

5.2.9 Marteiliasis and Marteilioidiasis of Shellfish

Ted Meyers

Alaska Department of Fish and Game
Commercial Fisheries Division
Juneau Fish Pathology Laboratory
P.O. Box 25526
Juneau, AK 99802-5526
(907) 465-3577

A. Name of Disease and Etiological Agent

The disease in shellfish known as Marteiliasis may result from infection of the upper digestive tract (rarely gills) by protozoan parasites in the phylum Paramyxea, class Marteiliidae of the genus *Marteilia*. Related parasites of the same class in the genus *Marteilioides* infect gills and oocytes. The type species is *Marteilia refringens* causing Abers Disease and “maladie de la glande digestive” in the European flat oyster *Ostrea edulis*. *Marteilia maurini* (Comps et al. 1982) was suggested to be a junior synonym of *M. refringens* based on the lack of significantly different ultrastructural morphologies (Longshaw, et al. 2001). Although this continues to make differentiation difficult, *maurini* has been validated by sequencing of the rRNA gene cluster (Berthe et al. 2000, Le Roux et al. 2001, Zrncic et al. 2001). *Marteilia sydneyi* is responsible for QX Disease in Australian Sydney rock oysters *Saccostrea commercialis (glomerata)*.

B. Known Geographic Range and Host Species of the Disease

Table 1. List of parasites, hosts and geographic ranges

<u>Agent</u>	<u>Host</u>	<u>Range</u>
<i>Marteilia refringens</i> (<i>maurini</i>)	<i>Ostrea edulis</i> , <i>O. angasi</i> , <i>Mytilus edulis</i> , <i>M. galloprovincialis</i> , <i>Cardium eduli</i> , <i>Tiostrea chilensis</i> ,	W. Brittany, France to N. Mediter. Sea
<i>Marteilia sydneyi</i>	<i>Saccostrea commercialis (glomerata)</i> <i>Saccostrea echinata</i>	Australia
<i>Marteilia lengehi</i>	<i>Saccostrea cucullata</i>	Persian Gulf Australia
<i>Marteilia christenseni</i>	<i>Scrobicularia piperata</i>	France (Ronces- les-Bains)

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Table 1 (contd)

<u>Agent</u>	<u>Host</u>	<u>Range</u>
<i>Marteilia</i> sp.	<i>Crassostrea gigas</i> , <i>C. virginica</i> <i>Ostrea puelchana</i> <i>Argopecten gibbus</i> <i>Tridacna crocea(maxima)</i>	France Florida, USA Fiji
<i>Marteilioides branchialis</i>	<i>Saccostrea commercialis</i>	Australia
<i>Marteilioides chungmuensis</i>	<i>Crassostrea gigas</i>	Korea, Japan
<i>Marteilioides-like</i>	<i>Venerupis philippinarum</i>	S. coast Korea (Lee et al. 2001)

C. Epizootiology

1. *Marteilia refringens*: “Maladie des Abers” was first described from Bretagne (Brittany), France in the European flat oyster *Ostrea edulis* in 1967, causing losses of up to 90% annually. The causative agent was first described by Comps (1970) from oysters held in “claires” in Marennes-Oleron Bay while Grizel et al. (1974) described the organism in more detail and named it *Marteilia refringens*. Epizootics generally have begun in May, peaking in June to August and gradually diminishing by December and January, leaving an eclipse period of March to April when the parasite cannot be found. In colder waters to the north, subclinical infestations have been observed throughout the winter. Observations have shown that old mature sporangia, present from May or June onward, are eliminated by the end of January and that young plasmodia persist during the winter to re-initiate new infestations during the following May. Transplantation experiments indicated that new infestations occur from May to August. Interestingly, oysters may become parasitized without the occurrence of disease and epizootic mortality that may be regulated, in part, by environmental conditions such as water temperature and high salinities. Increasing water temperatures in spring favor development of the organism while increasing salinities and water renewal may inhibit development and transmission. Nonetheless, these parameters alone cannot explain the apparent differences in virulence of this parasite for oysters in French and Dutch waters (van Banning 1979 a, b) and the reported geographic range of the parasite from as far north as Western Brittany, France to as far south as the northern Mediterranean Sea. Control of the disease has included: restriction of oyster transfers from infested areas; delay or reduction of oyster out-planting during May to August when disease transmission is high; and grow-out of oysters in areas with high salinities (35-37 ppt) to retard parasite development.

Experiments using cohabitation and inoculation have shown no strong evidence for direct transmission of the parasite. Instead, the parasite’s life cycle is likely complex, involving one or more intermediate hosts. Most recently, the calanoid copepod *Paracartia grani*

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Sars was found to be a host for *M. refringens* in French claires (Audemard et al. 2004). In *Ostrea edulis*, early stages of the organism are usually observed in the palps and stomach and less frequently in the gills. Sporulation of the parasite occurs in the digestive gland epithelium, generally from May to September. The sporulation process involves internal cleavage within a stem cell producing spores consisting of several cells enclosed inside one another, typical of paramyxean parasites. Specifically, the inner cell of a 2-celled plasmodium divides to form 8 sporangia (sporonts) within a sporangiosorus. Within each sporont, spore primordia divide internally to form 3-4 spores each containing 3 concentric cells (Roubal et al. 1989). Polymorphic birefringent inclusion bodies appear in the extraspore cytoplasm and become a dominant feature of the sporangia when viewed by light optics, hence the species name. Sporangia are discharged as propagules through the gut with fecal material. More detailed morphological and life-cycle descriptions of the *Marteilia* are provided by Lauckner (1983).

2. *Marteilia sydneyi*: QX Disease was first reported from Sydney rock oysters *Saccostrea commercialis (glomerata)* in Moreton Bay, Queensland, Australia by Wolf (1972). The causative agent was later identified as a *Marteilia* and named *M. sydneyi* by Perkins and Wolf (1976). QX Disease has caused oyster losses of up to 80% from southern Queensland and northern New Wales, Australia. The incubation period for the parasite is less than 60 days from early infestation to oyster death and development is favored by lower salinities. The developmental cycle and target tissues are similar to *M. refringens* but the disease is not seasonal whereby mortalities can occur anytime of the year. There also are morphological differences described in the diagnostic section E. The disease is limited to the subtropical and tropical waters of Australia and apparently does not occur in the colder waters south of Richmond River. Control measures used to avoid infestation or reduce pathogen loads include: growout of oysters at higher levels in the intertidal zone; delayed or reduced out-planting of oysters in areas of risk during the austral summer months of January to March; grow-out of juvenile oysters in high salinity waters until after the high risk period of infestation is past and harvest of larger oysters by late December before the beginning of the transmission period.

3. *Marteilia sp.* of Calico Scallops: In December of 1988, mass mortality of nearly 100% in a calico scallop *Argopectin gibbus* population occurring over a 2,500 square mile area of the eastern coast of Florida, USA was attributed to a *Marteilia sp.* resembling *M. refringens* (Moyer et al. 1993). This mortality resulted in the loss of 11-40 million pounds of adductor muscle meats and the population had still not recovered to harvestable quantities by the spring of 1992 when last reported (Bower et al. 1994). The parasite apparently filled the tubules of the digestive gland causing dysfunction and starvation followed by rapid mortality within a one month period.

4. Other *Marteilia*: Plasmodia 8-15 μm in diameter and presporulation stages of *M. lengehi* were reported in the stomach epithelium and the digestive gland of *Saccostrea cucullata* in the Persian Gulf (Comps 1976). Similar stages were also reported from the same oyster species in Australia (Hine and Thorne 2000). However, these can only be tentative identifications as mature spores were not found. *Marteilia christensenii* from *Scrobicularia piperata* apparently can be differentiated from other *Marteilia* by the

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characteristics of the cytoplasmic contents of sporangia and the morphology of the spore (Comps 1985). Nonetheless, the taxonomic validity of these *Marteilia* species will remain questionable until more investigations are completed. *Marteilia* sp. has also been described from the Argentina oyster (*Ostrea puelchana*) at French farming sites (Pascual et al. 1991).

5. *Marteilioides branchialis*: This organism, described by Anderson and Lester (1992), parasitizes the gills of Australian Sydney rock oysters *Saccostrea commercialis* that are often infested with *Marteilia sydneyi*. The resulting gill damage can cause significant losses during autumn. There are no known methods of control except for restriction of host transfer from areas where the parasite is known to occur.

6. *Marteilioides chungmuensis*: This organism, described by Comps et al. (1986, 1987), parasitizes the oocytes of Korean and Japanese Pacific oysters *Crassostrea gigas* with prevalences up to 16.3% reported in December from Gosung Bay, Korea (Ngo et al. 2003). Parasites could be found in developing and mature ova from June to January but intensity was highest in June when water temperatures were high. Parasitized ova were not observed from February to May. Because this parasite causes spawning failure and unacceptable market quality, serious economic losses to oyster farmers have resulted. There is no known control measure except to restrict transport of oysters from enzootic areas.

D. Disease Signs

1. *Marteilia refringens/maurini*:

Oysters - Parasitized oysters may appear normal but epizootic disease is characterized by glycogen depletion and tissue necrosis of the digestive gland with clinical signs of poor growth, brown to pale yellow discoloration of the digestive gland, loss of pigmentation and mucoid atrophy of the visceral mass, gaping valves followed by mortality, generally from sporulation of the parasite in the digestive tubule epithelium.

Mussels – No mortality in mussel populations has been reported that is attributable to *Marteilia* infestation. Disease signs in parasitized mussels are not described except for an overall lower condition factor and suspected disruption of the digestive gland epithelium in *M. edulis* and inhibition of gonadal development in *M. galloprovincialis*.

2. *Marteilia sydneyi*: Parasitized oysters appear similar to those infested with *M. refringens*. Glycogen depletion and tissue necrosis result in a pale brown to yellow digestive gland instead of the green color in healthy animals; an emaciated, shrunken and translucent visceral mass with complete resorption of the gonad may be visible. Mortality from starvation generally occurs in less than 60 days from initial infestation.

3. *Marteilia* sp.: Giant clam (*Tridacna crocea/maxima*) – Parasitized clams had chalky white multiple foci observed throughout a dark red-brown kidney (Norton et al. 1993).

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4. *Marteilioides branchialis* – Parasitized oysters have focal lesions in the gills
5. *Marteilioides chungmuensis* – Parasitized oysters have focal nodules in the ovaries during spawning season resulting in unacceptable appearance and marketability (Fig. I-A).

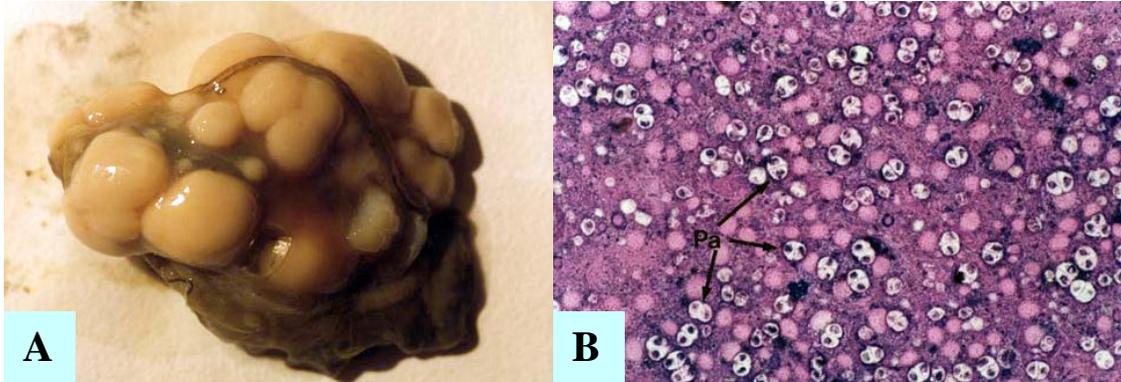


Fig. I A and B. A shows the gross appearance of a Pacific oyster infected with *Marteilioides chungmuensis*. B shows the stained appearance of the parasite in infected ova (example parasites are indicated by Pa). B, 200x, eosin, methylene blue stained wet impression smear. From Park et al. 2003. Provided courtesy of M.S. Park for use in the BlueBook.

E. Disease Diagnostic Procedures

Table 2. Marteiliasis/Marteilioidiasis - surveillance, detection and diagnostic methods: BF = bright field; LM = light microscopy; EM = electron microscopy; IFAT= indirect fluorescent antibody test; PCR = polymerase chain reaction

Methods	Screening		Presumptive	Confirmatory
	Juveniles	Adults		
Gross signs	-	-	+	-
Direct BF/LM	++	++	+++	++
Histopathology	+++	+++	+++	++
Bioassay	-	-	-	-
Transmission EM	++	++	+++	+++
IFAT^a	+	+	+	+
PCR^b	+++	+++	+++	+++
DNA probe-<i>in situ</i>^c	+++	+++	+++	+++

^a IFAT has been used only for *Marteilia sydneyi* and reagents are limited in availability

^b PCR reagents available for differentiation between *Marteilia refringens* and *M. maurini* and specific identification of *M. sydneyi*

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^c In situ hybridization available for *Marteilia sp.*, *M. refringens* and *M. sydneyi*

- = the method is presently unavailable or unsuitable
- + = the method has application in some situations, but cost, accuracy, or other factors severely limits its usefulness
- ++ = the method is a standard method with good diagnostic sensitivity and specificity

- +++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

1. Brightfield light microscopy: Presumptive diagnosis for *Marteilia* species can be made from smears of cut sections from fresh digestive gland tissue that has been blotted dry. The smears are dried and fixed in methanol for 2-3 minutes and stained according to instructions from any of several commercially available Giemsa or methylene blue-based staining kits (example; Dif-Quik). After staining, the rinsed and dried smears can be cover-slipped with resin and/or examined directly with oil for plasmodia of 5-8 μm in length during the early stages of the disease that can reach 40 μm in diameter during sporulation. The parasite cell cytoplasm stains variably basophilic with a bright red nucleus. The secondary cells or sporoblasts are surrounded by a bright halo.

2. Histopathology: Confirmative diagnoses for the genera *Marteilia* and *Martelioides*, based on internal cleavage to produce cells within cells, can be made by histopathological examination of stained sections of paraffin or resin-embedded bivalve tissues using standard histological fixatives (seawater Davidson's or Carson's solutions or 10% formalin in filtered seawater), procedures and stains (ie, hematoxylin-eosin, trichrome, etc.).

1. *Marteilia refringens*- sections of palps (rarely gills), stomach and digestive gland. The younger vegetative plasmodia may be present within the epithelium of the stomach, in the connective tissues surrounding the upper intestinal tract, between the epithelial cells or within lumens of these organs. Maturing presporangia or sporangial primordia are found in the epithelium of the digestive tubules, each of which develops into a sporangiosorus of up to 30 μm in diameter containing as many as 8 sporangia. Spore formation within sporangia or sporulation results in the formation of haplosporosomes in the cytoplasm peripheral to the spores that condenses into birefringent inclusion bodies visible by light microscopy. Spores are about 2.6 to 3.5 μm in diameter. A most important overall diagnostic feature is that the phylum Paramyxia (*Marteilia* and *Martelioides*) is distinguished from all other protozoans in shellfish by this unique morphology of internal cleavage to produce cells within cells during sporulation.

2. *Marteilia sydneyi*: - sections of digestive gland. *Marteilia sydneyi* can be distinguished from *M. refringens* by the following histological characteristics: 1) lack of striated inclusions in the plasmodia; 2) the formation of 8-16 sporangial primordia in each plasmodium instead of only 8; 3) the occurrence of 2 or rarely 3 rather than 4 spores in each sporangium and; 4) a heavy layer of concentric membranes surrounding mature spores that are lacking in spores of *M. refringens* (Bower et al. 1994).

3. Other *Marteilia sp* – little information is available to morphologically separate other *Marteilia sp* from *M. refringens* and *M. sydneyi*.

4. *Marteilioides branchialis* – sections of gills. Epithelial hyperplasia and focal inflammation surrounding parasite cells consisting of uninucleate primary cells containing 2-6, and rarely up to 12, secondary cells in the cytoplasm of epithelium, connective tissue cells, and occasionally hemocytes in the lesion.

5. *Marteilioides chungmuensis* – sections of ovary. The cytoplasm of parasitized ova and oocytes contain primary parasite cells that give rise to secondary cells that may have developing sporonts each producing one tertiary cell by endogenous budding. Each tertiary cell forms one tricellular spore by internal cleavage. The parasites can also be visualized in impression smears, which can be stained with eosin-methylene blue to differentiate the parasites (Fig. I B).

3. Transmission Electron Microscopy (TEM): Generally, histopathological characteristics are sufficient for confirmation of Paramyxia and for distinguishing *Marteilia refringens* from *M. sydneyi* and identification of *Marteilioides branchialis* and *chungmuensis*. If histological methods are unable to define morphological details described in section E-2 then standard TEM procedures for fixation (4% glutaraldehyde or 10% formalin in seawater), embedding, cutting and staining (uranyl acetate and lead citrate) are used to observe ultrastructural characteristics for confirmation of parasite species. Ultrastructural confirmation for *M. branchialis* may be necessary to determine that the spore contains two concentric cells rather than three and has 2-6, and rarely 12, sporonts per stem cell in comparison to only 2 or 3 for *M. chungmuensis*. Also, multivesicular bodies similar to those of *Marteilia sp.* occur in primary cells of *M. branchialis* but not *M. chungmuensis*.

4. IFAT: IFAT, as described by Roubal et al. (1989), has been used for *M. sydneyi* but is not yet a standard procedure.

5. PCR: PCR-restriction fragment length polymorphism is used for discrimination of *M. refringens/M. maurini* using methods described by Le Roux et al. (2001). Specific detection of *M. sydneyi* by PCR is described by Kleeman and Adlard (2000), and Kleeman et al. (2002a, 2002b).

1. Infected animals are frozen at -80°C and ground to powder for DNA extraction.
2. 10 volumes of extraction buffer at pH 8 (100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate) are added with 100 $\mu\text{g/ml}$ proteinase K for overnight incubation at 50°C .
3. DNA is extracted using a standard phenol/chloroform procedure and precipitated with ethanol.
4. A 50 μl volume of extracted DNA is denatured for 5 min at 94°C followed by 30 cycles of: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and

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elongation at 72°C for 1 min per kilo-base pair. This is followed by a final elongation step of 10 min at 72°C.

5. *M. refringens* is detected with primers (4 and 5) that target the internal transcribed spacer ITS1

4: 5'-CCG-CAC-ACG-TTC-TTC-ACT-CC-3'

5: 5'-CTC-GCG-AGT-TTC-GAC-AGA-CG-3'

6. *M. sydneyi* is detected with primers that target the ITS:

LEG1: 5'-CGA-TCT-GTG-TAG-TCG-GAT- TCC-GA-3'

PRO2: 5'-TCA-AGG-GAC-ATC-CAA-CGG-TC-3'

7. The restriction enzyme *Hha1* tests for cleavage to identify polymorphism among the PCR products that differentiate *M. refringens* from *M. maurini*. The restriction fragments for *M. maurini* produce an electrophoretic profile on 2% agarose gel corresponding to three bands of 156, 157 and 68 bp. The profile for *M. refringens* consistst of two bands of 226 and 156 bp.

The profile of *M. sydneyi* consists of a single fragment of 195 bp.

6. In-situ Hybridization (ISH): *In-situ* hybridization methods have been described for *M. refringens* by Mialhe et al (1995) and Le Roux et al. (1999). The latter investigators describe a probe specific for the genus *Marteilia* detecting both *M. refringens* and *M. sydneyi* while a probe specific for *M. sydneyi* targeting the ITS (internal transcriber spacer) is described by Kleeman and Adlard (2000) and Kleeman et al. (2002b).

1. The Smart 2 probe is used to detect *Marteilia* at the genus level and is obtained by PCR (section E-5) using the primers:

SS2: 5'-CCG-GTG-CCA-GGT-ATA-TCT-CG-3'

SAS1 : 5'-TTC-GGG-TGG-TCT-TGA-AAG-GC-3'

The PCR reaction is performed from *M. refringens* purified from *Ostrea edulis* DNA except the 1 µl of 25 mM digoxigenin-dUTP (DIG-dUTP) is added to the mix.

2. The ITS1 probe using the PRO2 and LEG1 primers (section E-5) is used to specifically detect *M. sydneyi* and is obtained by performing PCR from *M. sydneyi* purified from *Saccostrea glomerata* DNA, except the 1 µl of 25 mM DIG-dUTP is added to the mix.
3. In-situ hybridization including positive and negative controls:

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- a. The visceral mass of the sample mollusc, after fixation in Davidson's AFA (10% glycerine, 20% formalin, 30% of 95% ethanol, 30% distilled water and 10% glacial acetic acid) or 10% buffered formalin for 24 hr, is embedded in paraffin.
- b. 5 μm sections are cut and placed on aminoalkylsilane-coated slides and baked in an oven at 60°C overnight.
- c. Sections are deparaffinized by two 10-min soaks in xylene followed by immersion in two successive baths of 100% ethanol for 10 min each.
- d. Sections are rehydrated in an ethanol series and treated with 100 $\mu\text{g}/\text{ml}$ proteinase K in TE buffer (50 mM Tris, 10 mM EDTA) at 37°C for 30 min.
- e. Sections are dehydrated in an ethanol series, air dried and incubated with 100 μl hybridization buffer (4 X standard saline citrate [SSC], 50% formamide, 1 X Denhardt's solution, 250 $\mu\text{g}/\text{ml}$ yeast tRNA, 10% dextran sulfate) containing 10 ng (1 μl of the PCR reaction) of the DIG-labeled probe.
- f. Sections are covered with plastic cover-slips, placed on a heating block at 95°C for 5 min, then cooled on ice for 1 min and placed in a 42°C moist chamber overnight for hybridization.
- g. Sections are washed twice in 2 X SSC for 5 min each at room temperature and once for 10 min in 0.4 X SSC at 42°C.
- h. The sections are then rinsed in distilled water, counterstained with Bismark Brown Y, rinsed again in distilled water and cover-slipped with an aqueous mounting medium.
- i. *Marteilia* organisms are demonstrated by purple-black labeling of the parasite cells.

Note- additional procedural details for ISH or PCR can be found in the current O.I.E. (2003) Diagnostic Manual for Aquatic Animal Diseases. Most molecular procedures still await validation with the exception of the ISH and histology assays for *M. refringens* as recently published by Thebault et al. (2005).

F. Procedures for Detecting Subclinical Infections

Success for detection of subclinical infections may be more likely with the more sensitive assays such as PCR and ISH described in section E.

G. Procedures for Transportation and Storage of Samples

Optimum results can only be achieved by the performance of diagnostic methods on live shellfish. The procedures for transport and storage should include adequate insulated containers for shipping, refrigeration, reduced time out of seawater and consideration of animal health and condition when collected regarding survivability in transit.

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