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Carcinogenicity of Black Rock Harbor Sediment to the Eastern Oyster and Trophic Transfer of Black Rock Harbor Carcinogens from the Blue Mussel to the Winter Flounder

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The eastern oyster (*Crassostrea virginica*) developed neoplastic disorders when experimentally exposed both in the laboratory and field to chemically contaminated sediment from Black Rock Harbor (BRH), Bridgeport, Connecticut. Neoplasia was observed in oysters after 30 and 60 days of continuous exposure in a laboratory flow-through system to a 20 mg/L suspension of BRH sediment plus postexposure periods of 0, 30, or 60 days. Composite tumor incidence was 13.6% (49 neoplasms in 40, n = 295) for both exposures. Tumor occurrence was highest in the renal excretory epithelium, followed in order by gill, gonad, gastrointestinal, heart, and embryonic neural tissue. Regression of experimental neoplasia was not observed when the stimulus was discontinued. In field experiments, gill neoplasms developed in oysters deployed in cages for 30 days at BRH and 36 days at a BRH dredge material disposal area in Central Long Island Sound, and kidney and gastrointestinal neooplasms developed in caged oysters deployed 40 days in Quincy Bay, Boston Harbor. Oysters exposed to BRH sediment in the laboratory and in the field accumulated high concentrations of polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and chlorinated pesticides. Chemical analyses demonstrated high concentrations of PCBs, PAHs, chlorinated pesticides, and heavy metals in BRH sediment. Known genotoxic carcinogens, cocarcinogens, and tumor promoters were present as contaminants. The uptake of parent PAH and PCBs from BRH sediment observed in oysters also occurs in blue mussels (*Mytilus edulis*). Winter flounder fed BRH-contaminated blue mussels contained xenobiotic chemicals analyzed in mussels. The flounder developed renal and pancreatic neoplasms and hepatotoxic neoplastic precursor lesions, demonstrating trophic transfer of sediment-bound carcinogens up the food chain.

Introduction

Black Rock Harbor (BRH) is a polluted harbor selected by the U.S. Army Corps of Engineers (COE) and U.S. Environmental Protection Agency (EPA) Environmental Research Laboratory in Narragansett, Rhode Island (ERL/N), in an interagency program to study aquatic dredged material disposal. Black Rock Harbor was selected by the COE and EPA in a generic program to provide techniques and interpretive approaches to dredging and disposal operations and predictive hazard-assessment research strategies in the aquatic environment. Black Rock Harbor is located where Cedar Creek enters Long Island Sound after passing through Bridgeport, Connecticut (Fig. 1). It is heavily polluted by mixed industrial effluent, urban runoff, sewage, and shipping. The shipping channel was dredged by the COE, and dredge spoils were deposited in Central Long Island Sound (CLIS). Preliminary field and laboratory studies under an ERL/N–COE Field Verification Program (*1,2*) for aquatic disposal of the BRH dredged material in Long Island Sound suggested a relationship between tumor occurrence in the eastern oyster (*Crassostrea virginica*) and the winter flounder (*Pseudopleuronectes americanus*) with exposure to contaminated BRH sediment (*3,4*).

A National Cancer Institute (NCI) and EPA interagency agreement on environmental cancer supported the ERL/N in a 2-year research program to study the carcinogenic effects of BRH sediment on fish and mollusks. The molluscan portion of the NCI/EPA study being reported here used laboratory and field exposures to verify the preliminary results that bivalve mollusks exposed to BRH sediment developed tumors. The study also used feeding experiments to determine trophic transfer of suspected carcinogens up the food chain from laboratory-exposed bivalves to fish. Types and amounts of chemicals present in sediment and tissues from various locations were determined analytically; tumors were determined histologically.

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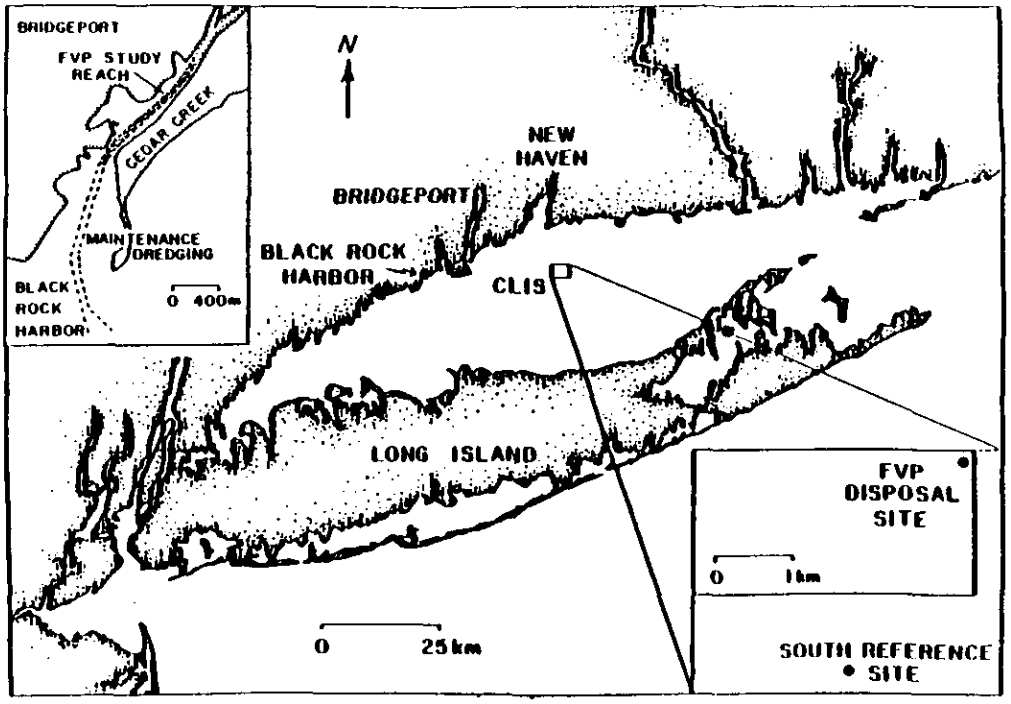

FIGURE 1. Black Rock Harbor sediment dredge and disposal site and Central Long Island Sound sediment reference site.

Materials and Methods

The effects of contaminant uptake and accumulation in oysters as a result of water-column filter-feeding were assessed following 30- and 60-day periods of continuous exposure and similar periods of postexposure. Laboratory research methodology followed recommended ERL/N Quality Assurance procedures and American Society for Testing Materials (ASTM) "Standard Practice for Conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Molluscs" (*5*) where practical.

Field studies were conducted for comparison with the laboratory results. Oysters were deployed at strategic locations in BRH and in CLIS at the dredged material disposal area. The oysters were recovered from BRH after 30 or 60 days and from CLIS after 36 days and evaluated to determine contaminant levels in tissues and the presence of tumors under natural environmental conditions.

Flounder were held in the laboratory for 120 and 365 days and fed a diet of blue mussels. There were three levels of direct exposure to bedded sediment: 100% reference sediment, a 50/50 mixture of reference and BRH sediment, and 100% BRH sediment as bedded sediment. Two regimes of feeding sediment-exposed mussels to winter flounder were used: feeding mussels exposed to reference sediment and feeding mussels exposed to BRH sediment.

Sediments

Sediment was collected from two locations using a Smith-McIntyre grab sampler (area sampled was 0.1 m^2). Reference sediment was collected in Long Island Sound on April 29, 1985, approximately 700 m south of the southern perimeter of the Field Verification Program disposal area (Fig. 1). Contaminated sediment was obtained from the channel of BRH near navigation marker buoy 12 on April 7, 1985.

Sediment collected on each date was returned to the laboratory, press-sieved wet through a 2-mm mesh stainless-steel screen, homogenized, and stored at 4°C until used. Reference and BRH sediments used in oyster tests were stored in 1-gallon wide-mouth glass containers.

Sediment Dosing System. Two identical dosing systems were constructed to deliver reference and BRH sediment to three replicate chambers. Each chamber used a transmissometer (SeaTech, Corvallis, OR) (10 cm) coupled with a microprocessor feed-back device to control a dosing airlift (*6*). The dosing apparatus consisted of a modified 6-L separatory funnel with an airlift and an 18-L polycarbonate reservoir. Sediment slurry was constantly circulated between the dosing funnel and reservoir with the airlift system. Seawater and sediment slurry were mixed in a common chamber, then distributed to the three replicate chambers using a three-way splitter to deliver equal amounts of sediment slurry to each replicate. Using the method described, along with modifications for the present tests, the reference- and BRH-suspended sediment concentration in the water column was maintained at 20 mg/L.

Transmissometers were placed in one reference and in one BRH replicate chamber to monitor sediment delivery to the three replicate chambers. Sediment loading for each replicate was confirmed by dry weight measurements of suspended sediment. Approximately 90 kg of BRH sediment was required for an oyster exposure test.

Organism Collecting/Holding

Adult oysters were obtained from the Cotuit Oyster Company (Cotuit, MA). All the oysters used in laboratory testing were

approximately 4 years in age. These animals were collected on August 15, 1985, from oyster bed 6 located in Cotuit Bay. Testing began September 10, 1985, following a short holding period for laboratory temperature acclimation.

Exposure Chambers. Oysters were exposed to sediment in six all-glass 152 L aquaria (91 long × 44 wide × 38 high cm). Three replicate chambers were used for both reference and BRH sediment. Seawater flowed through each chamber at a rate of 0.65 L/min to yield six daily turnovers of seawater. For the exposure tests, seawater was filtered in the laboratory, and for postexposure periods, seawater was unfiltered. The ambient seawater salinity was 32 ‰ and temperature was 20°C.

A uniform suspension of test sediments was achieved by aligning 30-cm long air stones near one side wall of each test container. Air bubbles provided a continuous lifting, circular motion that resulted in constant water movement, sediment suspension, and aeration without disrupting oysters. Suspended organic detritus and living bacteria contained in BRH and in reference sediments served as the nutritional source during the exposure experiments. Nutrition during postexposure holding was derived from natural food items (i.e., phytoplankton and bacteria) contained in unfiltered seawater.

Oysters were held at three levels within each chamber during exposure using a specially designed tier. The tier was constructed of nine polyethylene grids (34 cm × 26 cm with 1.3-cm square openings) held in position by 1.3-cm diameter threaded polyethylene rod with hex nuts. Horizontally, the three grids at each level were spaced 9 cm apart and approximately 3 cm from the inside walls of the chamber. A transmissometer was placed centrally on the bottom of one reference and one BRH test chamber.

Experimental Test Design. Replicate treatments of 30- and 60-day continuous exposures to reference and BRH suspended particulate were followed by 60 and 30 days of depuration (*3*). Each of the four exposure regimens consisted of three replicates. A minimal sample size of $n = 150$ ($n = 50$/replicate) was used for each continuous exposure regimen (30 and 60 days) and each exposure/postexposure regimen (60/30 and 30/60 days). The number of oysters exposed to each sediment (i.e., reference or BRH) was 600, for a combined total of 1200 animals. Upon conclusion of each treatment (30, 60 days for exposure, and 90 days for exposure/depuration), oysters were prepared for histopathological and chemical evaluations.

The number of oysters collected for histology depended on test organism survival rates. Thirty-five oysters from each of three reference ($n = 105$) and three BRH replicates ($n = 105$) in four treatments (reference $n = 420$; BRH $n = 420$) were allocated for histology (total $n = 840$), while 15 animals from each replicate ($n = 45$/treatment; total $n = 360$) were archived for chemical analyses, assuming 100% survival. Twenty-five oysters were processed for histology and 10 for chemistry as unexposed controls.

Caged Oyster Studies

Oysters were deployed in field tests inside polypropylene pipet baskets (15 × 15 cm or 23 × 23 cm) as a standard test chamber. Ten oysters per basket served as replicate tests. For deployment purposes, replicates were placed end-to-end in a custom-made tube constructed from a 5-cm mesh fish net. The nets were shaped to the pipet test chambers at the time of field deployment by sewing a seam along the longitudinal axis and tying off each end. Each tube could then be suspended from a fixed, above-surface object (as was done in BRH) or attached to a subsurface device for use in deep water (CLIS).

Black Rock Harbor. Two stations were selected for oyster deployment in BRH (Fig. 1): Buoy 12, located at the south side of the channel, and North Dock, on the inland side of the channel. North Dock was located 160 m above a sewage outfall near Cove Marina; buoy 12 was 300 m below the outfall. The two stations were approximately 460 m apart. A reference station was chosen at the Milford, Connecticut, NOAA National Marine Fisheries Laboratory; it was selected because its salinity, conductivity, and temperature measurements were similar to those at the two BRH stations. Oysters deployed at the two BRH stations and the Milford reference station tested effects of 30-day and 60-day exposures on two occasions. In the test, five replicates ($n = 50$) per treatment (i.e., 30-day or 60-day deployment) (total 100 oysters) were deployed at each station.

Central Long Island Sound. Four CLIS stations were selected for testing based on their location relative to the Field Verification Program disposal site for the BRH-dredged material. They had been selected by the COE in the Field Verification Program study. The stations used were the center of the mound, 400 m east of the center, 1000 m east, and a reference known as the "south reference site," approximately 3 km south of the sediment disposal location.

In the test, five replicates of $n = 10$ caged oysters (50 oysters) were deployed at each station for a period of 36 days. Divers deployed the oysters within 1 m of the sediment-water interface on July 7, 1985, and recovered them on August 13, 1985.

Histology/Tissue Preparation

Upon termination of experimental exposures, the oysters were opened and fixed according to procedures established at the ERL/N (*7*). Briefly, those procedures included fixation of tissues in Helly's fixative for 16 hr followed by washing overnight in a water bath tissue washer. Oysters were sagittally sectioned through the visceral mass during the first ½ hr of the fixation process. Adductor muscle was cut on the same longitudinal plane, although severance of the primary sagittal section was finalized only after complete fixation and during final trimming. A centrally located transection was then made, as well as parasagittal sections when tissue mass was large. Sections for embedding were trimmed to a thickness of approximately 3 mm. Thus, a minimum of four tissue blocks was generated for each oyster. Tissues were then trimmed, paraffin embedded, cut at 6 μm and stained with Harris hematoxylin and eosin (H&E) for examination. Heidenhain's stain was used in special cases for observations of connective tissue continuity.

Data Collection/Analyses

The experimental design used (i.e., three replicate aquaria containing 50 animals for each exposure) allowed testing of tank-to-tank variability in statistical analyses. In the absence of tank-to-tank variability, the sample size of $n = 150$ animals per

exposure was adequate to detect an absolute increase of 20% over control if the control incidence was 10% or less (*8*).

Chemical Analyses and Short-Term Testing

Chemical analyses supported histopathological evaluations by providing information on the presence and levels of known carcinogens and their metabolites in various biological tissues and carcinogens and mutagens present in sediment. Those data showed significant correlations between concentrations of tissue contaminants and tumor induction (*9*).

Methods described by Rogerson et al. were used to extract and fractionate BRH and reference sediments for testing in short-term assays (*10*). Whole and fractionated extracts were evaluated for mutagenicity in Salmonella (*11*), sister chromatid exchange in Chinese hamster V79 cells (*12,13*), and inhibition of gap-junctional communication (possible biomarker for tumor promoters), also in V79 cells (*14,15*).

Results

Laboratory Studies of Oysters

Histopathological examination of oysters exposed to BRH sediment in the laboratory demonstrated occurrences of neoplastic lesions involving renal tubular epithelial cells, gastrointestinal (GI) epithelium, respiratory and water tube epithelium, ventricular cardiac muscle, gonadal duct germinal epithelium, and elements of the nervous system. Polypoid and ulcerative lesions were also present in the stomach and mid-gut of some BRH-exposed oysters.

Overall, there were 49 neoplasms in 40 of 295 animals, or 13.6% of BRH sediment-exposed oysters. Multiple forms occurred in nine oysters as combinations that included kidney, GI, and gonad; kidney, GI, and neural; kidney and neural; kidney and gill; and GI and gonad. The highest prevalence of lesions occurred in renal tubule epithelium (Table 1). Twenty-eight of 295, or 9.5% of BRH-exposed oysters examined had renal lesions characterized by proliferative, indeterminant patterns of cellular growth. Eight occurred within interlamellar space and filaments of the respiratory organ, three in the rectal segment of the GI tract, three in heart cardiac musculature, four in gonadal ducts, and three in neural tissue elements.

Adult oysters surviving exposures in three replicates for each of four treatments (i.e., 30, 60, 60/30 and 30/60 days) to two different sediments totaled 864 of 1200 (Table 1). Of these, 643 were examined histologically, and 221 were archived for chemical analyses. Oysters surviving exposures to reference sediment totaled 455 of 600; 338 were examined for histopathology, and 117 were archived for chemistry. Oysters surviving exposures to BRH sediment totaled 409 of 600; 295 were examined for histopathology, and 114 were archived for chemistry. Mortality in oysters from both sediment treatments increased in parallel. Neoplastic or degenerative lesions were not detectable in 338 oysters examined from 30, 60, 60/30, and 30/60 day reference sediment exposure treatments.

Table 1. Oyster tumor induction summary.

	Tumors, number/percent					
	Days exposure/days depuration				Total	
Organ	30/00	60/00	60/30	30/60	*n*	Percent
BRH treatment						
Kidney	8/8	4/4.7	10/15.4	6/13.3	28	9.5
GI	2/2	0/0	0/0	1/2.2	3	1.0
Gill	5/5	2/2.4	0/0	1/2.2	8	2.7
Heart	0/0	2/2.4	0/0	1/2.2	3	1.0
Gonad	2/2	1/1.2	0/0	1/2.2	4	1.3
Neural	0/0	1/1.2	1/1.3	1/2.2	3	1.0
n[a]	100	85	65	45	295	
Total	17	10	11	11	49	16.6
NA[a]	12	10	10	8	40	13.6
BRH control						
n[a]	103	90	74	71	338	0
Total	0	0	0	0	0	0
NA[a]	0	0	0	0	0	0

[a]*n*, number exposed in all replicates per treatment; total, total number of tumors; NA, total number of different animals with tumors.

Neoplastic Disorders in Oysters

Renal Cell Tumors. Renal cell tumors were most frequently observed in the central part of the excretory organ located between the pericardium and adductor muscle. Normal nephridial tubules have a simple low cuboidal to columnar epithelium progressing from anterior to posterior portions of the kidney. Epithelial cells that composed the functional unit of the kidney had an agranular cytoplasm and a small, central to apically located nucleus (Fig. 2). Nuclei arranged in a regular pattern are uniformly hypochromatic with dusty-appearing chromatin and a small nucleolus.

In all renal neoplasms, the prominent contrasting features were hypercellularity of tubular epithelium associated with pattern alteration and nuclear hyperchromasia (Fig. 3). Lesions progressed from isolated to coalescing clusters or nidi of these mitotically active epithelial cells in well-formed tubules to complete tubular involvement with piles of disorganized renal excretory epithelial cells forming distended tubules. Further progression was accompanied by rupture of the basement membrane, diffusion of neoplastic cells into renal sinusoids, and in two cases, disseminated neoplastic cells invaded the outer sheath and large neurons of the visceral ganglion (Figs. 4 and 5).

Water Tube and Gill Filament Tumors. Water tube and gill filament tumors developed in interlamellar septa and gill filamental epithelium of eight oysters exposed to BRH sediment in the laboratory and in two BRH and one CLIS caged oyster (Table 1). Up to six foci occurred in one histological section from a specimen caged 36 days 400 m east of the BRH sediment mound center in CLIS. Early stages consisted of short stretches of thickened basophilic, hyperplastic epithelium. Protoplasm was condensed, and mitotic figures were present. As proliferation continued, lesions arising from gill epithelium developed into large frond-shaped papillomas, and lesions from water tube epithelium developed into glandular adenomas. The most advanced adenomas appeared to invade connective tissue elements of interlamellar septa that divide gill lamellae and form part of water tubes and extended to functional respiratory filamental epithelial surfaces (Fig. 6). Cytologically, hyperchromatic interlamellar water tube and gill filamental epithelia transformed by BRH exposures consisted of tightly packed cuboidal to columnar cells containing small nuclei with basophilic chromatin. An outstanding characteristic of gill water tube papillomas/adenomas was the replacement of epithelial cells by variably

differentiated cells with basophilic tinctorial properties. Clear cells that appeared mucoid usually occurred peripherally in the epithelial neoplasm. Those cells were usually located in a peripheral transition zone between normal and basophilic neoplastic elements. Indistinct cellular membranes, scanty eosinophilic cytoplasm, and a high nuclear to cytoplasmic ratio characterized small cells. A distinct cellular outline, basophilic staining cytoplasm, and a variable nuclear to cytoplasmic ratio characterized the more differentiated, elongated neoplastic cells.

***Gastrointestinal Adenocarcinoma* In Situ.** Adenocarcinomas *in situ* were striking glandular thickenings of the rectal mucosa in three oysters experimentally exposed to BRH sediment (Fig. 7). These grossly visible thickenings ran linearly with the gut but were not circumferential, so that both normal and tumor tissues were present in the same section. The normal rectum consists of simple columnar epithelium with cilia projecting into the lumen. The tumors consisted of up to 25 times as many cells in thickness. Tumor cells were columnar with hyperchromatic nuclei, and many were undergoing mitoses. Many adenomatous structures were formed within the tumor masses, but cilia were generally absent from the luminal borders of these abortive glands. One rectal tumor protruded through the anus and was continuous with the outside body wall (Fig. 8).

Neuroblastomas. Multiple encapsulated neuroblastomas were identified in three BRH-exposed oysters. These focal lesions were variably located in proximity to the alimentary tract, in vesicular connective tissue, in open sinusoidal vasculature, in the outer sheath of circumpallial connective and branchial nerve fibers, and in the gill axial region. Neoplastic development commenced as discrete areas of embryonic neuroblasts with random to rosette-appearing cellular organization. Embryonic tumor cells were round to slightly oval with indistinct cytoplasm. Nuclei were vesicular with scattered clumps of dense-staining chromatin, a prominent nucleolus, and a dense hyperchromatic membrane.

Circulatory System Tumors. The oyster heart is a three-chambered structure suspended at three points in the pericardium. A dorsal aorta supports the ventricle, while two efferent veins support two auricles. A large myxoid mass occurred in the vicinity of the auriculo-ventricular valves in two oysters after 60 days of exposure to BRH sediment in the laboratory. These lesions consisting of stellate-shaped cells with an abundant hyaline ground substance in the intervening spaces were interpreted as myxomas (Fig. 9). Huge papillary polypoid growths originating in the ventricle protruded into the pericardial cavity of three other oysters. Elsewhere in the circulatory system, numerous small anomalies developed in the vasculature. In the adductor muscle vessels, for example, anomalies originated from endothelial lining cells and protruded into the lumens as polypoid masses.

Gonadal Papillary Lesions. Oyster gonadal follicles near the surface of the reproductive organs were affected by exposure to BRH sediment. Four of the oysters experimentally exposed to BRH sediment had multiple polyps that originated in germinal epithelium and projected into lumens of gonadal follicles (Fig. 10). These polyps were pedunculate, and some had a papillary pattern. No polyps were present in control oysters.

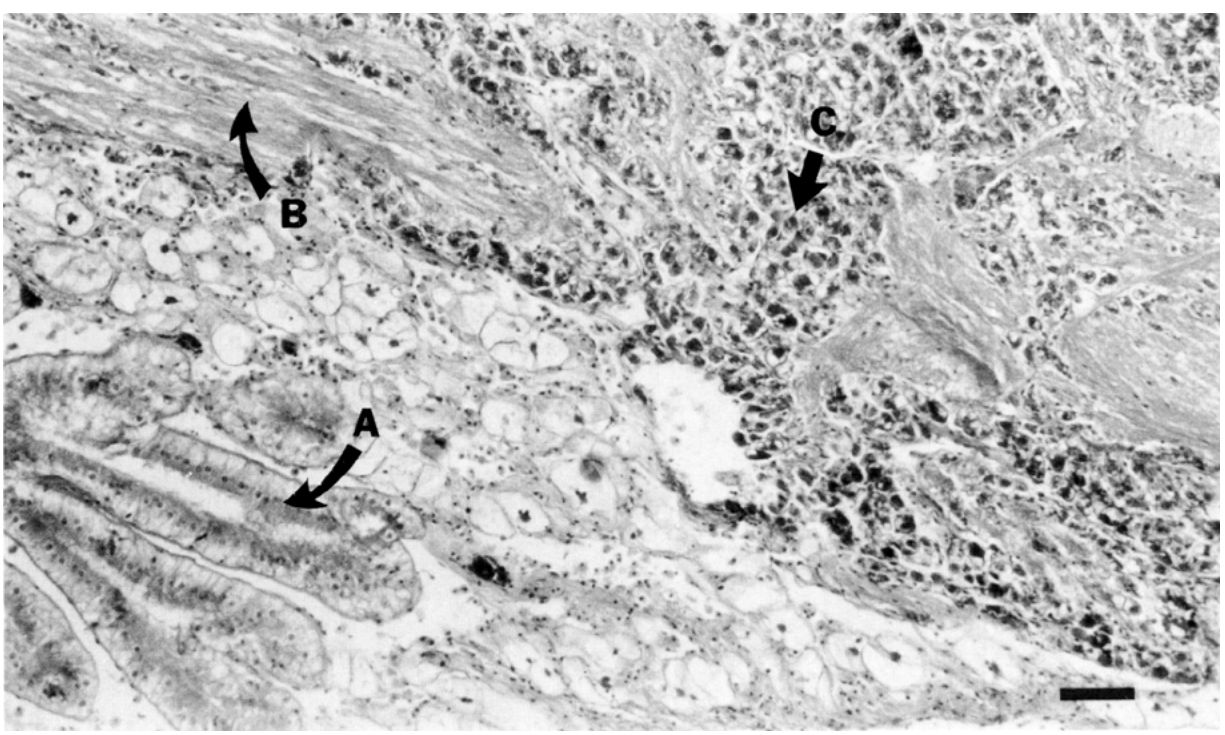

FIGURE 2. Light micrograph of kidney and visceral ganglion of a reference or control oyster held in suspended CLIS (reference) sediment for 30 days. Normal kidney tubules consist of a clear, simple columnar epithelium (arrow *A*), normal visceral ganglion and nerve tract (arrow *B*), normal large neurons (arrow *C*). Bar = 64.3 μm.

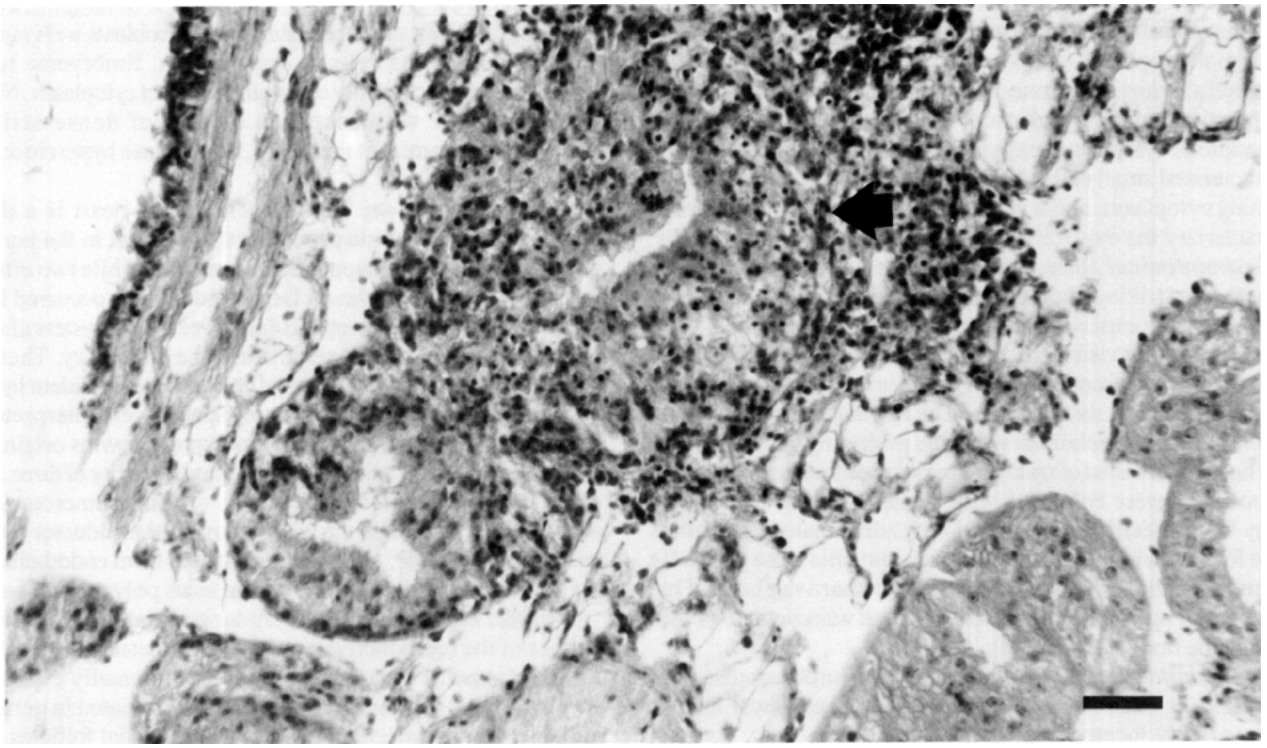

FIGURE 3. Oyster kidney tubules with a neoplastic lesion following continuous exposure to suspended BRH sediment for 30 days. Normal kidney epithelium is replaced by proliferating, hyperchromatic kidney epithelial cells. Arrow indicates area where limiting membrane was breached and neoplastic cells diffused into the renal sinusoidal spaces. Note normal tubules lower portion of micrograph. Bar = 28.6 μm.

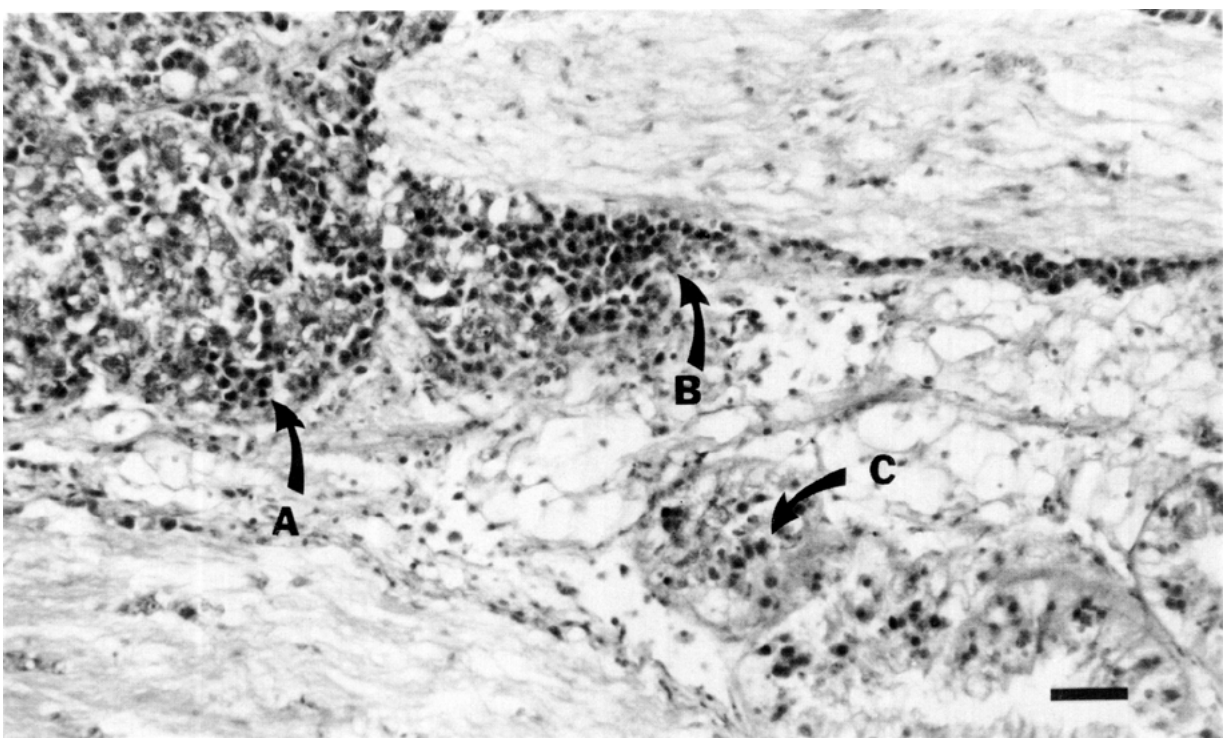

FIGURE 4. Invasion into visceral ganglion and attendant nerve fiber by neoplastic cells. Clusters of proliferating hyperchromatic cells in the visceral ganglion are highlighted (arrow *A*). These cells have replaced the larger neurons of the ganglion. (Arrow *B*) a representative area of the nerve outer sheath having invasive neoplastic cells. Kidney tubules with clusters of neoplastic excretory epithelial cells are located nearby (arrow *C*). Bar =27.4 μm.

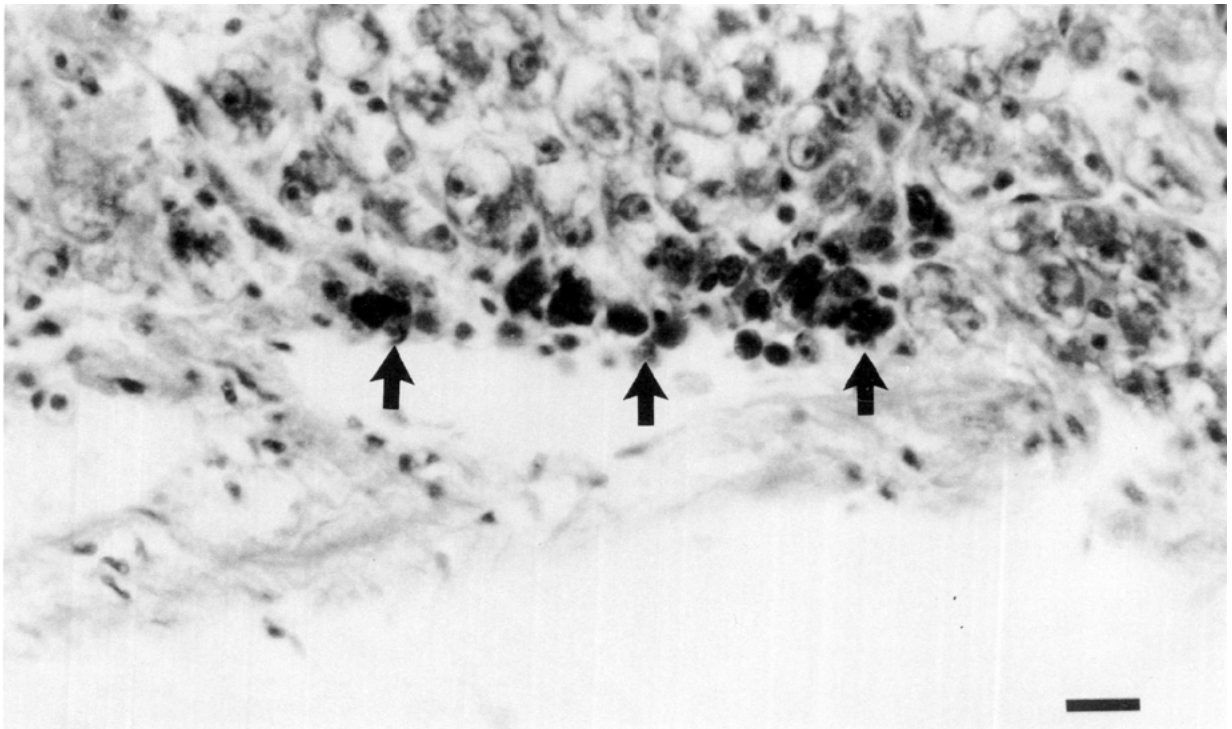

FIGURE 5. Neoplastic cells in the visceral ganglion of an oyster following 60 days of exposure and 30 days postexposure to suspended BRH sediment. Arrows delineate clusters of neoplastic cells near a vascular channel. Bar = 11.4 μm.

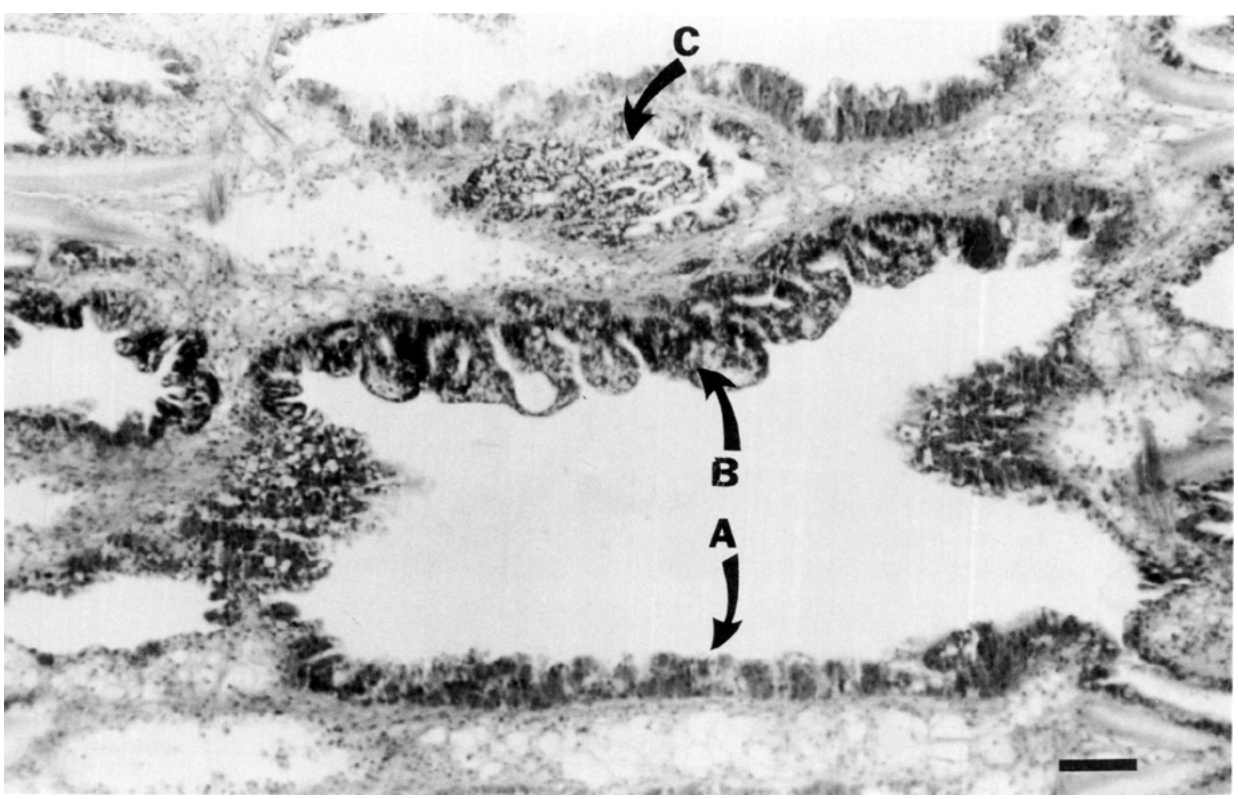

FIGURE 6. Adenomatous lesions on gill water tube and plicate. Compare normal water tube epithelium (arrow *A*) to lesion on interlamellar wall (arrow *B*) and septa (arrow *C*) of a gill water tube. Adenomatous formation also extends along gill plicate of the 30-day BRH sediment-exposed specimen. Bar = 74.3 μm.

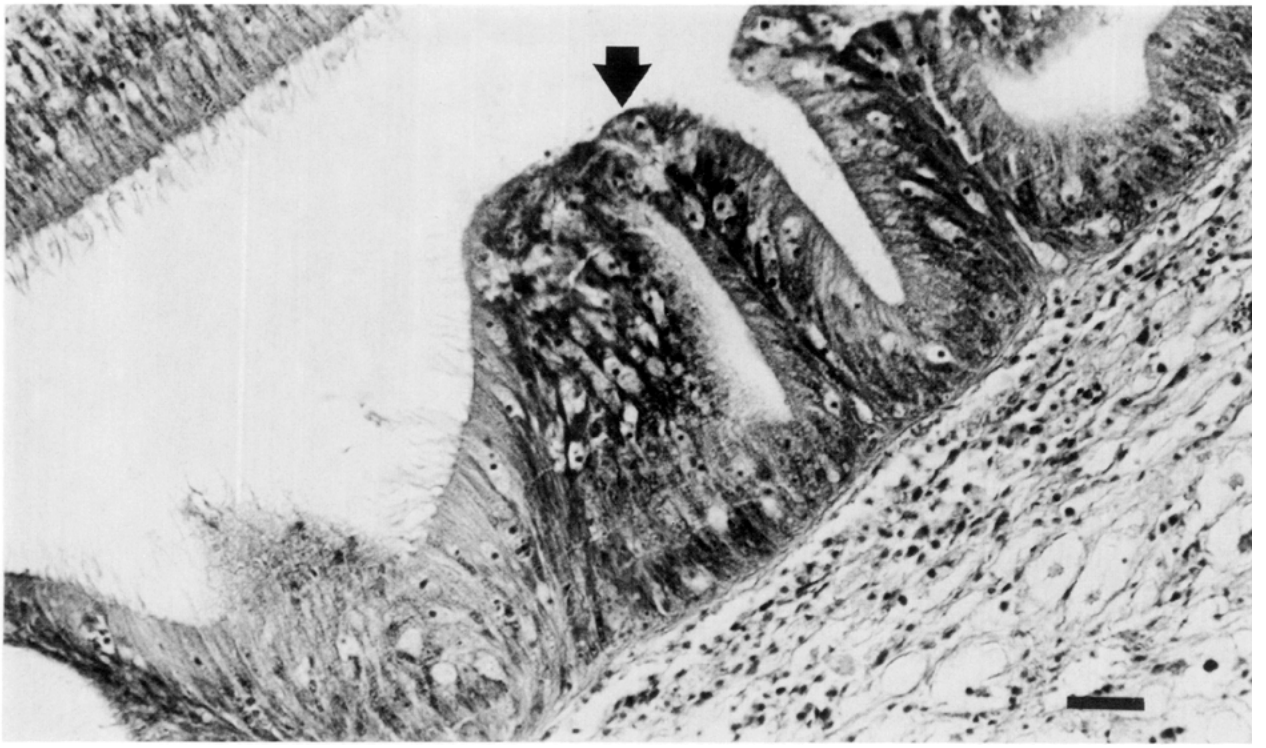

FIGURE 7. Adenocarcinoma *in situ* in rectum of an oyster exposed to BRH sediment for 30 days. Arrow indicates area of hyperchromatic neoplastic cells. Compare the glandular formations with normal ciliated columnar epithelial surface at top of micrograph. Bar = 27.0 μm.

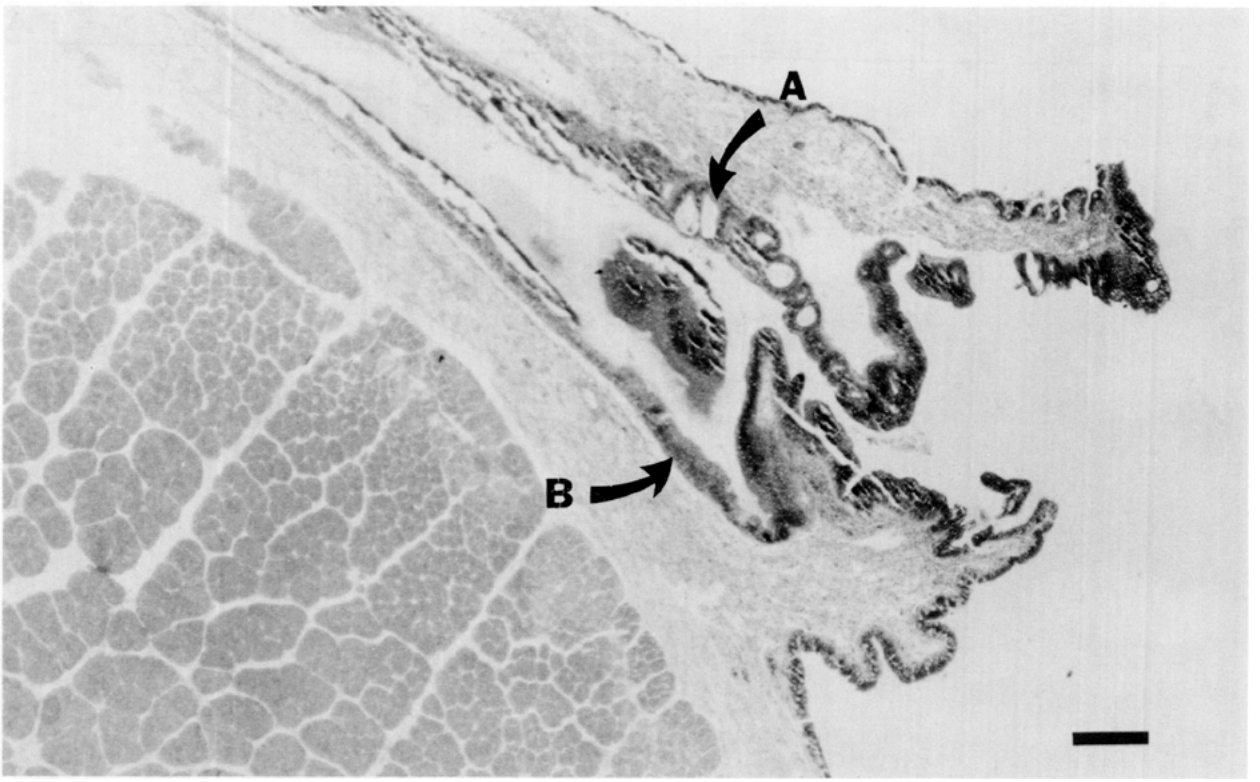

FIGURE 8. Adenocarcinoma *in situ* in rectum and anal rosette of a 30-day BRH-exposed oyster. Glandular formations that characterize the adenomatous lesion (arrow *A*), normal epithelium (arrow *B*). Bar = 17.5 μm.

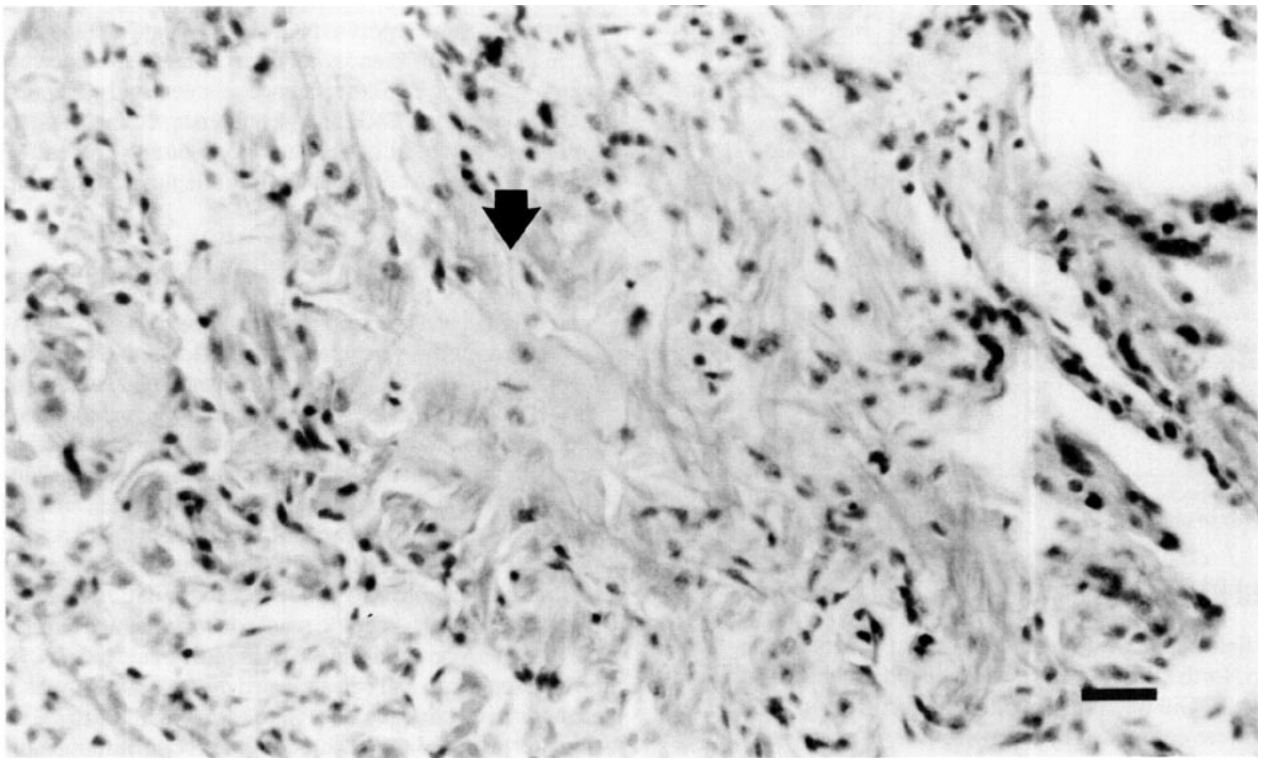

FIGURE 9. Heart atrioventricular valve with swollen cardiac muscle. Note ground substance and stellate-shaped cells (arrow). The lesion is interpreted as a myxoma. BRH 60-day exposure. Bar = 243 μm.

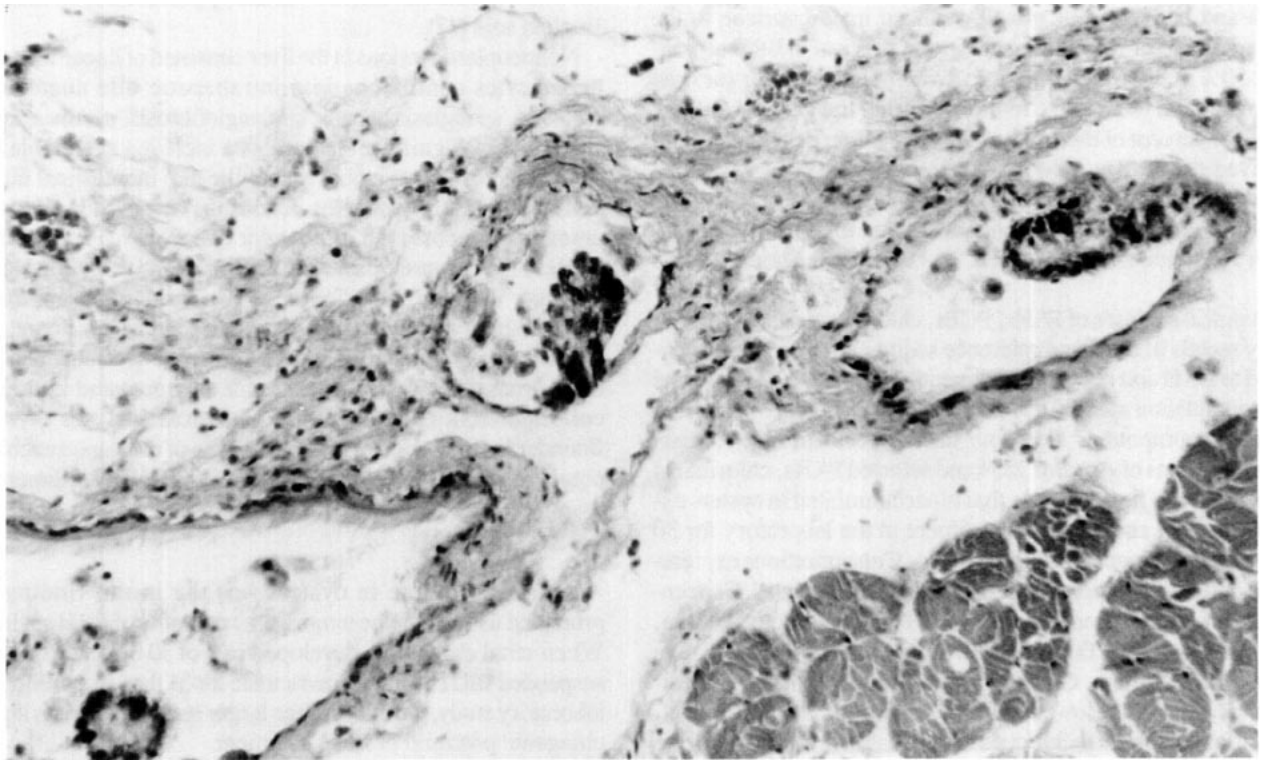

FIGURE 10. Multiple polyps protruding into gonadal duct.

Table 2. Neoplasms in oysters deployed *in situ*.

Site	Oysters, *n*	Neoplasms, *n*		
		Gill	Kidney	GI
Pre-exposure controls[a]	25	0	0	0
Black Rock Harbor				
North dock	46	2	0	0
Buoy 12	48	0	0	0
NOAA reference[b]	63	0	0	0
Central Long Island Sound				
Center	26	0	0	0
400 East	25	1	0	0
1000 East	25	0	0	0
South reference[b]	25	0	0	0
Quincy Bay				
Rainsford	50	0	0	5
Peddocks	50	0	0	1
Nut Island	50	0	1	1
Veazie Rock	50	0	3	1
The Graves[b]	50	0	0	0

[a]Cotuit oysters.
[b]Oyster *in situ* controls.

Field Studies of Oysters

In field deployment studies, two caged oysters held for 30 days at the North Dock in BRH developed gill water tube papillomas (Table 2). Gill water tube papillomas occurring in multiple locations were confirmed by serial sections in one oyster deployed 400 m east of the CLIS disposal mound center where PCB and chlorinated pesticide concentrations were highest. Renal (n = 4) and GI tract (n = 8) tumors developed in 6% of oysters deployed at four stations in Quincy Bay, Boston Harbor, after 40 days of exposure.

Survival of caged oysters held at the two BRH stations (North Dock and buoy 12) was greatly reduced in comparison to the reference site at Milford estuary. Oyster survival in BRH averaged < 50% after 30 days and < 25% after 60 days. Poor survival was attributed to anoxic conditions during the period of study. Sixty-six percent of the oysters deployed at four stations in CLIS survived the 36-day exposure period. The survival in Quincy Bay-deployed oysters was > 90%. Neoplasms were not observed in oysters deployed at reference locations in Milford estuary, south reference site (CLIS) and The Graves in Massachusetts Bay.

Chemical analyses of PAHs, PCBs, chlorinated pesticides, and heavy metals in BRH and reference sediments and in oysters exposed to BRH and reference sediments demonstrated uptake and bioaccumulation after 30 and 60 days. Depuration of organic and inorganic compounds did occur during postexposure studies. Concentrations of Aroclor 1254 and selected PAHs, chlorinated pesticides, and heavy metals that bioaccumulated in oysters exposed to BRH and reference sediment in the laboratory for 30 days are summarized in Tables 3 and 4. Concentrations expressed for oyster tissue represent a mean of three replicates. Concentrations of selected compounds in BRH and reference sediment, also represented in Table 3, allow for a comparison of relative contaminant loading. Chemical analytical procedures for sediment and tissue followed standards adopted at the ERL/N. Details of analytical techniques are available in a final report to the National Cancer Institute for a study on the carcinogenic effects of BRH sediment on mollusks and fish (*9*).

Sediment and tissue chemical concentration data were obtained from the same report to NCI (*9*). The data included in Table 3 demonstrates differences between contaminant concentrations when comparing BRH to reference sediment and oysters exposed in the laboratory to BRH and/or reference sediment and the carcinogenic potential of selected compounds present. Tissue chemical concentration data for field-deployed oysters were included in Table 4.

Laboratory Studies of Winter Flounder

Neoplastic, and nonneoplastic lesions were observed in young-of-the-year and 1-year class winter flounder exposed in the laboratory to BRH sediment contaminants after 3 and 12 months. These lesions consisted of renal capillary hemangiomas, small nephroblastomas or islands of retained blastema, renal cysts, a renal adenocarcinoma, and adenomas in pancreatic islets. Incidence of capillary hemangiomas ranged between and 38% and nephroblastomas between 5 and 25% in flounder exposed to the five BRH treatments. No capillary hemangiomas were observed in flounder exposed to reference sediment and fed uncontaminated mussels. A cystic renal adenocarcinoma developed in the kidney of one animal in the worst-case treatment, 100% BRH bedded sediment, and contaminated mussels, after 1 year. Cystic adenomas in pancreatic islets were observed in 11 to 67% of those flounder exposed to the three highest concentrations of BRH sediment treatments (50% BRH sediment and BRH-contaminated mussels, 100% BRH sediment, and both uncontaminated and BRH-contaminated mussels). Hepatocytic megalocytosis, developed in livers of flounder in all five BRH treatments and persisted after 60 days postexposure holding. The lesion has been correlated with the appearance of neoplasia in English sole (*17*).

Nonneoplastic lesions in the liver consisted of linear vacuolated hepatocytes continuous into intrahepatic bile ductule/duct epithelia, spongiosis hepatis, cholangiofibrosis, necrosis, and inflammation. Significant hyperplasia included nesidioblastosis and multiple adenomatous, basophilic nidi in intestinal mucosa and submucosa. Most of the conditions observed in laboratory animals were observed in flounder collected from Black Rock Harbor, Narragansett Bay, New Bedford Harbor, Boston Harbor, Martha's Vineyard, East Cape Cod Bay, and Long Island Sound. None of the conditions discussed occurred in winter flounder exposed in the laboratory to bedded reference sediment and fed a diet of mussels exposed to reference sediment and in flounder collected from Georges Bank. Chemical analyses of winter flounder have demonstrated the presence of the same xenobiotics observed in BRH-contaminated mussels and BRH sediment (*9*).

Discussion

Renal carcinoma in oysters was the initial finding that prompted us to study the tumorigenic potential of BRH sediment. When renal carcinoma developed in 2 of 10 oysters exposed to suspended BRH sediment particulate for 31 days in a preliminary laboratory study, we initiated the larger study to confirm the carcinogenic potential of BRH sediment.

The etiopathic relationship established between BRH sediment and neoplastic disorders in oysters indicates that

Table 3. Selected chemical concentrations identified in BRH sediment and in oysters exposed to BRH sediment for 30 days in the laboratory.[a]

Chemical	Sediment, ng/g dry weight		Oyster, ng/g dry weight	
	BRH	Reference	BRH	Reference
Sufficient evidence of carcinogenicity[b]				
Benz(*a*)anthracene	3450 ± 336	122.0 ± 15.7	694.8 ± 61.8	10.8 ± 4.8
Benzo(*a*)pyrene	3160 ± 311	243.0 ± 46.8	88.3 ± 14.3	2.1 ± 0.5
Benzfluoranthene	5970 ± 542	470.0 ± 106.0	364.3 ± 49.8	18.5 ± 2.8
Indeno(1,2,3-*cd*)pyrene			22.1 ± 4.8	1.4 ± 0.9
Dibenz(*a,h*)anthracene			9.0 ± 1.8	0.8 ± 0.2
Hexachlorobenzene			0.2 ± 0.3	0.0 ± 0.0
Chlordanes (alpha-, gamma-chlordane)			100.5 ± 11.0	8.6 ± 3.1
Nickel[c]	170 ± 16	25.5 ± 2.6	1.3 ± 0.2	0.7 ± 0.4
Lead[c]	420 ± 29	54.6 ± 4.2	2.0 ± 0.6	1.1 ± 0.2
Cadmium[c]	23.7 ± 1	0.2 ± 0.1	1.9 ± 0.4	0.9 ± 0.1
Chromium[c]	1480 ± 104	50.3 ± 13.8	2.2 ± 1.1	0.4 ± 0.2
Limited evidence of carcinogenicity				
Chrysene	4450 ± 377	174.0 ± 23.0	1260.0 ± 79.4	21.9 ± 4.6
Inadequate evidence of carcinogenicity				
Benz(e)pyrene	2880 ± 270	217.0 ± 44.8	264.3 ± 35.4	15.4 ± 2.4
Fluorene	635 ± 113	4.9 ± 0.6	41.7 ± 4.5	4.6 ± 0.9
Phenathracene	4020 ± 617	70.4 ± 7.8	553.3 ± 37.1	19.7 ± 3.7
Perylene	504 ± 127	66.7 ± 2.2	16.9 ± 6.3	1.5 ± 0.4
Benz(*ghi*)perylene			37.1 ± 7.7	2.3 ± 1.1
Coronene			1.3 ± 1.5	0.8 ± 0.4
No evidence of carcinogenicity				
Anthracene	1330 ± 288	10.8 ± 1.5	191.3 ± 23.0	2.8 ± 0.3
Fluoranthene	5800 ± 372	12.3 ± 1.9	1776.7 ± 25.2	44.6 ± 8.3
Promoter				
DDT (*p,p'*-DDD; *p,p'*-DDE; *p,p'*-DDT)			1183 ± 135.6	67.0 ± 23.6
PCBs (Aroclor 1254)	7170 ± 566	39.8 ± 4.4	1143 ± 162.9	307.3 ± 173.5
Pyrene	7250 ± 449	249.0 ± 27.9	2950 ± 130.0	39.1 ± 10.4

[a]Data from Gardner et al. (*9*).
[b]Evidence of carcinogenicity in experimental animals from International Agency for Research on Cancer (*16*).
[c]Metals expressed as microgram per gram dry weight.

Table 4. Bioconcentration of selected chemicals identified in oysters deployed *in situ* at BRH for 30 days and CLIS for 36 days.[a]

Site	PCBs	PAHs, ng/g				Metals, μg/g		Pesticides, ng/g
	Aroclor 1254	BaA	BaP	InD	DA	Cd	Cr	DDT series
Cotuit[b]	284	10	0.5	0.6	0.1	0.7	0.3	31.9
Black Rock Harbor								
North Dock	249	234	31.6	9.6	4.3	1.6	0.4	154.6
Buoy 12	305	233	30.9	9.5	4.5	1.2	0.3	171.0
NOAA reference	325	85	11.3	6.4	2.8	2.0	1.0	88.8
Indigenous	840	80	2.9	5.7	0.0	5.6	0.5	234.7
Central Long Island Sound								
Center	473	53.3	11.5	14.7	5.0	4.1	1.2	47.3
400 East	683	33.4	12.2	12.3	4.2	2.8	0.7	83.6
1000 East	337	23.0	14.0	12.2	4.6	2.5	0.8	30.9
South reference	514	24.1	5.3	9.0	3.4	3.3	0.8	45.4

[a]Values expressed as dry weight. BaA, benz(*a*)anthracene; BaP, benzo(*a*)pyrene; Ind, indeno(1,2,3-*cd*)pyrene; DA, dibenz(*a,h*)anthracene; DDT series, *p,p'*-DDD, *p,p'*-DDE, and *p,p'*-DDT.
[b]Pre-exposure controls.

industrially polluted Black Rock Harbor is a source of biologically available oncogenic substances. Experimentally induced neoplasia in oysters represents the first demonstration of a causal relationship between neoplasms in marine molluscs and exposures to contaminated marine sediment (*4,8*). These results also represent the first field investigations concurrent with laboratory studies to verify neoplasia in a marine bivalve mollusc exposed *in situ*. Chemical characterization of BRH sediment that confirmed the presence of carcinogens, co-carcinogens, and tumor promoters (*10*) was further supported by positive Ames test results for genotoxic agents and the V79 metabolic cooperation assay for tumor promoters (*9*). Increased frequencies of sister chromatid exchange were also observed in a marine worm, *Nephtys incisa*, exposed to whole BRH sediment in laboratory tests and at the field disposal site (*19–21*).

The success of these investigations was due in part to a unique approach in aquatic animal laboratory experimentation that departed from the traditional method of testing water column pollutants to one focused on sediment contaminants. Organic compounds from several major chemical classes occur in BRH sediment. Compounds quantified include PCBs, PAHs, polycyclic aromatic ketones, polycyclic aromatic quinones, and carbazoles (*9,10*). Several inorganic elements were also quantified, including iron, chromium, copper, zinc, cadmium, lead, nickel, manganese, and mercury. Traditional short-term (28 day) tests with various species of invertebrate and vertebrate marine

fauna during laboratory Field Verification Program investigations failed to demonstrate overt toxicity after exposures to BRH sediment with the exception of amphipods.

Major characteristics of the renal carcinomas were irregular basophilic tubules that usually infiltrated interrenal sinusoids. One case had invaded the visceral ganglion as well as the outer sheath and associated nerve tracts. These are the first renal carcinomas in bivalve mollusks except for a single, poorly differentiated carcinoma reported in a fresh water mussel following water column exposures to *N*-nitroso compounds (*22*).

While tumors in renal excretory epithelium provided the strongest demonstration of a causal relationship with BRH sediment, five other types of experimental tumors were also expressed within 30 and 60 days after exposure to BRH sediment. Frequency data demonstrated that the other experimental tumors also occurred most often in organs performing excretory roles such as gill, heart, and GI tract. All these lesions persisted without diminution after discontinuation of exposures to BRH sediment and after 30 and/or 60 day latency postexposure periods. Therefore, the induced lesions were autonomous and fulfilled an important criteria for neoplasia. Furthermore, the neoplastic disorders reported here were reproduced in a second, identical laboratory test of $n = 1200$ animals.

Excretory tissue tumors were not restricted to nephridial tubules *per se*, but often involved renopericardial openings, funnels, and canals as well as epithelial surfaces of the renal reservoir that retains waste fluids until discharge to the external environment. Neoplastic disorders in the heart may be related to the elimination of BRH contaminants as the organ passes wastes into kidney channels during ultrafiltration. Papillary polypoid formations observed on the ventricles and on the roots of anterior and posterior aortas, appearing as gross lesions approximately one-third the dimension of the ventricle itself, demonstrates the potential for rapid growth following exposure to BRH sediment. The blue mussel, *Mytilus edulis*, exposed to BRH particulate (10 mg/L for 28 days) in the same manner as oysters during Field Verification Program studies at ERL/N, also exhibited disorders of the heart (*23*).

Gill and rectal adenomatous lesions occured in nonciliated and ciliated epithelial surfaces that have constant physical contact with organic and inorganic particulate matter from the moment the oyster filters it as a potential food item until elimination to the surrounding aquatic environment. The nature of lesions in these tissues suggested that prolonged contact with chemically laden BRH particulates, whether diverted to digestive diverticula or passed through the gastrointestinal tract, was a factor in the development of neoplasms. Presumably, additional exposure of those surfaces to toxic substances may occur as a result of increased removal of heavy metals through gill filaments and rectal midgut by diapedesis (*24*). Toxic action of BRH sediment transported into the alimentary canal also manifests papillary and polypoid formations and occasionally massive stomach ulcerations that suggest the mechanics of food collection, digestion, and waste elimination in combination with physical and chemical properties of the particulate are important keys in the rapid uptake and possible accummulation of BRH contaminants.

Barry et al. (*25*) reported gill and kidney hyperplasia in the soft-shell clam, *Mya arenaria*, but established no relationship with chemical pollutants. Lesions in oyster gills have been reported several times as gill hyperplasia (*26,27*). Harshbarger and co-workers reported four cases of gill hyperplasia in 20,000 *C. virginica* collected from the Maryland portion of the Chesapeake Bay, and Farley (*26*) induced a 48% prevalence of hyperplasia in gills of the Olympia oyster (*Ostreola conchaphlia*, formerly *Ostrea lurida*) after 3 months of an 18-month *in situ* study in Yaquina Bay, OR. The thickened segments of gill with increased cellularity, nuclear size, and cytoplasmic basophilia that characterized the hyperplasia in Chesapeake Bay oysters and Olympia oysters introduced in Yaquina Bay were also observed in BRH-exposed oysters, but what we induced in oyster gills with BRH contaminants was more extensive. BRH sediment produced basophilic, mitotically active cellular components in lesions with adenomatous, papillary, and frond patterns. Gill tumors in oysters deployed at BRH and CLIS locations were histologically characteristic of those in oysters exposed to BRH sediments in the laboratory. The gill water tube tumor with multiple neoplastic papillomas observed in an oyster deployed 36 days at 1 m above the BRH-dredged material 400 m east in CLIS and in two oysters deployed 30 days in BRH in combination with high tissue levels of carcinogens known to occur in BRH sediment suggests an etiological linkage to BRH dredged/disposed sediment. The evidence in those field verification studies provided an impetus for *in situ* studies with oysters in a congressionally mandated evaluation of Quincy Bay, Boston Harbor. The Quincy Bay study (Table 2) supported our observations in the NCI program as 6% of those experimental oysters deployed at four locations (two near sewage outfalls) in Quincy Bay for 40 days had GI tract and renal neoplasms. Oysters deployed at a reference site in Massachusetts Bay for the same period of time did not have neoplasms (*28*).

The identification of neoplasms representing nearly all tissue systems in the several dozen different bivalve mollusk species over the past three decades has stimulated scientific and public interest in their significance, although etiology of these conditions remains elusive. Much of the literature can be found in the following proceedings, reviews and selected research papers (*26,29–37*). Reviewers also note that molluscan neoplasms are predominantly of hemic origin and that the evidence favors a viral rather than a chemical etiology. That conclusion is supported by our work because no hemic neoplasms were induced in our oysters exposed to sediments containing known genotoxic car inogens, co-carcinogens, and tumor promoters.

Short-term testing of fractionated tumorigenic extracts provided evidence for the presence of tumor initiators and promoters. Potential initiators required metabolism to active forms in those tests, whereas potential promoters appeared to be direct acting (*9*). Also, there is now evidence that the oyster can metabolize some procarcinogens [e.g., benz(*a*)pyrene] identified in the tumorigenic sediment (*38,39*). Other procarcinogens (e.g., aromatic amines) perhaps present in the sediment but not yet identified can be activated to bacterial mutagens by other bivalves (*40–42*). At sufficient concentrations, these might act as complete carcinogens; at lower concentrations, they may be more important as initiators. Other compounds (e.g., chlordanes, DDTs, and PCBs) found in the tumorigenic sediment may be more important as promoters (*43–45*).

Our study demonstrates the carcinogenic effects of a specific contaminated sediment in two bivalve mollusk species and a

benthic fish, the winter flounder. These results support what investigators have long suspected in fish cancer epidemics, that sediments are an important source of carcinogens (*46*). We have also shown that sediment-bound carcinogens are trophically transferred. We agree with the view of Farrington et al. that future research should place more emphasis on sediment (*47*). Research in progress is addressing tumor induction with specific carcinogens identified in tumorigenic sediments.

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