5.2.2 Haplosporidiosis of Oysters

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A. Name of Disease and Etiological Agent

Haplosporidiosis is a chronic to acute disease in oysters and perhaps other marine and freshwater bivalve molluscs caused by a variety of species in the protistan phylum Haplosporidia. There are four species of *Haplosporidium* and one species of *Minchinia* that occur in bivalve molluscs. Species in the enigmatic genus *Bonamia* are also now considered to be in the phylum Haplosporidia, but these important pathogens are considered in chapter VII. Although the end result of *Haplosporidium* spp. and *Minchinia* spp. infections is likely death of the host, most species are rare and are not considered important disease agents because of their very low prevalence. However, *Haplosporidium nelsoni* and *Haplosporidium costale* are important pathogens of the eastern oyster, *Crassostrea virginica*. *Haplosporidium nelsoni* was first known as Multinucleate Sphere Unknown and the acronym MSX has persisted as the name of the disease. *Haplosporidium costale* was called Seaside Organism and the disease is still known by the acronym SSO.

B. Known Geographic Range and Host Species of the Disease

Haplosporidium nelsoni infections occur in *C. virginca* along the entire East Coast of the US and in the Bras d'Or Lake system in Nova Scotia, Canada; prevalence is often over 50%. Infections also occur at low prevalence (< 5%) in *Crassostrea gigas* in Japan, Korea, California and France (Burreson et al. 2000).

Haplosporidium costale infections occur in *C. virginica* from Virginia to Maine and throughout Atlantic Canada. This parasite has also been reported from *C. gigas* on the West Coast of the US, but prevalence is very low.

C. Epizootiology

Based on data from Delaware Bay and Chesapeake Bay, the infection cycle of *H. nelsoni* begins each year in May and continues through the summer with maximum oyster

mortality in late July or early August. Infections acquired late in the summer usually overwinter and then proliferate when temperature increases in the spring, resulting in a second mortality event in April or early May. MSX disease is acute and oyster mortality can reach > 90% the first summer of exposure. Parasite distribution and abundance is controlled by temperature and salinity. Maximum abundance occurs at temperatures above 20°C and salinities above 15 psu. The parasite does not occur where salinities are consistently less than 10 psu. There is some evidence that winter water temperatures below 4°C limit *H. nelsoni* abundance the following summer. The life cycle of *H. nelsoni* is unknown. Attempts at direct transmission using either plasmodia or spores have been unsuccessful and most investigators think that an as yet unidentified intermediate host is a required component of the life cycle.

Infections of *H. costale* are acquired in early summer but do not become patent until the following March. Plasmodia multiply rapidly with synchronous sporulation in late May/early June causing host death. Prevalence of *H. costale* and, thus, oyster mortality is usually less than 20%, but can reach 40% in some years along the Virginia coast. Oyster mortality from SSO disease seems to be much lower in the northeast. *Haplosporidium costale* is restricted to high salinity coastal bays; it does not occur where salinity is less than about 25 psu. The life cycle of *H. costale* is unknown.

D. Disease Signs

There are no reliable gross clinical signs of infection. Both MSX and SSO disease are acute and mortality is so rapid that infected oysters can appear healthy.

Tissue sections show 4-30 µm spherical, multinucleate plasmodia. Plasmodia of *H. nelsoni* (Figure 1) occur systemically in both epithelium and connective tissue; plasmodia of *H. costale* occur only in connective tissue. Sporulation in *H. nelsoni* occurs only in the epithelium of the digestive tubules (Figure 2). Spores measure about 8 µm in length. Sporulation in *H. costale* occurs throughout connective tissue (Figure 2). Spores measure about 4 µm in length. There is often intense hemocyte infiltration associated with infections of both species, but phagocytosis of plasmodia is rare.

E. Disease Diagnostic Procedures

Table 1. *Haplosporidiuim* spp. surveillance, detection and diagnostic methods.

Method	**Surveillance**	**Presumptive diagnosis**	**Confirmatory diagnosis**
Gross signs	-	-	-
Histopathology	+++		
Plasmodia only		++[a]	-
Spores present		+++	+++
PCR	+	++	++[b]
In situ hybridization	n/a	n/a	+++

a: histopathology can establish that an infection is present, but cannot reliably distinguish *H. nelsoni* and *H. costale* if only plasmodia are present.
b: useful as a confirmatory diagnosis only if plasmodia have been visualized by histopathology.
n/a: not applicable, assay used only after a presumptive diagnosis.

– = 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.

1. Surveillance

Fixation with Davidson's fixative for 24 hours followed by standard paraffin histology and hematoxylin and eosin (H&E) staining is used for surveillance. Tissue sections of oysters should include digestive gland, gill and mantle. Sections are cut at 5-6 µm. Histology is reasonably sensitive for routine surveillance, but can miss very light plasmodia infections. PCR, if properly validated against histology, can be used for surveillance, but no information is acquired on host response or intensity of infection.

2. Presumptive diagnosis

Histology

If only plasmodial stages are present histology cannot be used for presumptive diagnosis in coastal waters where *H. nelsoni* and *H. costale* overlap in range because the plasmodia of *H. nelsoni* and *H. costale* cannot be distinguished morphologically (Stokes and Burreson, 2001). Within Chesapeake Bay and Delaware Bay in areas

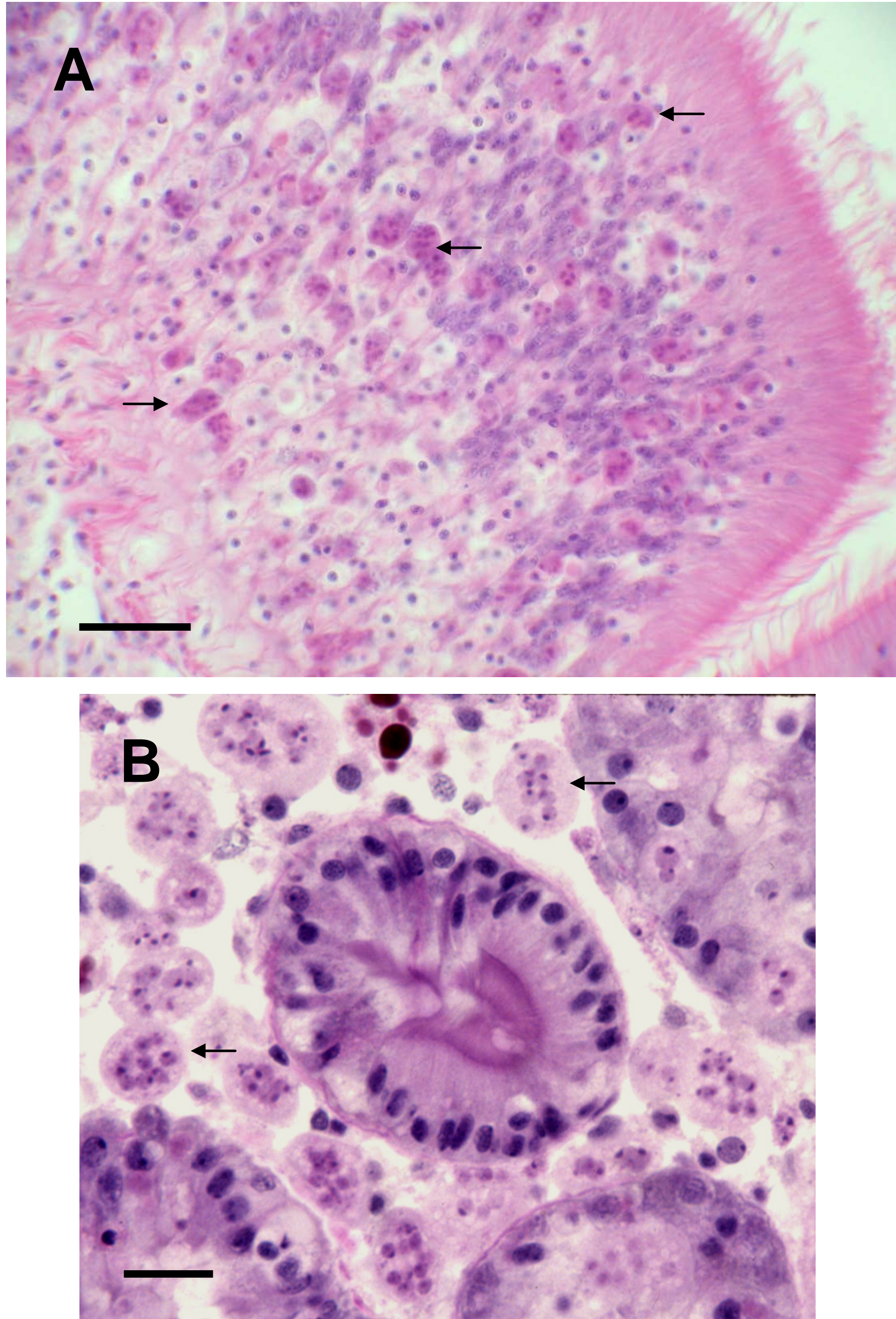

Figure 1. Multinucleate plasmodia of *Haplosporidium nelsoni*. A. 6 µm paraffin section showing plasmodia (arrows) in the gut epithelium, H&E stain. Scale bar = 30 µm. B. 1 µm plastic section stained with toluidine blue illustrating plasmodia (arrows) in connective tissue and the eccentric nucleolus of the nuclei. Scale bar = 15 µm.

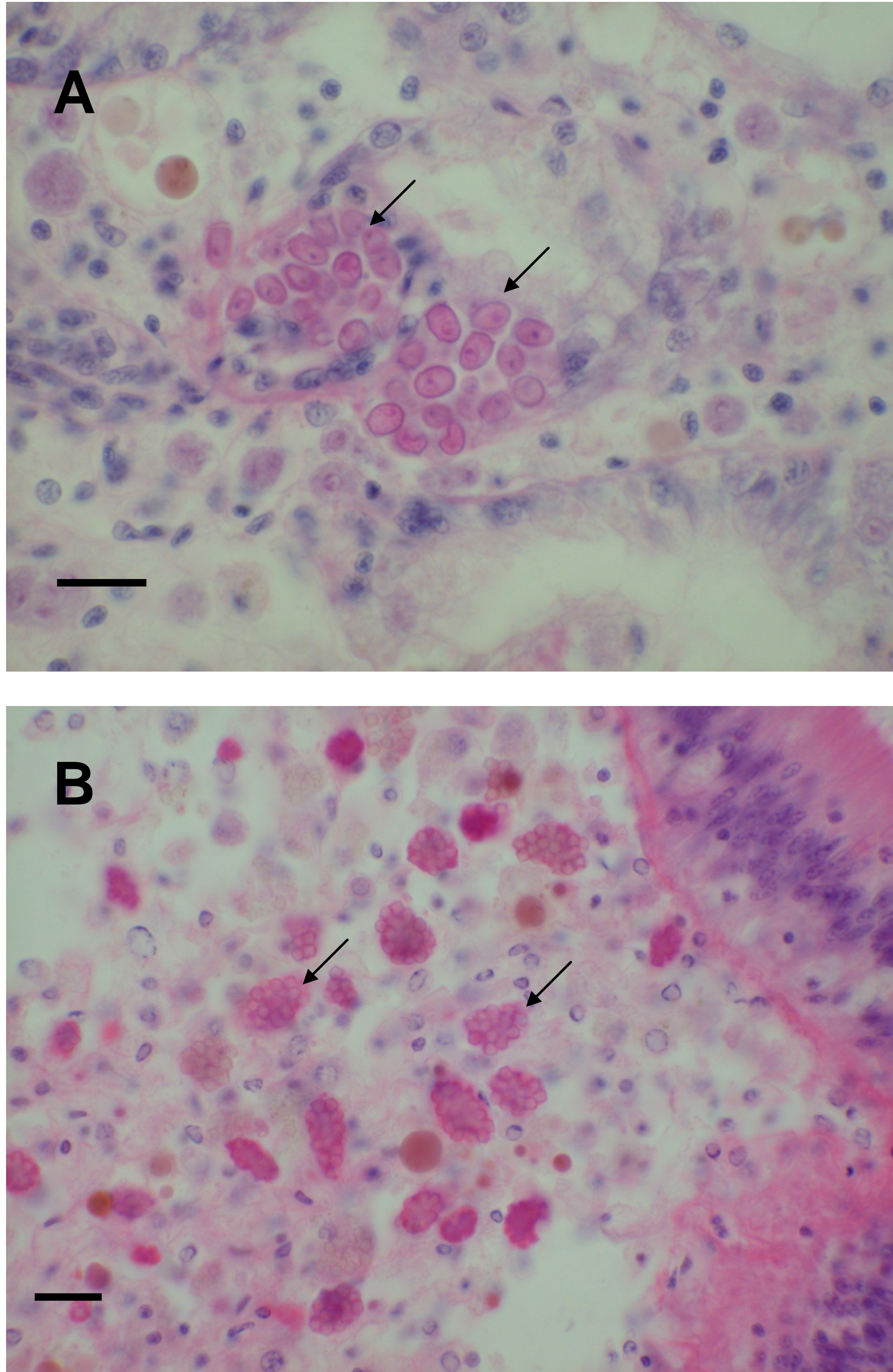

Figure 2. H&E-stained 6 µm paraffin sections of oyster tissue. A. Spores (arrows) of *Haplosporidium nelsoni* in epithelium of digestive tubules (scale bar = 20µm). B. Spores (arrows) of *Haplosporidium costale* in connective tissue (scale bar = 20µm).

where salinity is consistently below 25 psu, plasmodia can be presumptively identified as *H. nelsoni* because *H. costale* does not occur in low salinity waters. If spores are present histology can be used for both presumptive and confirmatory diagnosis because spores of *H. nelsoni* are 8 µm in size and occur only in the epithelium of the digestive tubules, and spores of *H. costale* are 4 µm in size and occur throughout the connective tissue. Unfortunately, spores of *H. nelsoni* are rarely present in adult oysters.

PCR assay

PCR can be used for presumptive diagnosis, or confirmatory diagnosis if plasmodia have been observed in histological sections. Specific PCR primers have been developed for both *H. nelsoni* and *H. costale* (Stokes et al. 1995, Renault et al. 2000, Stokes and Burreson 2001). Positive controls must be used.

a. Extract genomic DNA from gill/mantle and digestive gland tissue using the Qiagen DNeasy tissue kit (Qiagen™, Valencia, California).

b. Quantify DNA spectrophotometrically.

c. PCR amplification

1. Primers:
   H. nelsoni: MSX-A' (5'CGACTTTGGCATTAGGTTTCAGACC3')
   MSX-B (5'ATGTGTTGGTGACGCTAACCG3')

   H. costale: SSO-A (5'CACGACTTTGGCAGTTAGTTTTG3')
   SSO-B (5'CGAACAAGCGCTAGCAGTACAT3')

2. Reagent concentrations for 25 µl reactions.
   Reaction buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl, 10 µg/ml gelatin)
   400 µg/ml bovine serum albumin
   200 µM each of dATP, dCTP, dGTP, dTTP
   0.6 units Ampli*Taq* DNA polymerase
   25 pmoles of each primer (MSX-A' and MSX-B or SSO-A and SSO-B)
   200-400 ng template DNA

3. Cycling parameters for *H. nelsoni*
   Initial denaturation of 4 min at 94°C
   35 cycles of:
   30 sec at 94°C
   30 sec at 59°C
   1.5 min at 72°C
   Final extension of 5 min at 72°C

4. Cycling parameters for *H. costale*
   Initial denaturation of 4 min at 94°C
   10 cycles of touch-down PCR:
     30 sec at 94°C
     30 sec at 65°C, -1°C per cycle
     1.5 min at 72°C
   30 cycles of:
     30 sec at 94°C
     30 sec at 55°C
     1.5 min at 72°C
   Final extension of 5 min at 72°C

d. PCR products are electrophoresed on 2% agarose (in 1X TAE or TBE) gels, stained with ethidium bromide, and visualized using UV light. Expected PCR products are 573 bp for *H. nelsoni* and 557 bp for *H. costale*.

3. Confirmatory Diagnosis

If plasmodia infections have been observed in histology, PCR can be used as a confirmatory diagnosis. However, it is easier to cut additional sections and use an in situ hybridization assay as a confirmatory diagnosis. Mixed infections of *H. nelsoni* and *H. costale* occur in high salinity coastal waters, so it is important to assay for both pathogens. Positive and negative controls must be used in in situ hybridization assays. DNA probes that target SSU rRNA sequences have been developed for both *H. nelsoni* and *H. costale* (Figure 3) (Stokes and Burreson 1995, 2001). The use of two specific probes in a hybridization cocktail increases assay sensitivity for *H. costale*.

In situ hybridization assay

H. nelsoni DNA probe: MSX1347 (5'ATGTGTTGGTGACGCTAACCG3')
H. costale DNA probes: SSO1318 (5'CGAACAAGCGCTAGCAGTACAT3')
SSO785 (5'CAAAACTAACTGCCAAAGTCG3')
All probes are 5' end-labeled with digoxigenin.

a. Cut a transverse section through the visceral mass that includes mantle, gill and digestive gland and place it in Davidson's AFA fixative (glycerin [10%], formalin [20%], 95% ethanol [30%], dH_2O [30%], glacial acetic acid [10%]) for approximately 24 hours, then transfer to 70% ethanol until processed by histological procedures (step b). The ratio must be no more than one volume of tissue to ten volumes of fixative.

b. The samples are subsequently embedded in paraffin by conventional histological procedures. Sections are cut at 5–6 µm and placed on positively-charged slides or 3-aminopropyl-triethoxylane-coated slides. Histological sections are then dried overnight in an oven at 40°C.

c. The sections are deparaffinized by immersing them in xylene or another less toxic clearing agent for 10 minutes. The solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each and rehydrated by immersion in an ethanol series. The sections are then washed twice for 5 minutes in phosphate buffered saline (PBS).

d. The sections are treated with proteinase K, 50 µg/ml in PBS, at 37°C for 18 minutes. The reaction is then stopped by washing the sections in PBS with 0.2% glycine for 5 minutes. The sections are then placed in 2X SSC (20X SSC = 3M NaCl; 0.3M Na-citrate; pH 7.0) for 10 minutes.

e. The sections are prehybridized for 1 hour at 42°C in prehybridization solution (4X SSC, 50% formamide, 5X Denhardt's solution, 0.5 mg/ml yeast tRNA, and 0.5 mg/ml heat-denaturated salmon sperm DNA).

f. The prehybridization solution is then replaced with prehybridization buffer containing 2 ng/µl of the digoxigenin-labelled *H. nelsoni* probe or 3 ng/µl each of the digoxigenin-labelled *H. costale* probes. The sections are covered with *in-situ* hybridization plastic cover-slips and placed on a heating block at 90°C for 12 minutes. The slides are then cooled on ice for 1 minute before hybridization overnight at 42°C in a humid chamber.

g. The sections are washed twice for 5 minutes each in 2X SSC at room temperature, twice for 5 minutes each in 1X SSC at room temperature, and twice for 10 minutes each in 0.5X SSC at 42°C. The sections are then placed in Buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) for 1–2 minutes.

h. The sections are placed in Buffer 1 (see step g) supplemented with 0.3% Triton X-100 and 2% sheep serum for 30 minutes. Anti-digoxigenin alkaline phosphatase antibody conjugate is diluted 1/500 (or according to the manufacturer's recommendations) in Buffer 1 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue sections. The sections are covered with *in-situ* hybridization cover-slips and incubated for 3 hours at room temperature in the humid chamber.

i. The slides are washed twice in Buffer 1 for 5 minutes each (see step g) and twice in Buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 minutes each. The slides are then placed in color development solution (337.5 µg/ml nitroblue tetrazolium, 175 µg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 240 µg/ml levamisole in Buffer 2) for 2 hours in the dark. The color reaction is stopped by washing in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA [ethylene diamine tetra-acetic acid]).

j. The slides are then rinsed in dH_2O. The sections are counterstained with Bismarck Brown Y, rinsed in dH_2O, and cover-slips are applied using an aqueous mounting medium. The presence of *H. nelsoni* and/or *H. costale* is demonstrated by the purple-black labelling of the parasite cells.

F. Procedures for Detecting Subclinical Infections

Because of its high sensitivity, PCR amplification is the best method for detecting early infections.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability or Preservation of the Etiological Agent

1. **Histological/in situ hybridization assays**
   a. Transport or ship live oysters humidified and cooled to the analytical facility.

   b. Shuck oysters and excise a 5 mm-thick tissue section that includes digestive gland, gill and mantle. Place section in labeled histological cassette and fix in Davidson's fixative for 24-48 h. Transfer immediately to 70% ethanol. Embed in paraffin as soon as possible.

2. **PCR assays**
   a. Aseptically excise a small piece of gill/mantle and digestive gland and place into 1.5 ml microcentrifuge tubes containing 10 volumes of 95-100% ethanol. Do not use denatured alcohol. Dip dissection equipment in ethanol and flame between oysters to prevent cross contamination.
   b. Store samples at room temperature or in a refrigerator until processed.

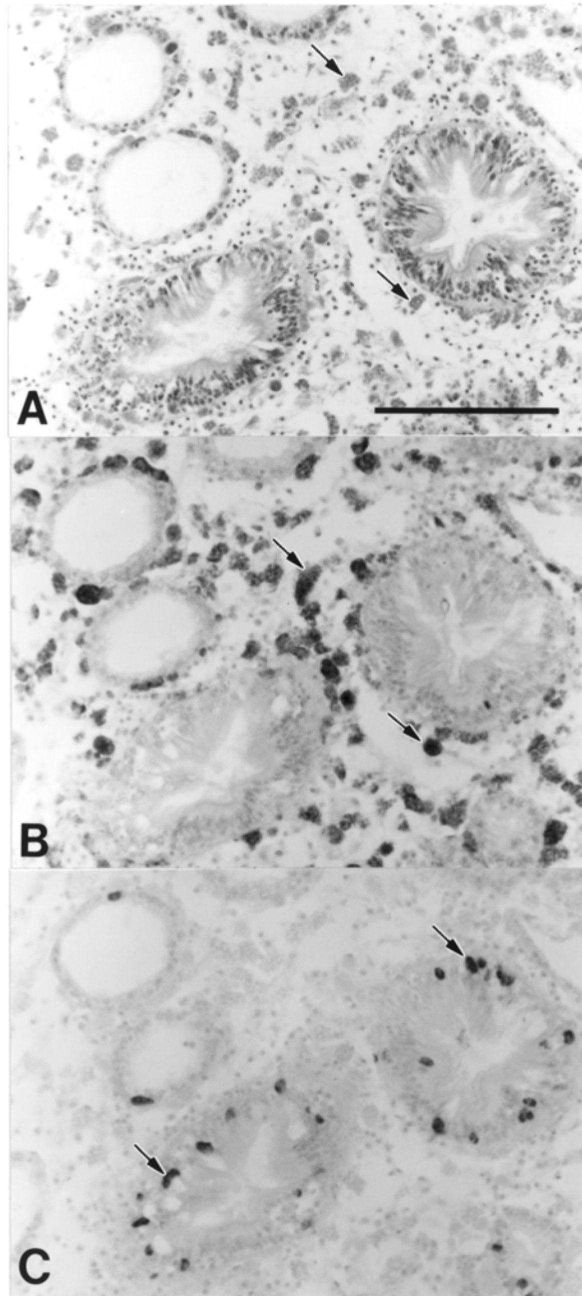

Figure 3. In situ hybridization of mixed haplosporidian infection in *Crassostrea virginica*. A, B and C are consecutive sections from the same region of the oyster. A. H&E stained section showing many plasmodia in connective tissue (arrows). B. In situ hybridization with the *H. costale* DNA probe showing positive reaction with plasmodia in connective tissue (arrows). C. In situ hybridization with the *H. nelsoni* DNA probe showing positive reaction with plasmodia in digestive tubule epithelium (arrows), but not with plasmodia in connective tissue.

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