5.2.5 Mikrocytosis (Denman Island Disease of Oysters)

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A. Name of Disease and Etiological Agent

Mikrocytosis (also referred to as Denman Island disease) is caused by *Mikrocytos mackini*, a small unicellular organism of unknown taxonomic affiliation that is commonly referred to as a microcell. However, the use of the term "microcell" is discouraged because this term has been used to refer to *Bonamia* spp. which are distinctly different microorganisms that also parasitize oysters.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The disease and associated mortality were first reported from Henry Bay on Denman Island in British Columbia, Canada in the early 1960s. It has subsequently been observed at other sites in the southern part of British Columbia. Although *M. mackini* has recently been detected in older beach cultured oysters in the northern parts of the state of Washington, mortalities attributable to *M. mackini* have not been reported in oysters from this area. The disease has been found only in old feral oysters at prevalences of less than 6%, using both histology and PCR detection methods.

2. Host Species

Mikrocytos mackini is infective to at least four species of oysters: Pacific oysters, *Crassostrea gigas*; eastern oysters, *Crassostrea virginica*; flat oysters, *Ostrea edulis*; and Olympia oysters, *Ostrea conchaphila* (=*lurida*).

C. Epizootiology

Although juvenile oysters are susceptible to infection, the disease caused by *M. mackini* is only apparent in older oysters in some locations in British Columbia and associated mortality only occurs in the spring (March through May). Diseased oysters that survive the spring infection period may express the disease again the following spring. Young oysters (spat or seed) can acquire the parasite when held with diseased oysters during the spring. Data suggest that most newly acquired infections do not develop into a clinical disease until the following spring.

The life cycle of *M. mackini* is temperature dependent. Exposed oysters, held at 18 °C, can retain *M. mackini* at subclinical levels for at least 6 months and *M. mackini* is only capable of causing disease in

oysters held at less than 10 °C for at least 3 months (Hervio et al. 1996). This apparent requirement for long periods at low temperatures for pathogenic expression and the prolonged prepatent period at warm temperatures may explain why the disease only occurs during the spring and is expressed in oysters from more northerly locations. Possibly, subclinical infections occur in oysters from enzootic areas throughout the year. *Mikrocytos mackini* can be directly transmitted between oysters but transmission seems to be limited to periods when the infection is active in diseased oysters. To date, no evident mechanisms of protection in *M. mackini* for existence outside the oyster host (i.e., no spore-like stage) and no alternate hosts have been detected.

D. Disease Signs

1. Gross Signs

Disease signs consist of the formation of green or yellow-green focal pustules up to 5 mm in diameter throughout the body but are most frequently observed on the surface of the body, labial palps and occasionally within the adductor muscle. Often, brown scars occur on the white nacre layer of the shell adjacent to pustules on the mantle surface. Similar lesions occur with other infectious diseases of the oyster. For example, the pustules caused by *Nocardia crassostreae*, a bacterial pathogen of oysters, are grossly indistinguishable from those caused by *M. mackini*. Thus, microscopic examination is required to identify the etiology of pustules.

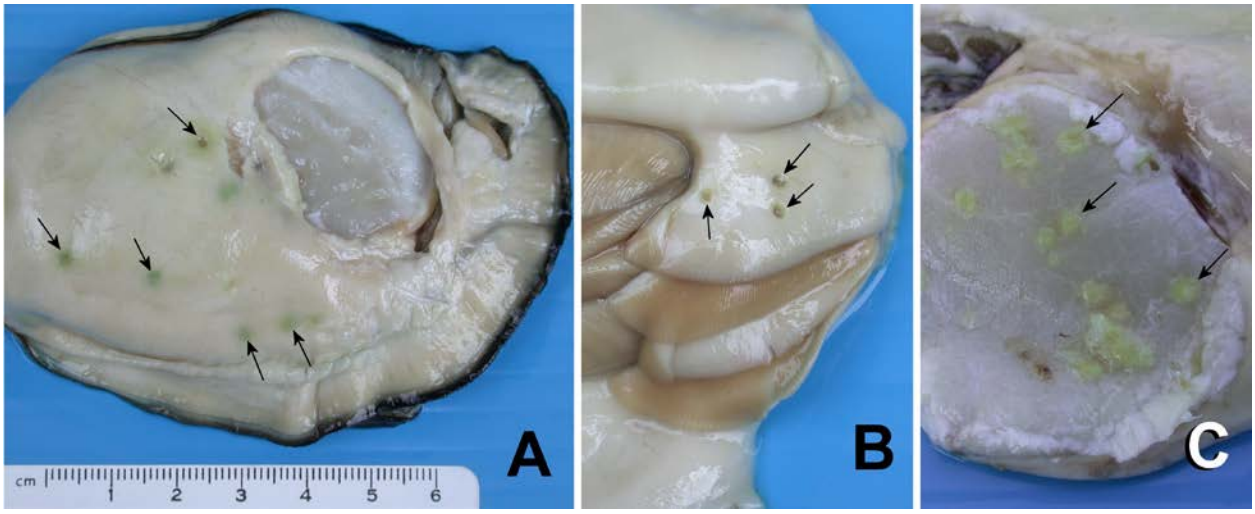

Figure 1. *Crassostrea gigas* removed from the shell and illustrating pustules (arrows) characteristic of *Mikrocytos mackini*. Figure 1A. Typical pustules on the mantle surface. Figure 1B. Pustules on the surface of the labial palps. Figure 1C. Obvious green pustules in the adductor muscle. Usually, the pustules are not as evident as shown in this adductor muscle but are often more diffuse and pale in color.

2. Microscopic Signs

Diagnosis is based on the characteristic histological signs (see below). Pustules consist of focal infiltration of hemocytes associated with *M. mackini* in the tissues (Figures 3A and 6B). Infected vesicular connective tissue cells contain the spherical unicellular parasites about 2 to 3 µm in diameter with a central nucleus (Figures 3B, 4A and 6D). *Mikrocytos mackini* also occurs in hemocytes and adductor muscle cells, and extracellularly within the pustules. Recently, the application of a digoxigenin-labelled DNA probe *in situ* hybridization technique (Meyer et al. 2004) has revealed the presence of *M. mackini* in the tissues of the gills and digestive tract (Bower et al. 2004). However, in these locations, the parasite is very difficult to discern because of its tiny size and pale staining characteristics and is thus usually masked by the denser staining of the oyster tissues. As the lesions resolve in surviving oysters late in the infection period, *M. mackini* is usually not evident in the tissues.

E. Disease Diagnostic Procedures

1. Stained Tissue Imprints

Imprints of pustules, air dried, stained with Wright, Wright-Giemsa or equivalent stain (e.g. Hemacolor, Merck; Diff-QuiK, Baxter) and examined under high power 1000× (oil immersion) for 3-5 µm spheroid or ovoid organisms with a central nucleus. Note: in tissue imprints, *M. mackini* is usually found outside of host cells and is slightly enlarged in size by this method when compared to *M. mackini* in histological preparations. Also, in imprints, *M. mackini* can not be differentiated from other microcell parasites of oysters.

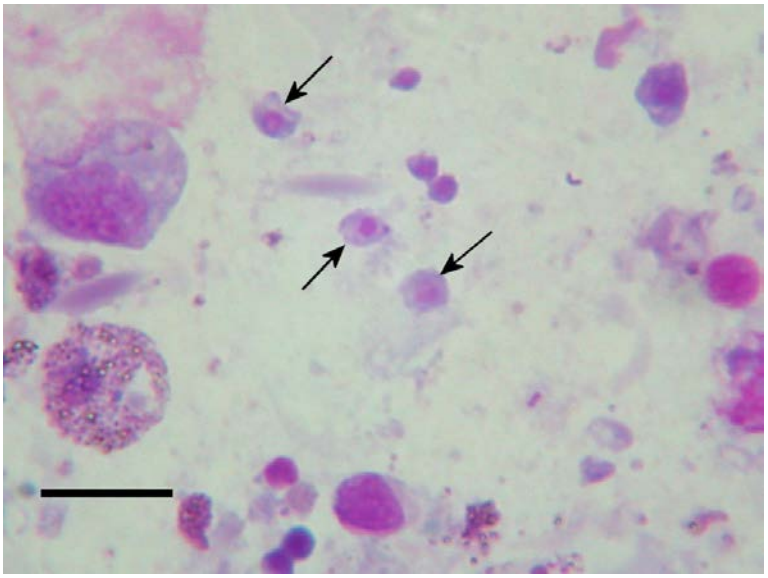

Figure 2. Imprint of a lesion in the adductor muscle of *Ostrea edulis* containing various hemocytes, tissue debris and *Mikrocytos mackini* (arrows). Hemacolor stain. Scale bar = 10 µm.

2. Histopathology

Standard histological tissue sections stained with hematoxylin and eosin stain and examined under high power 1000× (oil immersion) for the presence of intracellular protozoa 2-3 µm in diameter within vesicular connective tissue cells immediately adjacent to foci of hemocytic infiltration. This parasite also occurs in muscle cells (myocytes) and hemocytes within the lesions but is difficult to detect in these tissues. Other microcell parasites of oysters (e.g., *Bonamia* spp. which commonly occur in hemocytes) are not known to occur in the cytoplasm of vesicular connective tissue cells and myocytes. Thus, the occurrence of microcells in these host cells of oysters from enzootic areas can be used to identify *M. mackini*. Apart from host cell differences, *M. mackini* can not be reliably differentiated from other microcells based on parasite morphology at light microscopic levels of magnification (Fig. 4).

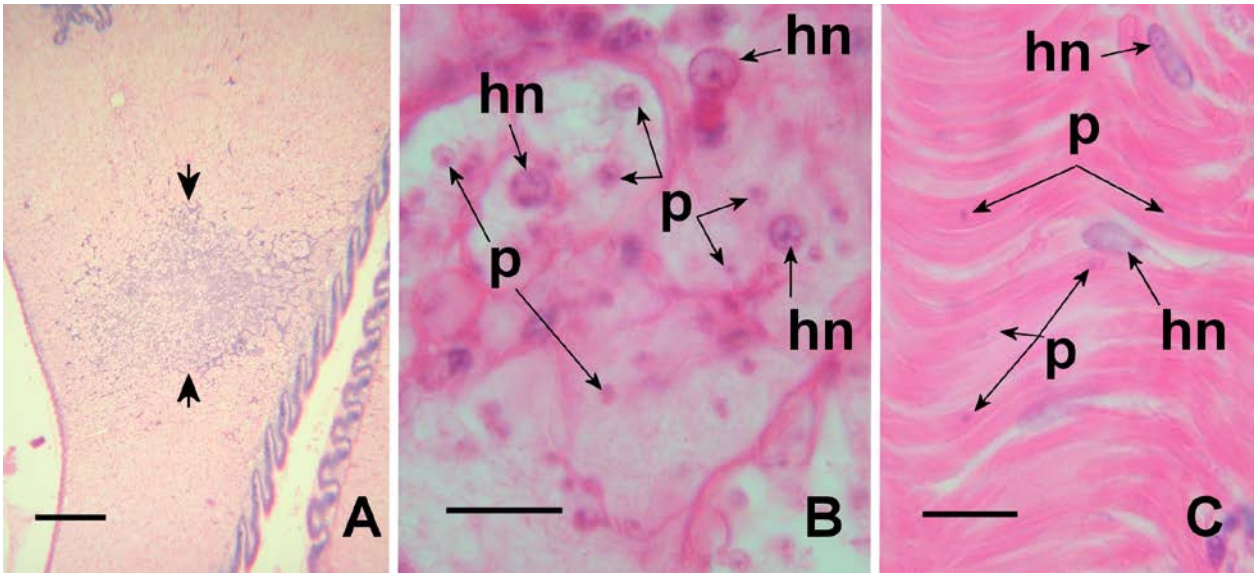

Figure 3. *Mikrocytos mackini* in the tissues of *Crassostrea gigas*. Figure3A. Foci of hemocyte infiltration (between arrows) in the labial palps. Scale bar = 0.5 mm. Figure 3B. Many *M. mackini* (p) in the cytoplasm of the vesicular connective tissue cells (hn indicates the nuclei of the host cells) on the periphery of the foci of hemocyte infiltration. Scale bar = 10 µm. Figure 3C. Cryptic *M. mackini* between the fibers of the adductor muscle (hn indicates the nuclei of the myocytes). Hematoxylin and eosin stain. Scale bar = 10 µm.

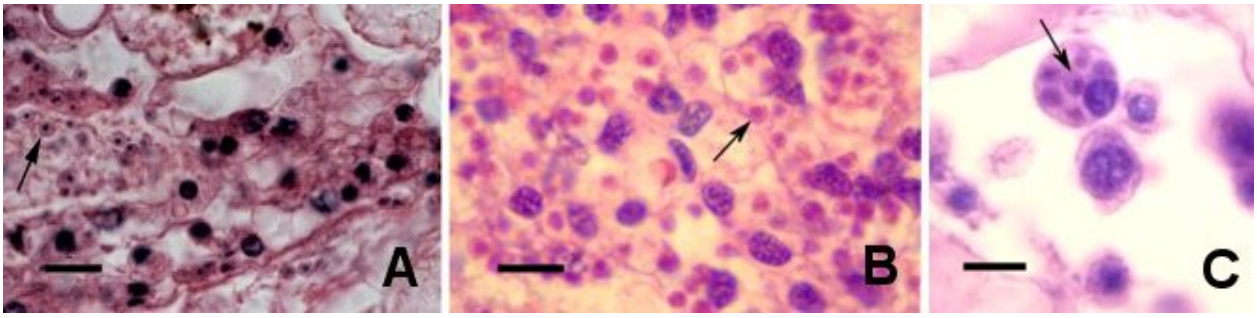

Figure 4. Images of various microcells illustrating the difficulty of differentiating between species at light microscopic levels of magnification. Figure 3A. *Microcytos mackini* (courtesy of Ralph Elston, FHS Blue Book 2004). Figure 3B. *Bonamia exitiosa* (courtesy of Ben Diggles, www.digsfish.com), Figure 3C. *Bonamia ostreae*. Hematoxylin and eosin staining variations. Scale bars = 10 µm.

3. Transmission Electron Microscopy (TEM)

Ultrastructural details of *M. mackini* from various host cells has facilitated the description of a sequence of development of this parasite in its oyster host (Hine et al. 2001). However, the salient features that distinguish *M. mackini* from all other microcells are the absence of mitochondria and haplosporosomes and its frequent close association with the mitochondria, nucleus and golgi cisternae of its host cell.

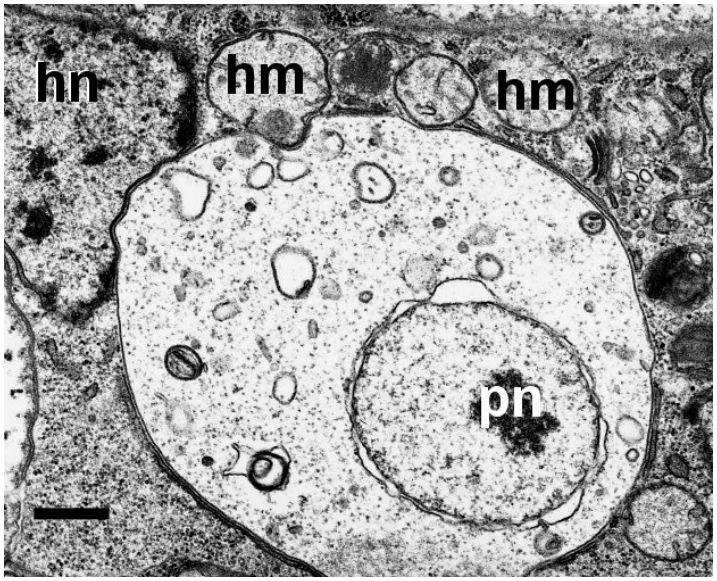

Figure 5. Transmission electron micrograph of a *Mikrocytos mackini* in close association with mitochondria (hm) and the nucleus (hn) of a hemocyte of *Crassostrea gigas*. Note the lack of mitochondria in the cytoplasm of *M. mackini* and presence of a centrally located osmophilic nucleolus (pn). Lead citrate and uranyl acetate stain. Scale bar = 0.5 µm.

4. Antibody-based Methods

Hybridomas that produce monoclonal antibodies specific for *M. mackini* have been produced (Hervio et al. 1995). However, they have not been applied to the development of diagnostic tool(s).

5. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was developed from a 1457 base pair segment of the small-subunit ribosomal DNA gene of *M. mackini* (GenBank assession number AF477623). Details on the PCR procedure are described by Carnegie et al. (2003). This *M. mackini*-specific PCR detects 3 to 4 times more *M. mackini* infections than standard histopathology (Carnegie et al. 2003).

6. *In Situ* Hybridization

Although a fluorescent *in situ* hybridization (FISH) assay was developed (Carnegie et al. 2003), this technique proved problematic because fluorescence quenched during examination, stained preparations had a short storage time, and background staining was inadequate for oyster tissue orientation. These problems were solved by labeling the 3' end of one of the oligonucleotide probes developed for FISH (MACKINI-1-OG: AGCCCACAGCCTTCAC) with digoxigenin. The digoxigenin *in situ* hybridization (DIG-ISH), specific for *M. mackini* binds to the parasites in tissue sections and are visualized using standard immunohistochemical procedures (Meyer et al. 2005). With this technique *M. mackini* stain a dark (blue/black) color and are more visible than with routine histopathology.

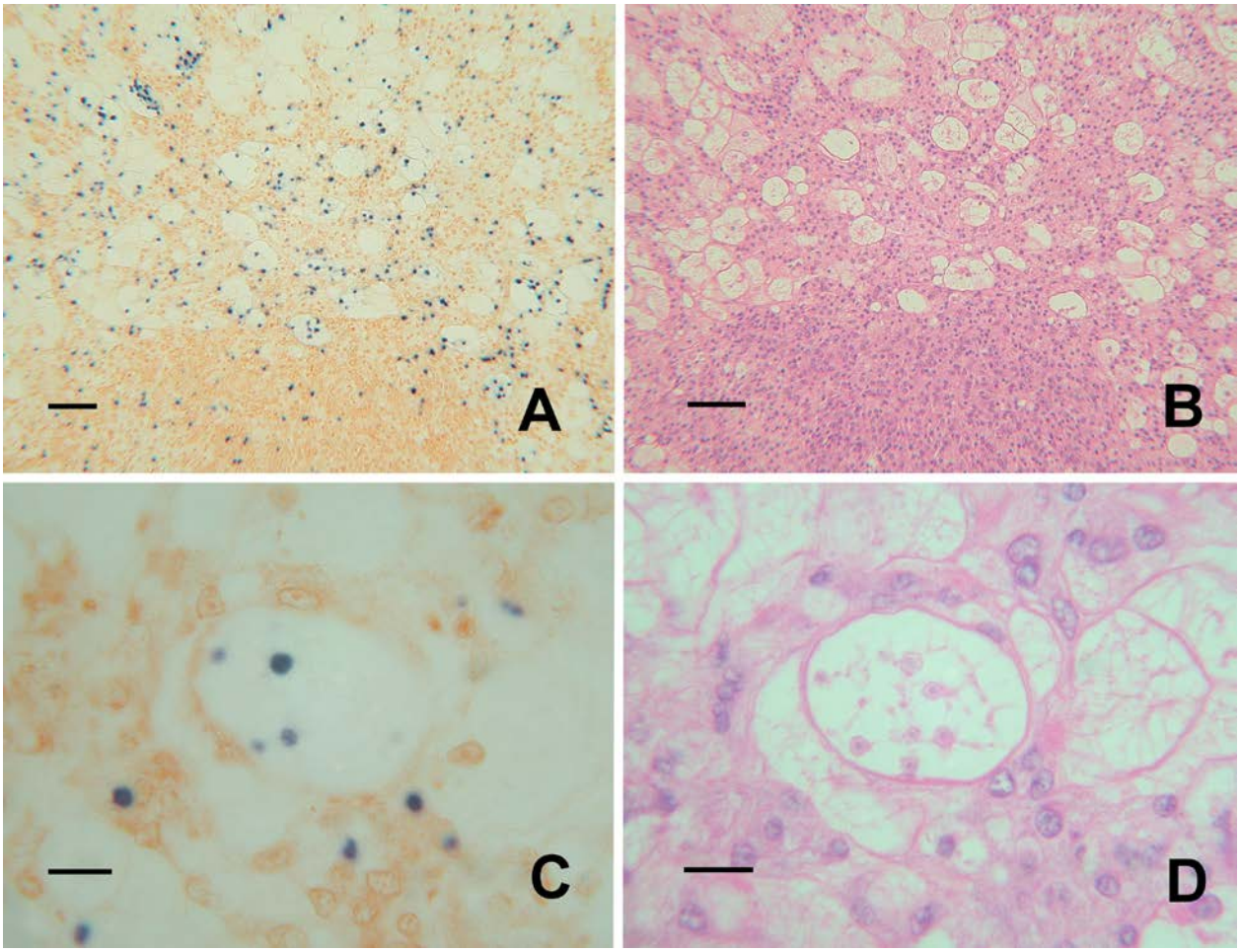

Figure 6. Comparison DIG-ISH and routine histology staining techniques for the detection of *Mikrocytos mackini* in serial sections of *Crassostrea gigas*. Figures 5A and 5B. At low magnification, staining with DIG-ISH reveals *M. mackini* as blue/black dots within the connective tissue and accumulated hemocytes at the bottom of the image (Fig. 5A). At this same magnification stained with hematoxylin and eosin stain, *M. mackini* is not visible (Fig. 5B). Scale bars = 50 µm. Figures 5C and 5D. At high magnification, DIG-ISH stained *M. mackini* are evident within a vesicular connective tissue cell and adjacent hemocytes (Fig. 5C) but are only apparent within the same vesicular cell when stained with hematoxylin and eosin stain.(Fig. 5D) Scale bar = 10 µm.

7. Summary of Diagnostic Procedures

A summary of the methods currently available for surveillance, detection, and diagnosis of mikrocytosis infections is listed in Table 1.

Table 1. Mikrocytos mackini *surveillance, detection and diagnostic methods*

Method	Screening			Presumptive	Confirmatory
	Larvae	Juveniles	Adults		
Gross signs	–	–	+	+	–
Stained tissue imprints	–	–	++	++	–
Histopathology	–	+	+++	+++	+++
TEM	–	–	–	–	+++
Antibody-based methods	–	–	–	–	+
PCR	–	++	++	++	–
DIG-ISH	–	+++	+++	+++	+++

TEM = transmission electron microscopy
PCR = polymerase chain reaction (assessment of reliability on-going)
DIG-ISH = digoxigenin *in situ* hybridization
– = the method is presently unavailable or unsuitable
+ = the method has application in some situations, but cost, accuracy, or other factors severely limits its usefullness
++ = 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.

F. Procedures for Transportation and Storage of Samples

Samples collected from apparently normal or moribund oysters should be kept cold (but not frozen) and moist, and preserved for diagnosis within 48 hours of the time of collection. For histological examination, fresh tissue samples should be preserved in fixative containing formalin (e.g. Davidson's Solution or 10% neutral buffered formalin). For PCR assays, tiny samples (about 2 mm^3) of the labial palps, mantle or pustules should be preserved in 95% ethanol.

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