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HAPLOSPORIDIUM COSTALE (SEASIDE ORGANISM), A PARASITE OF THE EASTERN OYSTER, IS PRESENT IN LONG ISLAND SOUND

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ABSTRACT A haplosporidian parasite, *Haplosporidium costale* (seaside organism or SSO), is associated with high mortalities of eastern oysters (*Crassostrea virginica*) in seaside bays of Virginia and Maryland. Its presence in Long Island Sound has been tentatively suggested in several publications for the last 50 y. Positive identification of *H. costale* and differentiation from another haplosporidian parasite, *Haplosporidium nelsoni* (MSX), from histological sections is difficult and requires the presence of spores. We detected *H. costale* spores in 4 out of 5010 (0.08%) oysters collected from Long Island Sound in 1997–1999. *In situ* hybridization using an oligonucleotide DNA probe designed to detect small subunit ribosomal DNA from Virginia's *H. costale* reacted positively with tentative *H. costale* plasmodia in 5 oysters from Long Island Sound. In each case there was a coinfection of *H. nelsoni*. In Virginia and Maryland, *H. costale* has historically sporulated in all infected animals in May–June. In Long Island Sound, the rare sporulating cases were detected in October–December, suggesting a different infection cycle.

KEY WORDS: *Haplosporidium costale*, eastern oyster, *Crassostrea virginica*, *in situ* hybridization, Long Island Sound

INTRODUCTION

A haplosporidian parasite, *Haplosporidium costale*, was identified as the causative agent of seaside organism (SSO) disease, resulting in high mortalities of eastern oysters (*Crassostrea virginica*) on the Atlantic coast of Virginia (Wood & Andrews 1962). Prevalence, mortality and infection cycle of *H. costale* have been thoroughly studied in Virginia and Maryland, but information from other geographical locations is scarce. In seaside bays of the Delmarva Peninsula, the first plasmodia can be detected in the epithelia of digestive tubules in April–May. Plasmodia rapidly proliferate in May and sporulate synchronously. Peak mortality of the oysters is in May–June, when dying oysters release spores in seawater to initiate a new infection cycle. New infections remain subpotent until spring of the following year. Mortality of oysters has been 20% to 50% annually in the seaside bays of Virginia (Andrews 1988; Andrews & Castagna 1978).

Reports of *H. costale* distribution north of Virginia's Atlantic coast are inconsistent. Several reports suggest the presence of plasmodia resembling *H. costale* in eastern oysters in Long Island Sound. According to Andrews (1984, 1988), SSO disease ranges from Cape Charles, Virginia, to Maine, but is important only in high-salinity bays (>25‰) from Cape Henlopen, Delaware, to the Virginia capes. He stated that the pathogen is regularly present from New York to Massachusetts, but mortality has not been a serious problem. However, a mortality event in Long Island Sound in 1953 may have been caused by *H. costale* (Andrews 1988). Sampling locations, dates, prevalences, or possible presence of spores were not mentioned in these reports.

Newman (1971) studied 1,337 oysters from New Haven Harbor, Connecticut, from 1966 to 1967. He found five specimens (0.4%) infected with plasmodia morphologically similar to *H. costale*, with one of the oysters moribund with heavy infection. No sporulation was detected. Meyers (1981) described haplosporidia-like plasmodia (4%) in juvenile oysters (n = 68) collected from Oyster Bay, north shore of Long Island, New York, from 1975 to 1976. He did not find similar organisms in adult oysters (n = 145). No effort was made to distinguish between different haplosporidian species because sporulating stages were not present. In addition, plasmodia morphologically identical to *H. costale* were observed in oysters transplanted from the vicinity of New Haven, Connecticut, to Tomales Bay, California, in 1967–1968 (Katkansky & Warner 1970). Six specimens with tentative *H. costale* infection (four moribund and two living) were reported, one with spores. Total number of oysters studied was not mentioned.

Reports of tentative *H. costale* infections in Long Island Sound reviewed above are based on histological examinations. Reliable diagnosis of *H. costale* on histological sections is nearly impossible when sporulating forms are not present. *H. costale* plasmodia can be easily misdiagnosed as *Haplosporidium nelsoni* (MSX), another haplosporidian oyster parasite enzootic to the area (for review, see Ford & Tripp 1996). During routine monitoring for oyster diseases in Connecticut, we found several oysters with spores and plasmodia consistent with descriptions of *H. costale*. We used a DNA probe, designed to detect *H. costale* from Virginia as the *in situ* hybridization (ISH) probe for these specimens to verify the presence of *H. costale* in Long Island Sound.

MATERIAL AND METHODS

The State of Connecticut, Department of Agriculture, Bureau of Aquaculture routinely receives oyster samples for histological diagnosis from Connecticut's commercial oyster companies. Most of the seed originates from natural seed beds, though some hatchery-raised seed is also used. Every oyster is transplanted an average of four times before it is marketed, which exposes it to possible parasitic infections in several different sites. Seventeen oyster samples were studied in 1997, 63 in 1998, and 87 in 1999. Each sample consisted of 30 oysters, for a total of 5,010 oysters. Samples represented the entire Connecticut shoreline and the north shore of Long Island, New York. Of the 167 samples, 20 originated from New York. Fifty-six of the samples originated from oyster nursery systems (5 from upwellers, 51 from suspended cultures), 30 from off bottom cultures, and 81 from natural oyster beds. Tissues were fixed in Davidson's fixative in 20‰ artificial seawater. Six-micrometer-thick paraffin sections were stained with hematoxylin-eosin. Samples with *H. costale* or *H. nelsoni* pre-

spores and spores were stained also with Ziehl and Harris' hematoxylin according to Farley (1965).

The ISH procedure was conducted with 27 oysters, two with presumed *H. costale* spores and 25 oysters with haplosporidian plasmodia. Specimens were selected for ISH as follows: (1) specimens with haplosporidian plasmodia from samples in which *H. costale* spores were detected; (2) specimens with small plasmodia with central nucleoli, morphology that is considered to be characteristic for *H. costale*; and (3) specimens with plasmodia in the stomach, intestine, or digestive tubule epithelia, locations that are considered to be characteristic for *H. costale*. For the ISH procedure, 6-μm-thick sections from these oysters were deparaffinized and ISH was performed on consecutive sections as previously described (Stokes & Burreson 1995; Stokes & Burreson 2001). Two commercially synthesized digoxigenin-labeled oligonucleotide DNA probes were used: a 22-base oligonucleotide (SSO1318) specific for *H. costale* (Stokes & Burreson 2001) and a 21-base oligonucleotide (MSX1347) specific for *H. nelsoni* (Stokes & Burreson 1995). A negative control was performed by substituting DNA probes with distilled water during hybridization.

RESULTS

Haplosporidium costale was detected in three locations on Connecticut's shoreline: Norwalk, Branford, and Clinton (Fig. 1). *H. costale* was diagnosed in seven different specimens either by the presence of spores or by a positive ISH result. There were 17 oysters with haplosporidian spores among the 5,010 oysters studied (0.3%). Four oysters had a mixture of *H. costale* and *H. nelsoni* spores (0.08%). The remaining 13 contained only *H. nelsoni* spores. Locations, dates, and seed origin of oysters with spores are listed in Table 1.

H. costale and *H. nelsoni* spores differed in size, form, and location in oyster tissues. *H. costale* spores (3 × 4 μm), the sporoplasm of which stained bright red with acid-fast stain, were detected throughout the connective tissue. Prespores, which did not retain acid-fast stain, occurred inside sporocysts throughout the tissues. *H. costale* spores were found between vesicular connective tissue cells surrounding the digestive diverticula (Fig. 2A), in connective tissue of the gills, in the adductor muscle, in the heart, between neurosecretory cells in the ganglia, and between kidney tubules. On rare occasions, *H. costale* spores were detected inside digestive tubule or digestive duct cells, in the lumens of the digestive tubules and intestine, in epithelial cells of the intestine, or in the follicles.

H. nelsoni spores (5 × 7 μm), which also stained bright red with acid-fast stain, were in most cases restricted to digestive epithelial cells (Fig. 2B). However, during intense sporulation in four specimens there was an overspill to digestive duct cells and the connective tissue surrounding the digestive tubules. In cases with

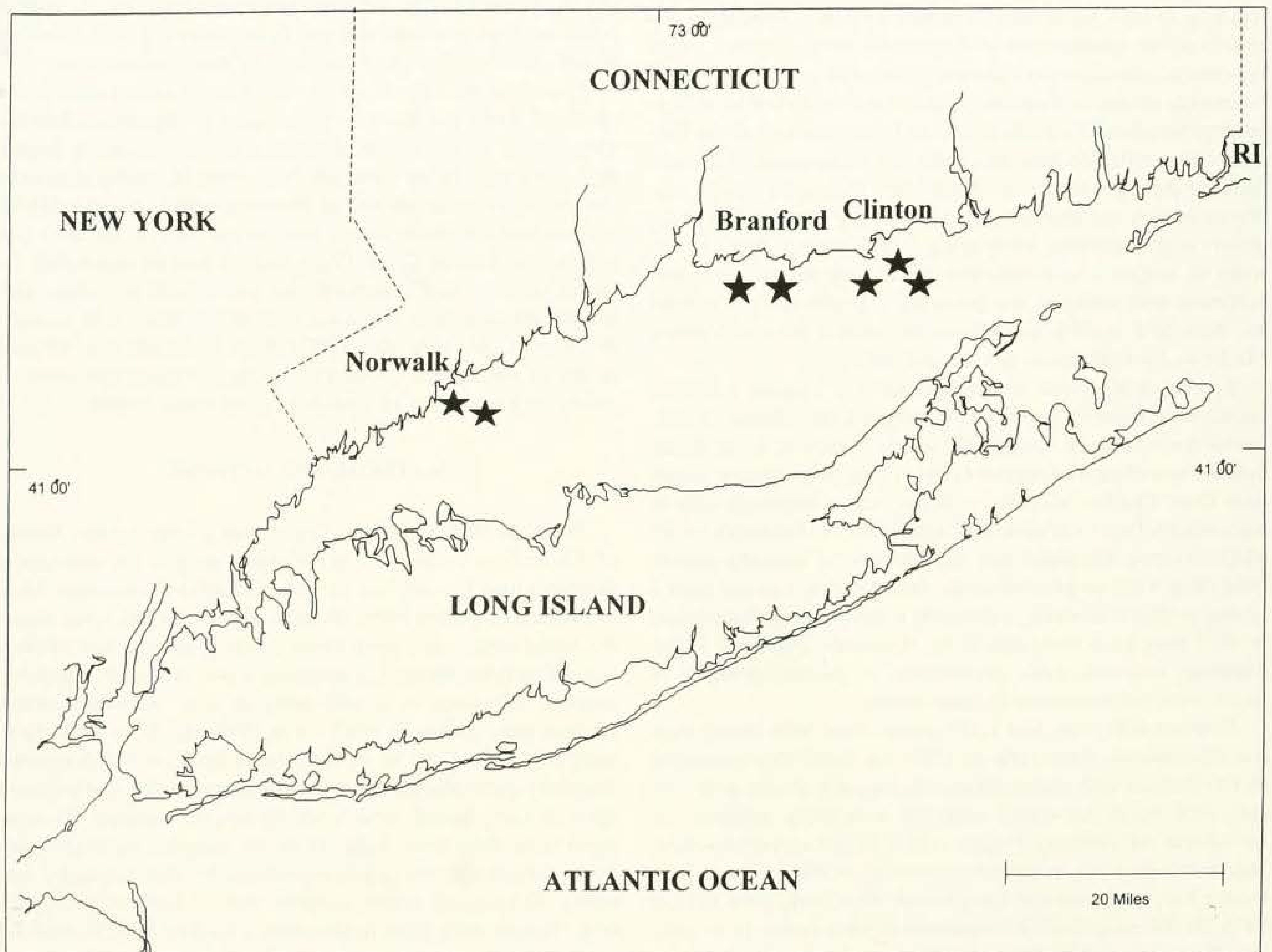


Figure. 1. A map of sampling stations with a positive diagnosis for *H. costale* in Long Island Sound.

TABLE 1.

***Haplosporidium costale* (SSO) and *Haplosporidium nelsoni* (MSX) spores in eastern oysters in Long Island Sound.**

Sampling Date	Sampling Site	Sampling Location	Shell Length (mm)	Spore Type	Seed Origin
09.28.97	Guilford, East River	41°16.05′N:72°39.62′W	104	MSX	Natural
10.14.97	Clinton, Cedar Island mud flat	41°15.97′N:72°32.00′W	78	MSX	Natural
10.14.97	Clinton, Cedar Island mud flat	41°15.97′N:72°32.00′W	101	MSX	Natural
12.17.97	Norwalk 1131	41°02.42′N:73°25.25′W	75	MSX	Natural
12.18.97	Norwalk 162	41°03.36′N:73°25.12′W	69	MSX	Natural
01.08.98	West Haven Lot 1B	41°15.47′N:72°55.26′W	42	MSX	Hatchery raised
09.18.98	Stratford 709	41°07.41′N:73°09.13′W	70	MSX	Natural
09.22.98	Milford 612	41°11.10′N:73°00.05′W	73	MSX	Natural
10.17.98	Branford 316	41°15.42′N:72°44.40′W	75	SSO, MSX	Natural
10.17.98	Branford 168	41°15.77′N:72°45.95′W	42	MSX	Natural
10.17.98	Branford 179	41°15.73′N:72°45.63′W	64	SSO, MSX	Natural
11.21.98	West Haven Lot 1B	41°15.47′N:72°55.26′W	48	MSX	Hatchery raised
12.03.98	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	24	SSO, MSX	Hatchery raised
09.22.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	76	MSX	Hatchery raised
09.22.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	33	MSX	Hatchery raised
09.22.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	26	MSX	Hatchery raised
11.16.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	29	SSO, MSX	Hatchery raised

extremely heavy sporulation, phagocytosed *H. nelsoni* spores were observed in the vascular system hemolymph sinuses and veins, in the mantle lobes and the gills, and being carried through stomach or mantle epithelia via diapedesis. Spores that occurred in the connective tissue were usually surrounded by aggregates of granular hemocytes. *H. nelsoni* spores were detected inside the lumens of digestive tubules, digestive ducts, and the intestine. *H. nelsoni* prespores, which did not retain stain in acid-fast reaction, were detected exclusively inside digestive cells.

It was as probable that spores would be found in oysters originating from natural set as in oysters originating from hatchery-raised seed ($\chi^2 = 0.68$ [not significant] [NS]). Oysters with spores were detected in most areas of Connecticut's shoreline. No sporulating specimens were detected in the north shore of Long Island, New York. The size of an oyster with spores did not differ significantly from the average size of the sample from which it was taken ($t = 0.08$ [NS]). The size of an oyster with spores also did not differ significantly from the average sizes of all oysters sampled for this study ($t = 0.97$ [NS]).

ISH results with *H. costale* and *H. nelsoni* DNA probes are summarized in Table 2. Five specimens had mixed infections, the rest were infected only with *H. nelsoni*. Two specimens with mixed infections had both types of spores present; three had only plasmodia. (An additional two specimens were diagnosed positive for both *H. costale* and *H. nelsoni* on the basis of the presence of spores [Table 1], with seven positive specimens altogether.) The two specimens with both spore types (Branford lots 179 and 316) that were subjected to ISH had prominent *H. costale* infections. Approximately 90% of the plasmodia hybridized with the *H. costale* probe and 10% hybridized with the *H. nelsoni* probe in these samples (Fig. 3). Two other specimens with mixed infections (Clinton and Norwalk 1131) had very light *H. costale* infections (Fig. 4). More than 99% of the plasmodia hybridized with the *H. nelsoni* probe and the very rare *H. costale* plasmodia would not have been detected without the probe. *H. costale* plasmodia in the

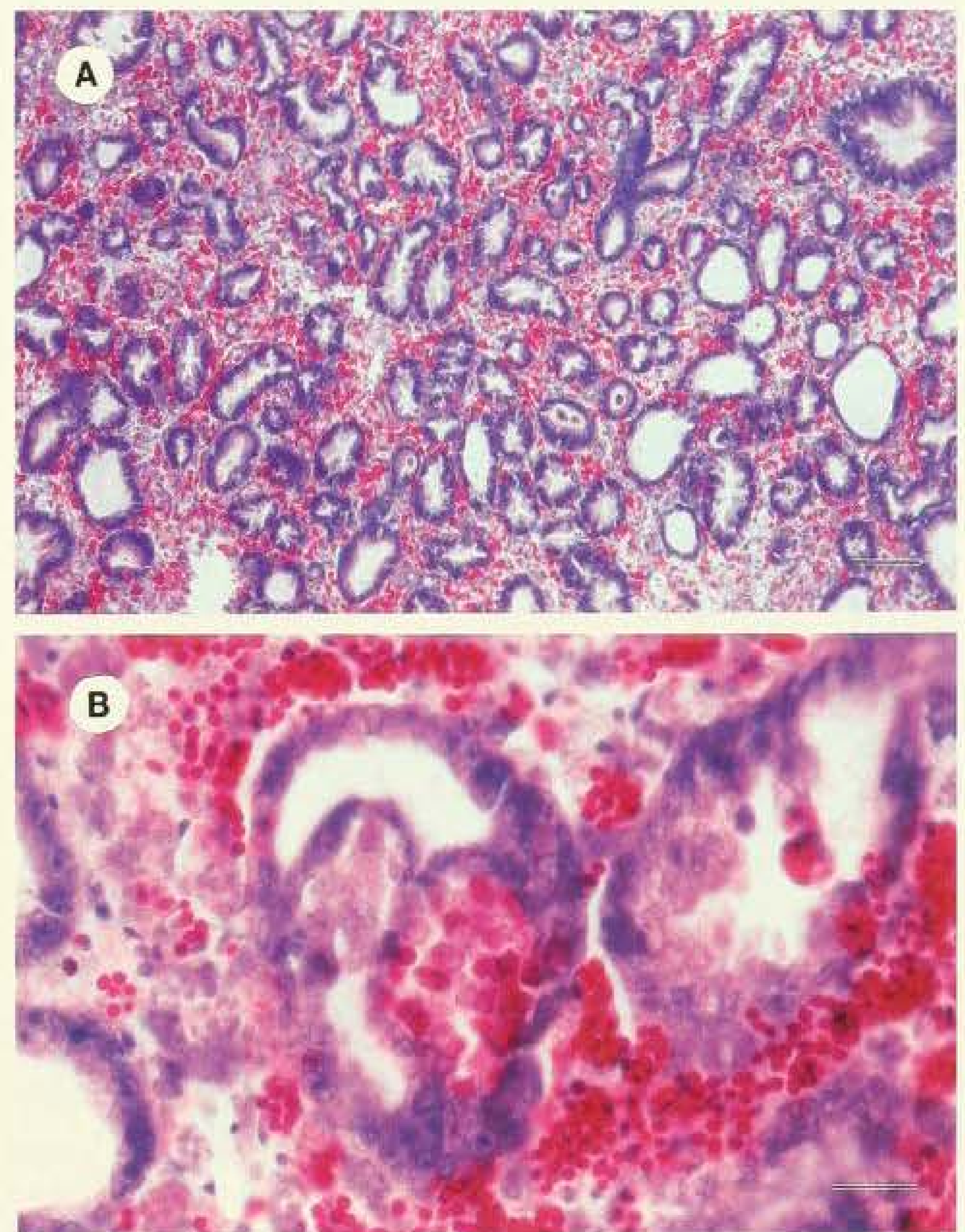


Figure 2. Sporulation of *H. costale* and *H. nelsoni* in Long Island Sound. (A) Digestive diverticulum of an oyster filled with acid-fast *H. costale* spores (Ziehl and Harris' hematoxylin). Scale bar 100 µm. (B) Cosporulation of *H. costale* and *H. nelsoni*. Small *H. costale* spores occur in the connective tissue surrounding digestive tubules; larger *H. nelsoni* spores occur inside digestive tubule. This is the same specimen as in item (A) (Ziehl and Harris' hematoxylin). Scale bar 20 µm.

TABLE 2.

ISH of *Haplosporidium costale* (SSO) and *Haplosporidium nelsoni* (MSX) in eastern oysters in Long Island Sound.

Sampling Date	Sampling Site	Sampling Location	Shell Length (mm)	ISH with SSO and MSX Probes (Positive Result)	Location of Plasmodia
09.16.97	Norwalk Natural Bed	41°04.85′N:73°23.55′W	110	MSX	Plasmodia throughout the tissues
10.14.97	Clinton, Cedar island mud flat	41°15.97′N:72°32.00′W	113	SSO, MSX	MSX plasmodia (>99%) throughout the tissues, rare SSO plasmodia (<1%) in the gills
12.17.97	Norwalk 1131	41°02.42′N:73°25.25′W	80	SSO, MSX	MSX plasmodia (>99%) throughout the tissues, rare SSO plasmodia (<1%) in the gills and mantle
12.17.97	Norwalk 162	41°03.36′N:73°25.12′W	66	SSO, MSX	MSX plasmodia (40%) throughout the tissues; prespores in digestive tubules; SSO plasmodia (60%) throughout the tissues
12.17.97	Norwalk Manresa Island	41°04.42′N:73°24.55′W	70	MSX	Plasmodia in the intestine, digestive duct and epibranchial chamber epithelia; rare plasmodia throughout the tissues
10.17.98	Branford 316	41°15.42′N:72°44.40′W	68	MSX	Plasmodia in the intestine and stomach epithelia; some plasmodia throughout the tissues
10.17.98	Branford 316	41°15.42′N:72°44.40′W	75	SSO, MSX	MSX spores and plasmodia (10%) in the digestive tubules; SSO spores and plasmodia (90%) everywhere in the connective tissue
10.17.98	Branford 168	41°15.77′N:72°45.95′W	41	MSX	A few plasmodia in stomach epithelium; several plasmodia throughout the tissues
10.17.98	Branford 168	41°15.77′N:72°45.95′W	41	MSX	A few plasmodia in stomach epithelium; several plasmodia throughout the tissues
10.17.98	Branford 168	41°15.77′N:72°45.95′W	60	MSX	A few plasmodia in the stomach, intestine, digestive tubule, and duct epithelia; several plasmodia throughout the tissues
10.17.98	Branford 179	41°15.73′N:72°45.63′W	62	MSX	Plasmodia exclusively in the stomach, intestine, digestive duct, and digestive tubule epithelia
10.17.98	Branford 179	41°15.73′N:72°45.63′W	70	MSX	Plasmodia throughout the tissues
10.17.98	Branford 179	41°15.73′N:72°45.63′W	64	SSO, MSX	MSX spores and plasmodia (10%) in the digestive tubules; SSO spores and plasmodia (90%) everywhere in the connective tissue
11.10.98	Stony Brook Harbor, NY	40°54.30′N:73°10.70′W	123	MSX	Plasmodia throughout the tissues
11.10.98	Stony Brook Harbor, NY	40°54.30′N:73°10.70′W	127	MSX	A few plasmodia in gill epithelia, several plasmodia throughout the tissues
11.10.98	Stony Brook Harbor, NY	40°54.30′N:73°10.70′W	107	MSX	Gaper; plasmodia throughout the tissues
11.12.98	Oyster Bay, H, NY	40°54.33′N:73°30.22′W	76	MSX	Rare plasmodia exclusively in the stomach, digestive tubule, and duct epithelia
11.12.98	Oyster Bay, H, NY	40°54.33′N:73°30.22′W	87	MSX	A few plasmodia in gill epithelia; several plasmodia throughout the tissues
11.12.98	Oyster Bay, W, NY	40°52.59′N:73°32.11′W	92	MSX	Rare plasmodia exclusively in stomach epithelium
11.12.98	Oyster Bay, W, NY	40°52.59′N:73°32.11′W	84	MSX	Rare plasmodia exclusively in digestive duct epithelia
12.01.98	Milford 305	41°11.15′N:73°04.80′W	78	MSX	Plasmodia throughout the tissues
12.01.98	Milford 305	41°11.15′N:73°04.80′W	24	MSX	Plasmodia throughout the tissues
12.15.98	Northport Bay, NY	40°55.87′N:73°22.87′W	92	MSX	Rare plasmodia exclusively in the stomach and gill epithelia
11.17.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	67	MSX	Plasmodia throughout the tissues
11.17.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	65	MSX	Plasmodia throughout the tissues
12.07.99	Oyster Bay, J, NY	40°53.50′N:73°30.23′W	90	MSX	Very rare plasmodia exclusively in intestine and gill epithelia
12.07.99	Oyster Bay, J, NY	40°53.50′N:73°30.23′W	68	MSX	Plasmodia in the stomach, digestive tubule, and gill epithelia; some plasmodia throughout the tissues

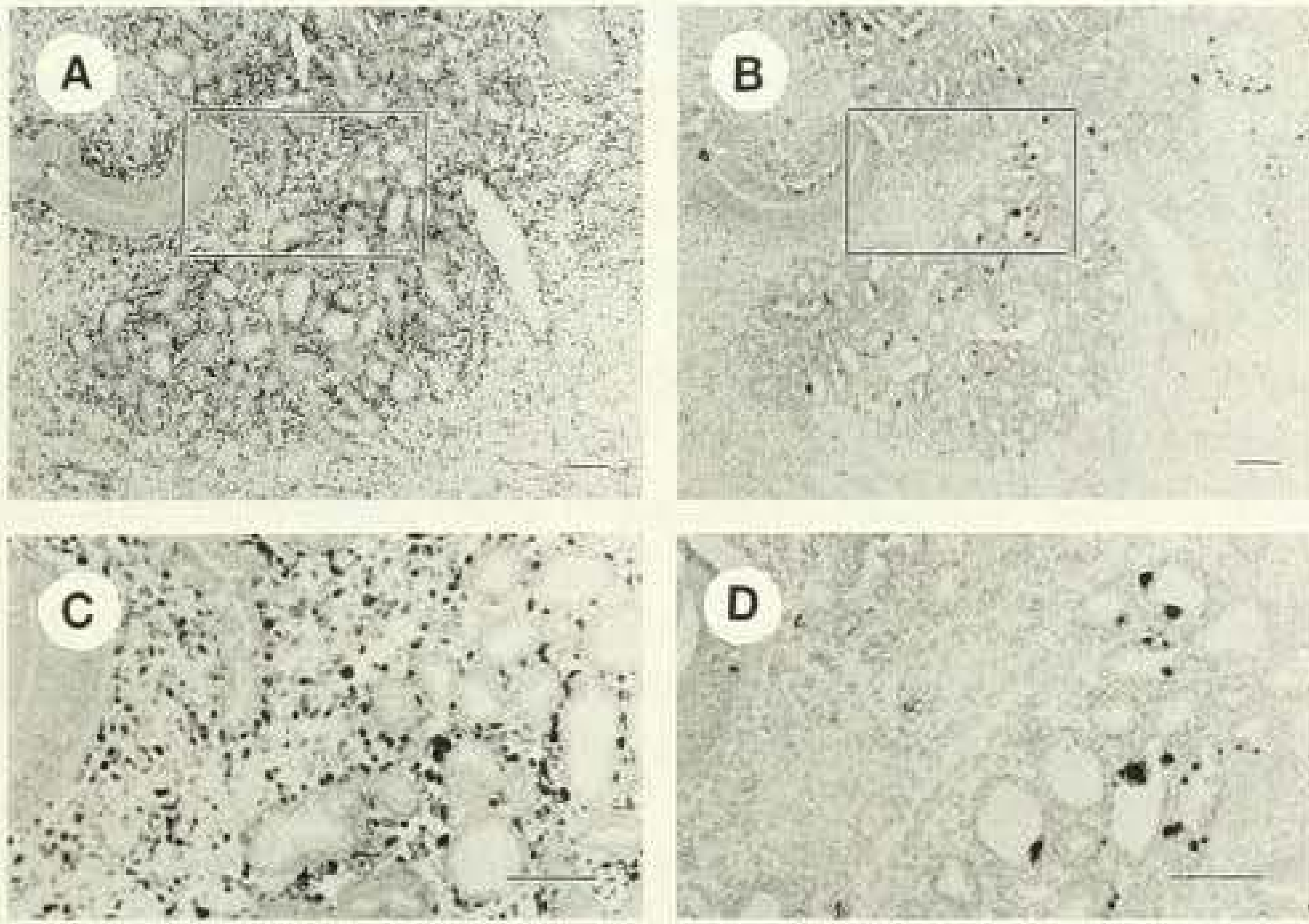


Figure 3. ISH of *H. costale*- and *H. nelsoni*-infected oyster from Long Island Sound. This is the same specimen as in Figure 2. (A) *H. costale* DNA probe hybridizing with *H. costale* plasmodia and prespores in the connective tissue surrounding digestive tubules. (B) *H. nelsoni* DNA probe hybridizing with *H. nelsoni* plasmodia and spores in digestive tubules. (C) Higher magnification of bracketed area in item (A). (D) Higher magnification of bracketed area in item (B). Scale bars 100 µm.

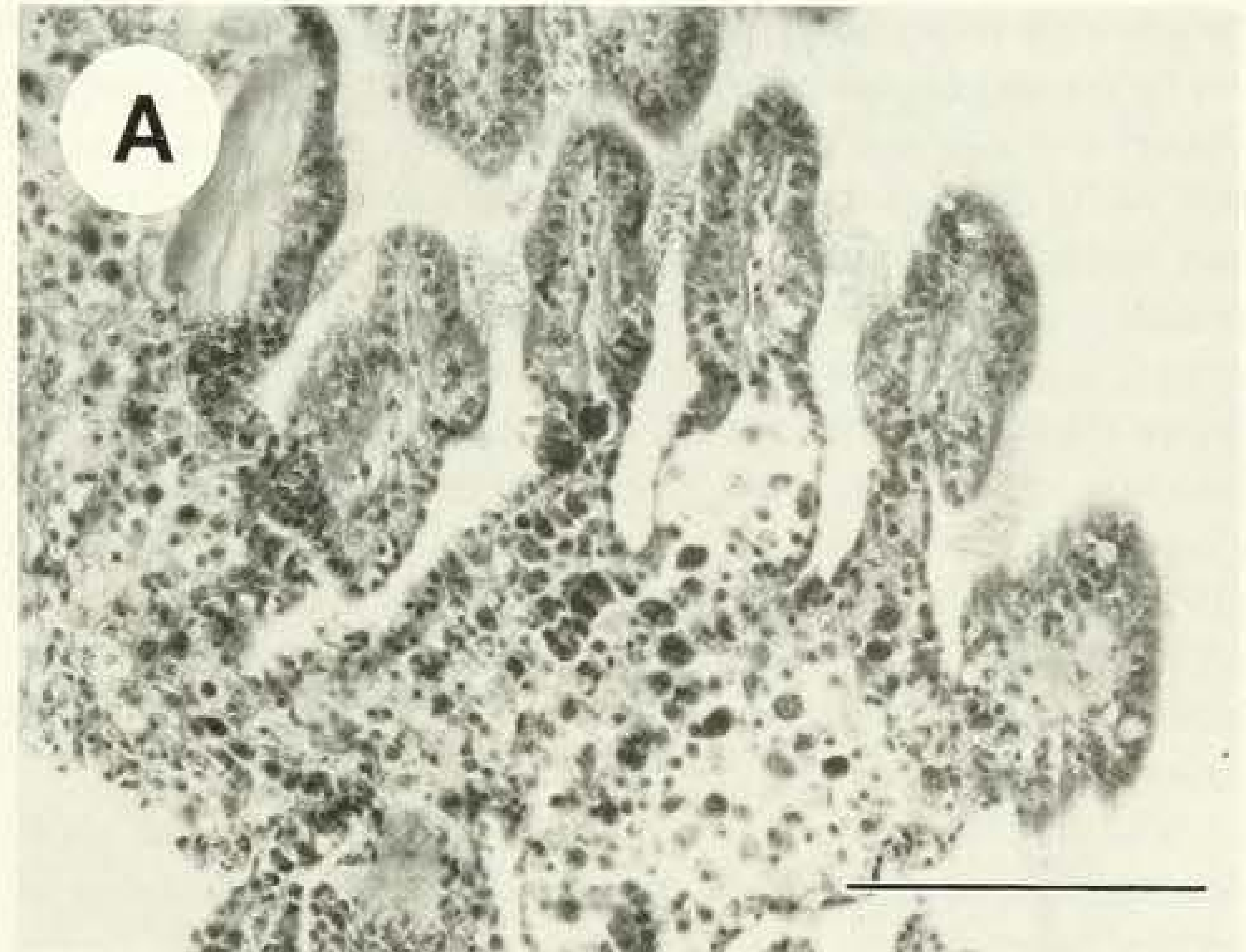
light infections were detected in the gills and the mantle, and in the heavy infections, were detected throughout the tissues.

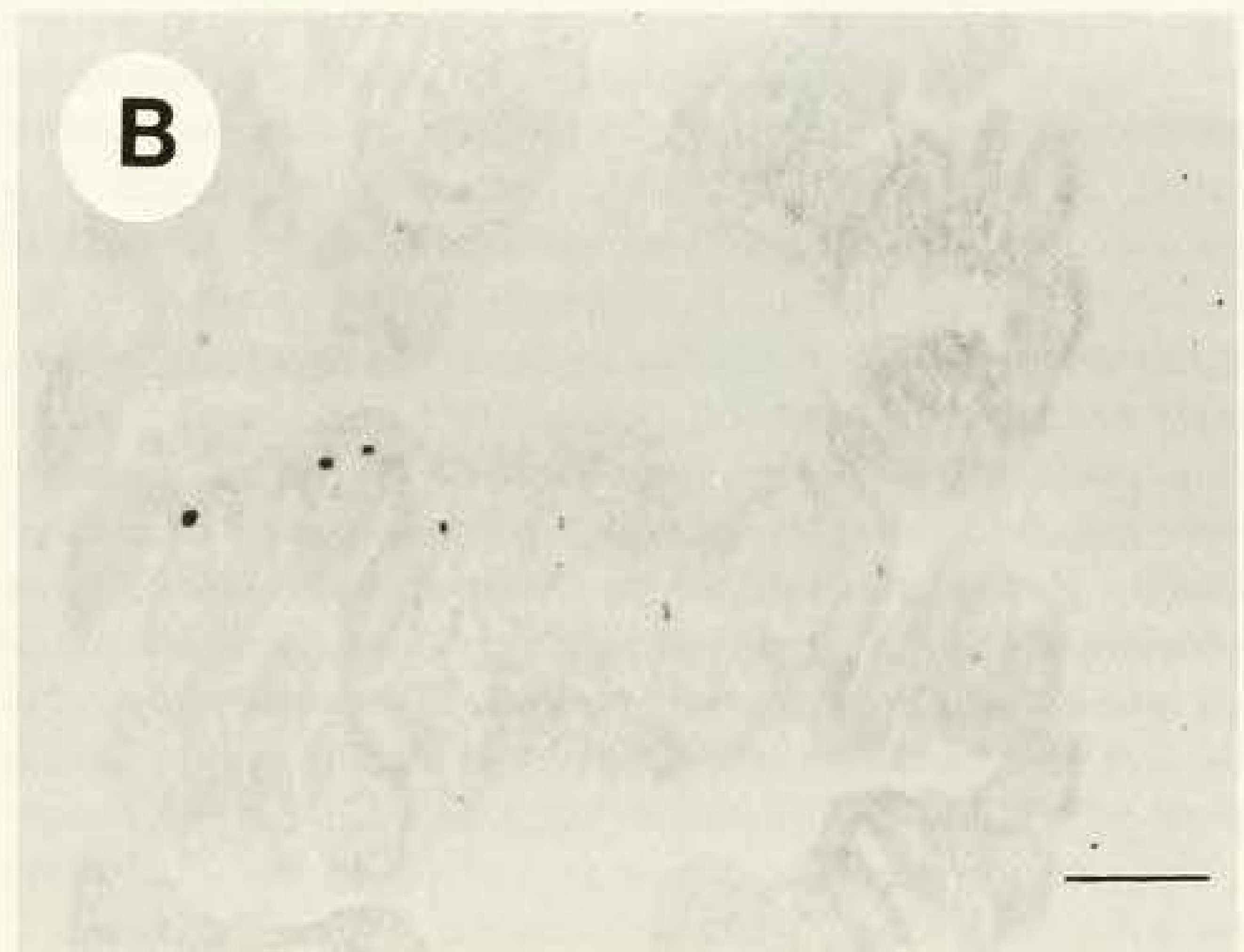
ISH of an oyster with both *H. costale* and *H. nelsoni* spores is illustrated in Figure 3. *H. costale* probe hybridized to plasmodia and prespores scattered in the connective tissue surrounding the digestive diverticula (Fig. 3A and C), in the gills, and the mantle, whereas *H. nelsoni* probe hybridized to plasmodia and prespores mainly in the digestive tubules (Fig. 3B and D). This specimen represented a terminal infection of *H. nelsoni*, when migration of plasmodia to digestive tubules had already occurred. Both probes outlined mature spores but did not completely penetrate them. Serial sections of an oyster with mixed infection of *H. costale* and *H. nelsoni* plasmodia stained with hematoxylin-eosin and ISH with the DNA probes are illustrated in Figure 4A, B, and C.

DISCUSSION

The presence of *H. costale* in Long Island Sound has been suggested in several earlier reports (Andrews 1984; Andrews 1988; Katkansky & Warner 1970; Newman 1971); however, in the absence of sporulating stages, positive identification has been impossible. Failure to detect sporulating stages is easily explained on the basis of the results of the present report: spores were detected in only 0.08% of the oysters studied. We can assume that *H. costale* has been enzootic to the area, but its presence was finally verified, not because of increased prevalence, but because of increased sampling effort and the use of species-specific diagnostic tools. Furthermore, absence of spores in 1997 and in any of the samples collected from the north shore of Long Island, New York, is most likely due to small sample sizes (510 and 600 oysters, respectively), which would give <0.5% probability to encounter a sporulating specimen. High occurrence of positive specimens detected in Branford-Clinton area (Table 1) was due to intensive sampling in that area.

Detailed descriptions of sporulation and spore structures of *H. costale* or *H. nelsoni* are presented by Couch et al. (1966), Rosenfield et al. (1969), and Perkins (1969). Although morphological characteristics of *H. costale* spores or plasmodia in our material





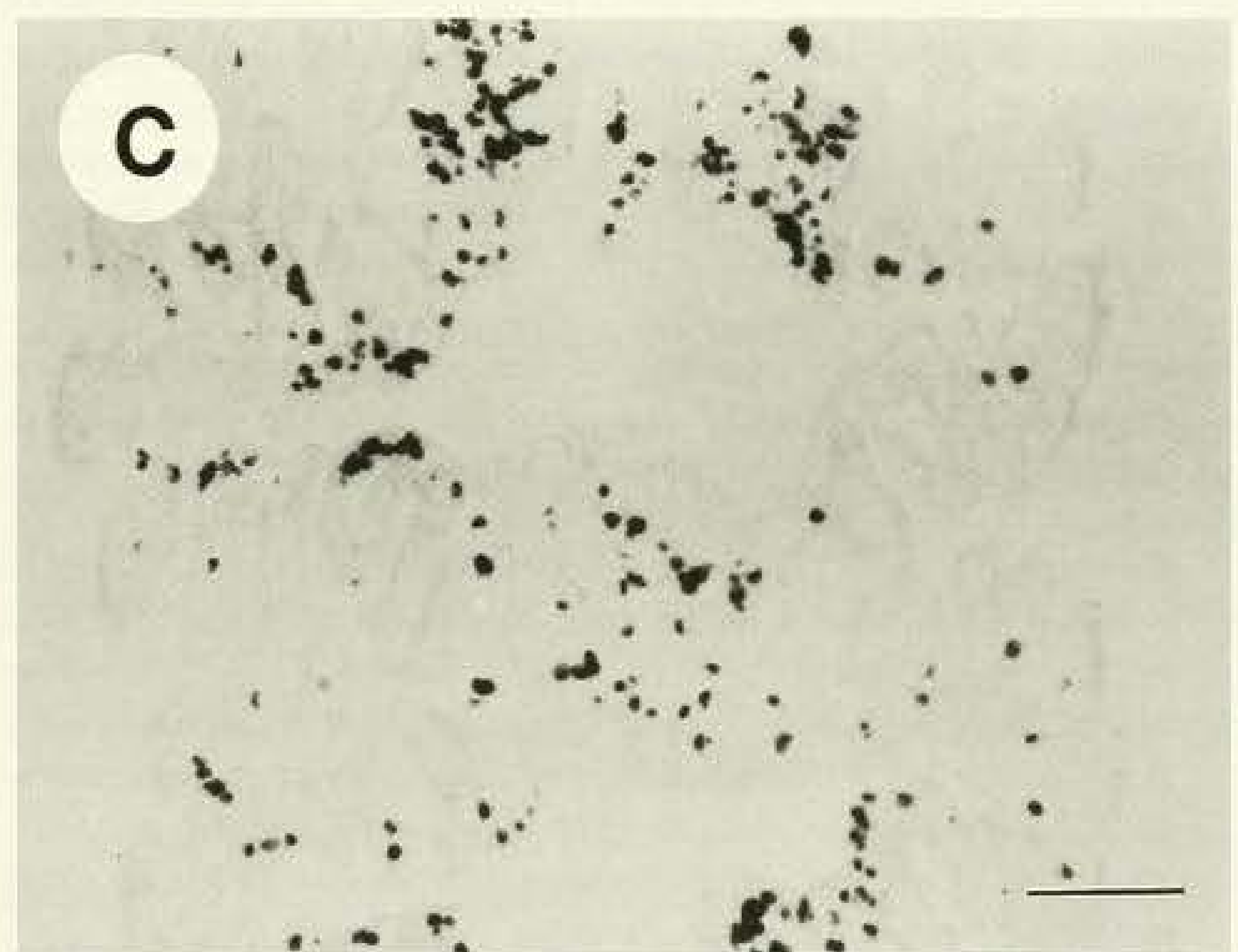


Figure 4. Serial sections of an oyster with mixed haplosporidian plasmodial infection. (A) Hematoxylin-eosin stain. (B) ISH with *H. costale* DNA probe detecting rare plasmodia in the gill. (C) ISH with *H. nelsoni* DNA probe of the same area shown in item (B). Scale bars 100 µm.

did not differ from those described in previous publications, *H. costale* in Long Island Sound appears to have a different pathogenesis than in the south. Gross signs as defined by Andrews (1988) (emaciation, failure of new shell growth in spring, high

epizootic mortality mid-May–mid-June, and discoloration of gapers by spores) did not apply to our samples. In Virginia, infected oysters sporulate synchronously in May–July, presenting an obvious, easily diagnosed stage. All plasmodia develop into sporonts, and oysters die promptly after sporulation (Andrews 1984). *H. costale* is considered to be a well-adapted parasite that infects new oysters via spores that are released during mortality season (Andrews 1982). Spores are usually detected in moribund oysters and infection causes 20%–50% yearly mortalities (Andrews 1988). Cosporulation with *H. nelsoni* was reported previously by Couch (1967) in oysters collected from Chincoteague Bay, Virginia. The six cases with both spore types were dead and dying oysters collected in May and June during the characteristic *H. costale* sporulation time for oysters in Virginia (Couch 1967).

SSO disease in Long Island Sound differed from the above description in several ways. First, plasmodia and sporulation in the present material were found in October to December. During this time period, SSO disease in Virginia is subpatent and no plasmodia are detected before spring (Andrews 1988). However, in a recent paper describing *H. costale* probes also used in the present publication, Stokes and Burreson (2001) reported the presence of a positive ISH to *H. costale* plasmodia in an oyster sampled in October 1994 in Virginia. In the present paper, spores were detected in live specimens with no evidence of a synchronous mortality event. Spores were very rare, and infection always occurred as a coinfection with *H. nelsoni*. Because *H. costale* plasmodia were also detected in the samples, sporulation may occur infrequently. Rare sporulation such as presented in this report cannot sustain a widespread infection. Actual prevalence of *H. costale* in the area is not known based on the results of the present study, but the rare sporulation and the difficulty of finding positive specimens with ISH suggest a low prevalence.

Possible mortality associated with *H. costale* is impossible to estimate because of an *H. nelsoni* epizootic that occurred in Long Island Sound in 1997–1998 (Sunila et al. 1999). Oyster production in Connecticut decreased from more than 500,000 bushels in 1996 to 170,000 in 1999, reflecting high MSX-associated mortalities. According to Andrews (1984), *H. nelsoni* kills oysters much quicker than *H. costale*, and depresses manifestation of SSO disease during MSX epizootics.

H. costale is usually restricted to high-salinity bays with salinities >30‰. Its lower limit is 25‰ and salinities <20‰ appear to cause disease regression (Andrews 1979). It is possible that salinity in Long Island Sound's oyster beds is not high enough to sustain full epizootic *H. costale* activity. In Branford lots (Table 1), salinity varies between 26‰ and 27‰; in the Clinton Cedar Island Marina (in the mouth of Hammonassett River), salinity varies from 13‰ to 28‰. Oysters in the area are transplanted four times before they are marketed. This exposes them to even lower salinities, which may have provided a control for the disease.

Classically, the diagnosis of *H costale* relies on the presence of sporulating stages and the site of initial infection, which for *H. costale* is the epithelium of the digestive system and for *H. nelsoni* is the gill epithelium. In addition, history of the sampling area relating to past *H. costale* or *H. nelsoni* infections directs the diagnosis. In the present report, we were able to diagnose *H. costale* in a new geographic area with a deviating sporulation time by using DNA probes (Stokes & Burreson 1995; Stokes & Burreson 2001). Further research to study the infection cycle, prevalences, and possible association with mortalities is under way.

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