au

- **Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish by Bower, SE McGladdery and IM Price (1994)**

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- **Mollusc Diseases: Guide for the Shellfish Farmer. 1990. by R.A. Elston. Washington Sea Grant Program, University of Washington Press, Seattle. 73 pp.**

- **A Manual of the Parasites, Pests and Diseases of Canadian Atlantic Bivalves. 1993. by SE McGladdery, RE Drinnan and MF Stephenson.**

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