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Marine Environmental Research 53 (2002) 17–35

MARINE
ENVIRONMENTAL
RESEARCH

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Cellular responses and disease expression in oysters (*Crassostrea virginica*) exposed to suspended field — contaminated sediments

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Received 22 January 2000; received in revised form 15 December 2000; accepted 30 January 2001

Abstract

Exposure of oysters to water soluble fractions derived from field-contaminated sediments (FCS) containing predominantly lower molecular weight organic aromatic compounds, has been previously demonstrated to enhance pre-existing infections caused by the protozoan parasite, *Perkinsus marinus* (Dermo), and the prevalence of experimentally induced infections. To further explore the role of pollution on the onset and progression of disease, effects of suspended FCS from an estuarine creek in Virginia, USA, dominated by higher molecular weight polycyclic aromatic hydrocarbons (PAHs) on cellular responses and Dermo disease expression in oysters (*Crassostrea virginica*) were examined. Sediments were collected from a PAH polluted estuarine creek in Virginia, USA. To test effects on cellular response, oysters from Maine were exposed daily to 0, 1.0, 1.5, or 2.0 g suspended FCS (corresponding to 0, 70.2, 105, or 140 µg PAHs, respectively) for 5, 10, 20, and 40 days. Hemocyte activities and plasma lipid, protein and lactate dehydrogenase (LDH) levels were then measured. Exposure stimulated neutral red uptake, MTT reduction, and ³H-leucine incorporation in oyster hemocytes at various exposure times, but did not affect the plasma protein, lipid and LDH levels. To test effects on Dermo expression, oysters from a Dermo enzootic area, with an initial estimated infection prevalence of 39%, were exposed daily to 0, 1.0, 1.5, or 2.0 g suspended FCS (corresponding to 0, 75.0, 113, or 150 µg PAHs, respectively) for 30 days. Exposure enhanced disease expression in oysters. However, no significant change was noted in any measured cellular or humoral parameters. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Oyster; Diseases; *Perkinsus marinus*; Polycyclic aromatic hydrocarbons; Effects-physiology; Hemocyte activities; Neutral red; MTT reduction; ³H-leucine incorporation

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1. Introduction

Pollution of the estuarine environment has been linked to a variety of effects on aquatic organisms. Mortalities have been observed in connection with episodic releases of xenobiotics. However, sublethal effects of pollutants are less well understood. Sublethal stress may affect normal physiological functions such as the immune system, resulting in reduced disease resistance (Mix, 1988; Sinderman, 1983; Sinderman, 1993; Vethaak & Rheinallt, 1992). It has been suggested that environmental degradation may contribute to the initiation and intensity of infectious disease epizootics in aquatic animals (Mix, 1988; Sinderman, 1983).

In aquatic ecosystems, both water- and sediment-borne contaminants are of concern. The bioavailability of sediment-associated pollutants is a critical factor mediating their toxicity (Adams, Kimerle, & Barnett, 1992). Generally, low molecular weight organic contaminants are well represented in the liquid phase and high molecular weight hydrophobic contaminants are preferentially retained in the sediment organic phase. Benthic filter-feeding bivalves, such as oysters are continuously exposed to a suite of contaminants associated with both the dissolved and particulate phases. Some interactions between pollution and the onset and progression of the infectious disease caused by the protozoan parasite, *Perkinsus marinus* (Dermo), in eastern oysters (*Crassostrea virginica*) have been previously investigated (Anderson, Unger, & Burreson, 1996; Chu & Hale, 1994; Fisher, Oliver, Sutton, Walker, Manning, & Lytle, 1999). The eastern oyster has historically supported a major fishery on the east coast of the United States. *P. marinus* is one of two parasites causing severe mortality in eastern oyster populations from the mid-Atlantic to the Gulf of Mexico since the 1950s. Our previous study demonstrated that exposure of oysters to a water soluble fraction (WSF) derived from creosote-contaminated sediments from the Elizabeth River, enhanced pre-existing *P. marinus* infection and the prevalence of experimentally induced infections (Chu & Hale, 1994). The WSF in that study contained predominantly low molecular weight and highly bioavailable polycyclic aromatic hydrocarbons (PAHs) and related heterocyclic compounds. To further explore the role of pollution on the onset and progression of infectious disease caused by *P. marinus*, we investigated here the hemocyte function, level of plasma lipid, protein, and lactate dehydrogenase, and *P. marinus* disease expression in oysters (*C. virginica*) exposed to different doses of suspended field contaminated sediments (FCS) dominated by higher molecular weight, hydrophobic PAHs.

2. Materials and methods

2.1. Contaminated sediments and chemical analyses

Surficial contaminated sediments for the two experiments were collected on two occasions, from approximately the same location in Scuffletown Creek, a small tidal creek located on the eastern bank of the Southern Branch of the Elizabeth River,

Virginia. This area is noted for its high sediment burdens of PAHs (Bieri, Hein, Huggett, Shou, Sloane, Smith, & Su, 1986). The creek is directly across the river from a recently closed creosote application facility (about 1300 m away), now being remediated, after being declared a federal “Super Fund” site. Each sediment collection was sieved to remove large inclusions, homogenized in a commercial cement mixer and separately stored in plastic buckets at 4°C until use. Each was re-homogenized immediately before use and representative aliquots chemically characterized.

To determine organic pollutant burdens, sediment aliquots and oysters were chemically desiccated with sodium sulfate and surrogate standards containing d_8 -naphthalene, d_{10} -fluorene, 1,1'-binaphthyl and polychlorinated biphenyl (PCB) congeners (IUPAC 30, 65 and 204) added. Sodium sulfate blanks were analyzed with the samples to monitor for laboratory contamination. Samples were extracted with methylene chloride at 100°C and 1500 psi for 10 min on an enhanced solvent extractor (Dionex ASE 200). Details of the extract purification and subsequent gas chromatographic (GC) analysis methods have been previously published (Chu & Hale, 1994). Briefly, coextracted lipids were removed by gel permeation chromatography and extracts further purified by normal phase liquid chromatography. Purified extracts were analyzed by GC on 60 m DB-5 (J&W Scientific) columns. Detection was by flame ionization (FID) and electrolytic conductivity detectors (ELCD). Compound identification was accomplished by application of a relative retention index, followed by mass spectral confirmation in the electron impact mode. Quantitation was by the internal standard method, using p-terphenyl and pentachlorobenzene, added immediately prior to GC analysis. The major organic compounds detected in the FCS were PAHs (Table 1). Composition was dominated by high molecular weight components, likely derived from historical releases from nearby industries. The relative contributions of individual PAHs to the total were consistent in the two sediment batches used in subsequent experiments. The total of the 13 most abundant PAHs in the sediments used for Experiment 1 and 2 were 70.2 (S.D. = 5.94) and 75.0 mg/kg (S.D. = 15.0) dry sediments, respectively (Table 1). PCB and DDT metabolite concentrations were more than two orders of magnitude lower than total PAHs.

FCS metal concentrations were also determined. Sediments were prepared with concentrated trace metal grade HCl and HNO₃. Standards of each element examined were prepared in 1% HNO₃ from sequential dilutions of commercially available standards. Samples were dried at 110°C and thoroughly ground. Aliquots were transferred to beakers and deionized water and then *aqua regia* (3:1 HCl:HNO₃) added. Beakers were covered and refluxed for 2 h at 90°C and additional *aqua regia* added to prevent evaporation to dryness. Deionized water was added after cooling and the solution, vacuum filtered. A Perkin–Elmer Model 1100B Atomic Absorption Spectrophotometer was used for all determinations via air/acetylene flame (Cu and Zn), graphite furnace (Ag, As, Cd, Cr, Ni, Pb, Sb, Se, Tl), or cold vapor reduction with 1% sodium borohydride (Hg). Sediment trace metal concentrations were elevated from background levels, as expected for an industrialized area (Table 1).

2.2. Cellular responses

Hemolymph (1–2 ml) was withdrawn from the adductor muscle sinus of individual oysters with a syringe inserted through a notch on the shell, and samples were placed in an ice bath. Total numbers of hemocytes, granulocytes and hyalinocytes were counted in each hemolymph sample using a Bright-Line hemocytometer (Reichert, Buffalo, NY). Cellular responses were evaluated, including neutral red (NR) uptake, the reduction of a tetrazolium dye (MTT reduction), and ^3H -leucine incorporation by hemocytes of individual oysters. NR uptake and MTT reduction assays have been used for monitoring cell viability and/or proliferation in culture (Borenfreund & Puerner, 1984; Buttke, McCubrey, & Owen, 1993; Hansen, Nielsen,

Table 1

Concentrations (mg/kg dry wt. basis) of metals and organic contaminants in Scuffletown Creek sediments used in Experiments 1 and 2

Analyte	Experiment 1	Experiment 2
Ag	1.90	2.20
As	13.0	27.0
Cd	2.70	2.60
Cr	43.0	54.0
Cu	95.0	164
Ni	2.20	2.40
Pb	107	148
Sb	<0.50	<0.50
Se	<0.50	<0.50
Tl	<0.30	<0.30
Zn	360	450
Hg	4.50	4.70
Phenanthrene	3.72±0.12	4.46±1.22
Anthracene	1.65±0.25	1.54±0.11
4H-cyclopenta(def)phenanthrene	1.35±0.17	1.78±0.44
Methylphenylnaphthalene	2.50±0.22	2.41±0.44
Fluoranthene	10.9±0.80	11.7±2.85
Pyrene	9.24±0.45	11.2±2.20
MW216 and 232 (several isomers)	13.5±1.88	13.0±1.83
Benz(a)anthracene	4.73±0.83	4.80±0.91
Chrysene	7.01±1.58	6.76±1.70
Benzo(e)pyrene	5.28±0.67	5.36±1.20
Benzo(a)pyrene	4.63±0.85	5.39±1.18
Indeno(1,2,3-cd)pyrene	2.99±0.56	3.38±0.67
Benzo(ghi)perylene	2.70±0.55	3.21±0.64
Total of these PAHs	70.2±5.94	75.0±15.0
Total PCBs	0.41±0.09	0.52±0.09
4,4-DDT	0.03±0.00	0.06±0.00
4,4-DDD	0.02±0.00	0.02±0.00
4,4-DDE	0.01±0.00	0.01±0.00

& Berg, 1989; Skarmeta, Bandin, Santos, & Toranzo, 1995). However, these two assays do not directly measure cell viability. The NR assay examines uptake of liquid droplets, commonly referred to as pinocytosis or fluid-phase endocytosis by living cells, thus it is a measure of membrane stability. NR can be taken up into living cells via membrane diffusion and/or pinocytosis, which involves membrane fusion. Thus, changes in NR uptake could be an indication of a change of function related to the cell membrane. The MTT reduction assesses the reduction of a tetrazolium dye (3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazan by mitochondrial dehydrogenase, coupled to the reduction of NADP (nicotinamide adenine dinucleotide phosphate (oxidised form)) or NAD (nicotinamide adenine dinucleotide (oxidised form)) to NADPH (nicotinamide adenine dinucleotide phosphate (reduced form)) or NADH (nicotinamide adenine dinucleotide (reduced form)), respectively (Dunigan, Waters, & Owen, 1995). ^3H -leucine incorporation by hemocytes is indicative of protein synthesis. Cell-free hemolymph (plasma) of individual oysters was assayed for protein, lipid and lactate dehydrogenase (LDH) levels. The LDH assay measures the amount of cytoplasmic LDH released into the medium (or plasma), and thus evaluates membrane integrity.

2.2.1. NR uptake

NR uptake by hemocytes was assessed using a “Neutral Red Bioassay” kit (Clonetics Corporation, San Diego, California, USA; Borenfreund & Puerner, 1984). Briefly, duplicate 200 μl of hemolymph from individual oysters were added to the wells of 96-well microtiter test plates. The plates were centrifuged for 5 min at $50\text{--}60\times g$ at 4°C , to facilitate hemocyte attachment. The supernatants (plasma) were discarded and 200 μl of NR solution ($50\ \mu\text{g}\ \text{ml}^{-1}$) added to the wells. The plates were then incubated for 1 h at 21°C , centrifuged to remove supernatant, and 200 μl of wash/fix solution (3.7% formaldehyde and 1% CaCl_2 in distilled water) added to each well. After 1 min of wash/fixation, the solution in the plates was removed by inversion. NR was extracted from lysosomes using 1% glacial acetic acid in 50% ethanol. After 20 min at room temperature, the plates were transferred to a Dynatech microplate reader, and absorbency at 540 nm recorded. Data were normalized ($\text{OD}/10^6$ hemocytes) using hemocyte counts of hemolymph samples from individual oysters.

2.2.2. MTT reduction

The reduction of MTT by hemocytes was assayed with 96-well microtiter plates according to Hansen et al. (1989). Triplicate aliquots of 100 μl hemolymph from individual oysters were added to the plates' wells. MTT solution (10 μl) in York River water (YRW, $5\ \text{mg}\ \text{ml}^{-1}$) were added to each well and the plates incubated for 3 h at room temperature. Following incubation, 100 μl of MTT solvent (0.1 N HCl in anhydrous isopropanol) were added to each well to solubilize the formazan deposits. Absorbencies of well solutions were read at 540 and 690 nm after 45 min and the latter (background) subtracted from the former. Data were normalized ($\text{OD}/10^6$ hemocytes) using hemocyte counts of hemolymph samples from individual oysters.

2.2.3. ^3H -leucine incorporation in hemocytes

Hemolymph (500 μl) from individual oysters was incubated with 25 μl of ^3H -leucine (10 $\mu\text{Ci ml}^{-1}$, in filtered YRW (York river water)) in microcentrifuge vials for 3 h. Following incubation, vials were centrifuged and supernatants removed. Each hemocyte pellet was then washed twice with YRW to remove unincorporated ^3H -leucine, transferred to 1.5 ml of YRW, and 5 ml of liquid scintillation counting fluid (ECOLITE, ICN) added. Radioactivity was measured using a Beckman (Model LS-5000TD) liquid scintillation system. Data were normalized with hemocyte counts obtained from individual hemolymph samples and reported as percent of DPM (disintegration per minute) incorporation in 10^6 hemocytes (DPM in ^3H -leucine incubated hemocytes divided by DPM of ^3H -leucine added to the hemocytes).

2.3. Plasma total lipid and lipid class composition

Plasma lipids of individual oysters were extracted according to Bligh and Dyer (1959). Lipid classes were separated on S-III chromarods (Iatron Laboratories, Tokyo, Japan) using hexane-diethyl ether-formic acid (85:15:0.04, v/v/v) and quantified using an Iatroscan TH-10, MK-3 analyzer (Iatron Laboratories, Tokyo, Japan) equipped with a flame ionization detector (TLC/FID) (TLC = thin layer chromatography). Cholesteryl esters, phosphatidylcholine, oleic acid, cetyl palmitic acid, and triolein were used as reference standards. Quantities of each lipid class were determined by comparison with standard curves constructed for each standard (1.0, 5.0, 10, and 20 μg). Lipid concentration was expressed as $\mu\text{g lipid ml}^{-1}$ of plasma.

2.4. Plasma protein

Plasma protein was determined by the method of Lowry, Rosebrough, Farr, & Randell (1951), using bovine albumin as a standard (Bio-Rad). Duplicate 10 μl of plasma (cell-free hemolymph) from individual oysters were assayed for protein.

2.5. Plasma lactate dehydrogenase (LDH)

Duplicate plasma samples (150 μl) from individual oysters were assayed for LDH in a 96-well plate using a LDH assay kit (Sigma, Saint Louis, Missouri, USA). Seventy-five μl of LDH assay mixture (25 μl each of LDH substrate, enzyme, and dye solution) were added to each well. After a 30-min incubation in darkness at room temperature, 25 μl of 1N HCl were added to the wells to terminate the reaction, and the plate was read at 490 nm and then corrected for background absorbency at 690 nm.

2.6. Condition index

Dry oyster meat weight and shell weight were obtained from individual oysters. The condition index (CI) was calculated as dry meat/dry shell weight $\times 100$ (Lucas & Beninger, 1985).

2.7. *Perkinsus marinus* diagnosis

The thioglycollate assay (Ray, 1952, 1966) was used for *P. marinus* diagnosis. Aliquots of rectal, mantle + gill, and adductor tissues were removed from individual oysters and incubated in fluid thioglycollate medium (FTM) for 4–5 days. These tissues were then removed from the FTM and examined individually for *P. marinus* infection by preparing a tissue smear of each on a glass slide. Lugol's working solution was added to each preparation. The stained tissue was then examined under a light microscope. Intensity of infection was ranked 0 (negative), 1 (light), 3 (moderate), and 5 (heavily infected) based on the number of stained *P. marinus* particles contained in the oyster tissue smear (Andrews, 1988; Ray, 1952).

2.8. Statistical analysis

Data were analyzed using the SAS program (SAS Inc., North Carolina, USA). Differences in PAH accumulation between groups provided different doses of suspended FCS (0, 1.0, 1.5, and 2.0 g) and over exposure times were analyzed using two factor analysis of variance (ANOVA). Non-parametric analysis (proc rank followed by two-way ANOVA using proc GLM (general linear model)) was used to compare the differences in hemocyte activities (i.e. MTT, NR, and leucine incorporation), CI, plasma protein, plasma lipid, and LDH between sediment dose treatments and exposure duration. The Student–Neuman–Kuel's multiple comparison test was used to compare means when ANOVA was significant. One factor ANOVA was used to test the differences in hemocyte activities, CI, plasma protein and lipids between infected and uninfected oysters pooled from all treatments. The Logistic regression test was used to determine the relationship between *P. marinus* prevalence (percent of infected oysters) and suspended FCS exposure dosage. Differences were considered statistically significant if $P \leq 0.05$.

3. Experiments

3.1. Preliminary experiments

Since the study involved daily exposure of oysters to different amounts of suspended FCS, it was necessary to determine if the sediment doses alone, proposed for use in the later experiments, affected the physiology/biochemistry of the oysters. Suspended particulates and siltation derived from erosion and other processes have been identified as the pollution category impairing the most water segments in the USA (US EPA, Office of Water 1998 Section (d) List Fact Sheet: National Picture of Impaired Waters). Two preliminary experiments utilizing varying dosages of nontoxic, clay particles (Illite 46E0315, Wards/Cenco, Rochester, New York) were conducted to examine their effects on: (1) phagocytosis of *P. marinus*, ^3H -leucine incorporation, plasma protein, plasma lipid, and condition index, and (2) *P. marinus* disease progression in oysters collected from the James River, Virginia. Experimental oysters were

maintained in 1.0 μm filtered YRW (temperature = 20–21°C, salinity = 20 ppt), and exposed daily to four doses (0, 1.0, 1.5, or 2.0 g suspended FCS per oyster) for 30 days.

3.1.1. Experiment 1: Hemocyte function of oysters exposed to suspended FCS

The aim of this experiment was to examine the effects of suspended FCS on oyster hemocyte function. Test organisms were obtained from the Damariscotta River, Maine, about 25 miles from the Atlantic coast. At collection (October 1996), the ambient temperature and salinity of the site were approximately 18–20°C and 30 ppt, respectively. Oysters ($n=360$) were acclimated to a salinity equivalent YRW (temperature 22–24°C, salinity 18 ppt) for 9 days in two 600 l tanks. After acclimation, 60 oysters were randomly selected for diagnosis of *P. marinus* infection. No disease was detected. Remaining oysters ($n=300$) were divided randomly into four treatment groups: Control, Dose 1, Dose 2, and Dose 3; corresponding to daily doses of 0, 1.0, 1.5, or 2.0 g of suspended FCS, respectively ($n=65$ per treatment). Oysters were maintained in individual 2-l glass jars with aeration. Each day the appropriate dosages of FCS particles, suspended in 10 ml of YRW, were delivered to the oysters, 30 min after feeding (algal paste, 0.2 g/oyster daily). The control group received no sediment particles. Water in the jars was changed every other day. Due to the aeration supplied and the pumping action of the oysters, sediment particles generally remained in suspension until removed by the oyster. Desorbed PAHs were not detected in the water ($<1.0 \mu\text{g/l}$). Fifteen oysters were chosen randomly from each treatment group after 5, 10, 20, and 40 days of exposure and hemolymph was sampled. Hemocyte activities from individual oysters were assessed on the day of sampling, and plasma samples were stored at -80°C for later analysis of lipid, protein and LDH. After completing hemolymph sampling, all experimental oysters were sacrificed to determine CI and prevalence and intensity of *P. marinus* infection. At each sampling date, three oysters from each treatment group were analyzed for PAH accumulation.

3.1.2. Experiment 2: *P. marinus* expression in suspended FCS-exposed oysters

The primary goal of this experiment was to examine the effect of suspended FCS on *P. marinus* expression in oysters. Experimental oysters were collected from Point of Shoals, James River, Virginia (December, 1996; ambient temperature = 8°C, salinity = 1 ppt). Oysters ($n=250$) were acclimated gradually in the laboratory to maintenance water salinity, 1 μm filtered YRW (salinity = 17 ppt, temperature = $20 \pm 1^\circ\text{C}$). Before exposure to suspended FCS, initial assessment of Dermo infection was performed on 50 randomly selected oysters. The remaining oysters (200) were randomly divided into four groups: Control, Dose 1, Dose 2, and Dose 3. These received daily doses of 0, 1.0, 1.5, or 2.0 g of suspended FCS, respectively. Control groups received no FCS (0 g). Oysters were maintained and fed as described previously (Experiment 1). After 30 days of exposure, hemolymph was withdrawn from individual oysters ($n=46-50$) from each treatment group. Hemocyte activities and plasma protein were then measured to determine any pathologic effects due to *P. marinus* infection and/or toxic effects due to suspended FCS exposure. After hemolymph withdrawal, oysters were sacrificed and *P. marinus* infection and CI determined. Three oysters

from each treatment group were analyzed for PAHs. To determine whether *P. marinus* progression continued after termination of exposure, the remaining oysters ($n = 10$) in each treatment group were maintained in filtered YRW for an additional 2 weeks. Oysters ($n = 3-4$) from each treatment group were diagnosed for *P. marinus* infection at 3, 7, and 14 days during this post-exposure period.

4. Results

4.1. Preliminary experiments

Results of preliminary experiments revealed no significant effects of suspended clay particles alone on hemocyte activities (e.g. phagocytosis of *P. marinus*, ^3H -leucine incorporation), plasma protein, condition index (Table 2), and disease progression of *P. marinus* in oysters (Table 3).

4.1.1. Experiment 1

4.1.1.1. *Bioaccumulation of PAHs in oysters.* PAHs in the oysters were tracked as they were the dominant contaminants in the sediments. PAH accumulation in oysters exposed to suspended FCS increased with both the amount of sediment particles provided (ANOVA, $P = 0.001$; $F_{0.05, 3} = 35.67$) and length of exposure time

Table 2

Preliminary Experiment 1: condition index and cellular responses in oysters ($n = 19-20$) exposed to uncontaminated suspended clay particles (0, 1.0, 1.5, or 2.0 g per oyster daily) for 30 days

Measured parameters	Treatments (g of suspended clay particles)			
	0	1.0	1.5	2.0
Condition index	1.23±0.23	1.27±0.30	1.26±0.37	1.29±0.23
Phagocytic index	0.39±1.20	0.41±0.22	0.36±0.19	0.50±0.24
Leucine incorporation (%)	3.63±3.46	2.75±1.05	3.11±1.92	2.85±1.36
Serum protein (mg/ml)	4.50±0.50	5.60±0.50	5.85±0.75	5.70±0.40

Table 3

Preliminary Experiment 2: *Perkinsus marinus* (Dermo) infection prevalence and intensity in oysters exposed to uncontaminated suspended clay particles (0, 1.0, 1.5, or 2.0 g per oyster daily) for 40 days^a

Dermo infection	Treatments (g of suspended clay particles)			
	0	1.0	1.5	2.0
Light No. of oysters	9	7	9	8
Moderate No. of oysters	1	1	1	2
Heavy No. of oysters	0	2	0	0

^a Initial infection prevalence (percent of infected oysters) was estimated to be 100% ($n = 10$; seven oysters were lightly infected (Light); two oysters were moderately infected (Moderate); one oyster was heavily infected (Heavy)).

($P < 0.001$; $F_{0.05, 2} = 9.115$; Fig. 1) effects. Oysters exposed to 2.0 g suspended FCS accumulated significantly higher concentrations of PAHs (140 $\mu\text{g/g}$) compared to the groups exposed to 0 (0 μg), 1.0 (70.2 μg), or 1.5 g (105 μg) daily after 5 and 10 days exposure. There was no significant difference in total PAHs accumulated between the latter two groups. After 20 and 40 days of exposure, total PAHs accumulated were significantly higher than after 5 and 10 days.

4.1.1.2. Hemocyte number and activities. Exposure to suspended FCS did not affect the concentration of total circulating hemocytes. However, the interaction between FCS exposure and exposure time was significant. Also, there were some significant differences in total numbers of hemocytes between sampling dates in oysters exposed to 1.0 g suspended FCS. Oysters sampled after 5 and 20 days of exposure to 1.0 g suspended FCS had significantly higher concentrations of total hemocytes ($16.99 \pm 2.45 \times 10^5$ hemocytes ml^{-1} hemolymph at 5 days exposure; $19.04 \pm 3.86 \times 10^5$ hemocytes ml^{-1} hemolymph at 20 days exposure) than those sampled after 10 days ($7.35 \pm 0.87 \times 10^5$ hemocytes ml^{-1} hemolymph). In contrast to total hemocyte numbers, there were no significant FCS dose or time effects on the percentage of granulocytes and hyalinocytes (data not shown).

There was a significant interactive effect between FCS dosage and exposure duration on all measured hemocyte activities. Exposure to suspended FCS appeared to stimulate NR uptake, MTT reduction and ^3H -leucine incorporation. Compared to

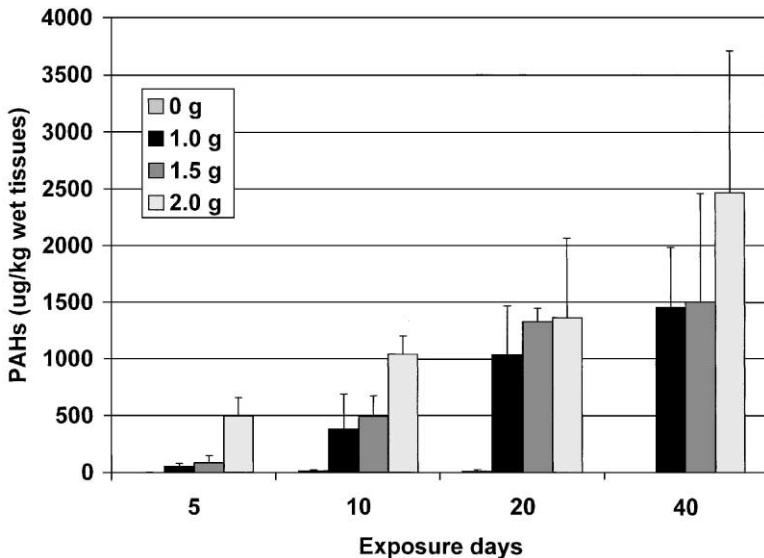


Fig. 1. Total PAH concentrations in control and suspended field contaminated sediment (FCS) exposed oysters from Experiment 1 ($n = 2-3$, after 5, 10, 20, and 40 days of exposure). Daily doses of 0, 1.0, 1.5, and 2.0 g FCS. Oysters exposed to 2.0 g suspended FCS accumulated significantly higher concentrations than the groups exposed to 0, 1.0 or 1.5 g daily after 5 and 10 days exposure. There was no significant difference in total PAHs accumulated between the latter two groups. After 20 and 40 days of exposure, total PAHs accumulated were significantly higher than after 5 and 10 days.

other treatments (including the control), exposure of oysters to 2.0 g for 10, 20, and 40 days resulted in stimulation of NR uptake (Fig. 2). However, only after 40 days of exposure was NR uptake in hemocytes from oysters at the 2.0 g dosage significantly higher than the control (Fig. 2). Generally, there was no significant difference in NR uptake in hemocytes of suspended FCS-exposed oysters between sampling dates. MTT reduction tended to increase in all exposed oysters after 5, 10, and 20 days exposure (Fig. 3), however the increase was only significant after 10 days of exposure. The MTT reductions in all treatments, including the control, decreased to the lowest levels at the end of the experiment (Day 40). The percent ^3H -leucine incorporation in hemocytes was higher in FCS dosed than control oysters after 10, 20, and 40 days exposure (Fig. 4). However, only hemocytes from oysters exposed to the 1.5 g dosage for 20 days exhibited significantly higher ^3H -leucine incorporation than control oysters. ^3H -leucine incorporation in hemocytes of exposed oysters did not change significantly with time (from 10 to 40 days).

4.1.1.3. Plasma protein, lipid, and LDH. Exposure to suspended FCS did not alter either the oyster's plasma protein concentration (mg ml^{-1} plasma), lipid concentration ($\mu\text{g ml}^{-1}$ plasma) or lipid class composition (data not shown). No significant difference was noted in plasma protein or lipid between samplings and among treatments. There were no significant treatment effects in plasma LDH level after oysters were exposed to suspended FCS.

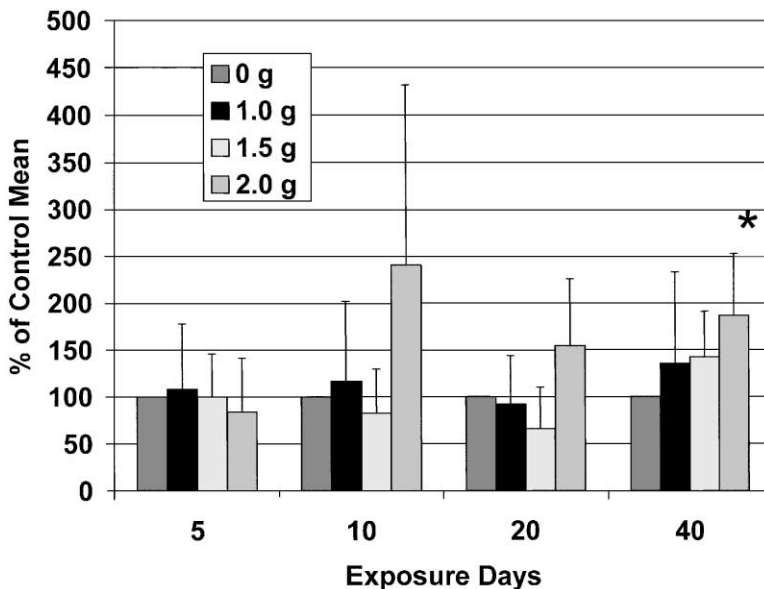


Fig. 2. Results of NR uptake by hemocytes (percent of control mean, $n = 14\text{--}15/\text{treatment}$) of control and field contaminated sediment (FCS) exposed oysters. Daily doses of 0, 1.0, 1.5, and 2.0 g FCS. * Indicates significantly different from the control, 0 g FCS.

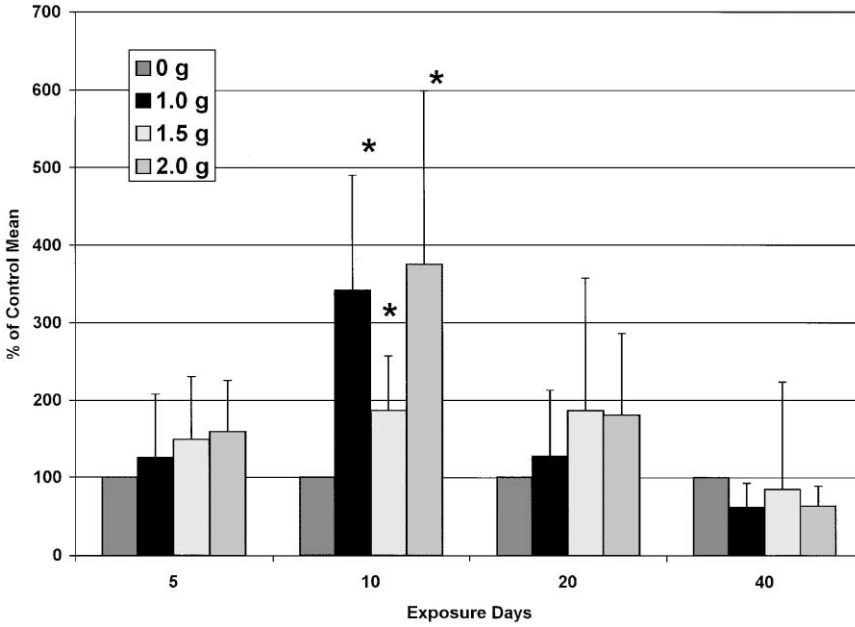


Fig. 3. Results of MTT reduction in hemocytes (percent of control mean, $n=15$ /treatment) of control and field contaminated sediment (FCS) exposed oysters. Daily doses of 0, 1.0, 1.5, and 2.0 g FCS. * Indicates significantly different from the control, 0 g FCS.

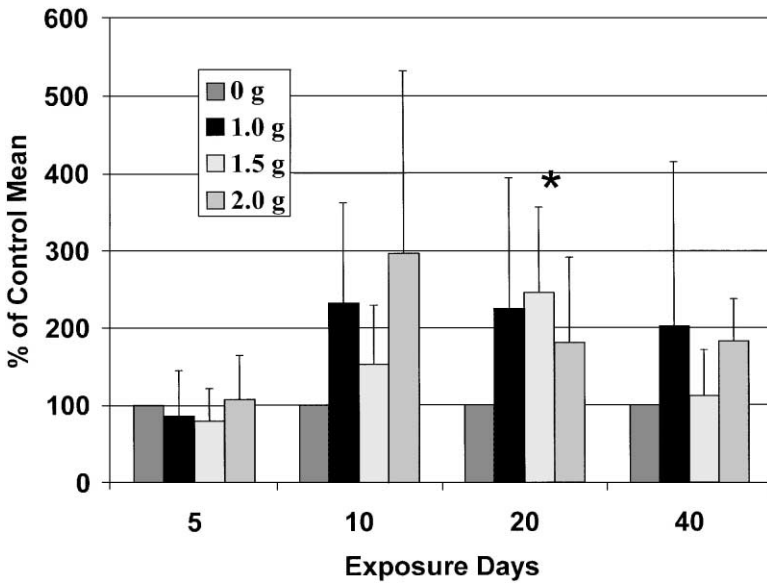


Fig. 4. Results of ³H-leucine incorporation in hemocytes (percent of control mean, $n=15$ /treatment) of control and field contaminated sediment (FCS) exposed oysters. Daily doses of 0, 1.0, 1.5, and 2.0 g FCS. * Indicates significantly different from the control, 0 g FCS.

4.1.1.4. *Condition index (CI) and P. marinus infection diagnosis.* No significant difference was found in oyster CI between treatment groups. Oyster CI did not change over time. *P. marinus* infections were not detected in any oysters sampled at 5, 10, 20 and 40 days of exposure.

4.1.2. Experiment 2

4.1.2.1. *Bioaccumulation of PAHs in oysters.* After 30 days, while oysters exposed to 1.0 or 1.5 g of suspended FCS accumulated similar amounts of PAHs (Fig. 5), both groups had lower mean PAH concentrations than oysters exposed to 2.0 g. However, the difference was statistically insignificant ($P=0.075$; $F_{0.05, 3} = 3.372$). Sediments used in this experiment contained a similar concentration of total PAHs as in Experiment 1.

4.1.2.2. *P. marinus expression.* Initial assessment indicated that 39% of the experimental oysters were lightly infected by *P. marinus*. Exposure to suspended FCS increased *P. marinus* expression in oysters. After 30 days of exposure to 0, 1.0, 1.5, or 2.0 g of suspended FCS, infection prevalences in oysters were 44, 67, 53, and 70%, respectively. The relationship between oyster *P. marinus* prevalence and FCS exposure was nearly significant ($P=0.0582$). When control and dosed (i.e. pooled from all suspended FCS-exposed groups) oysters were compared, the latter showed significantly higher *P. marinus* prevalences than either the controls or the oysters examined at the beginning of the experiment (Fig. 6, $P=0.0025$). *P. marinus* prevalence in control and initial oysters were similar. Cessation of suspended FCS exposure did not alter the pattern of *P. marinus* progression in oysters observed during the exposure phase. Of the ten depurated oysters in each treatment, six (Control), six (Dose 1), four (Dose 2), and nine (Dose 3) oysters were found to be

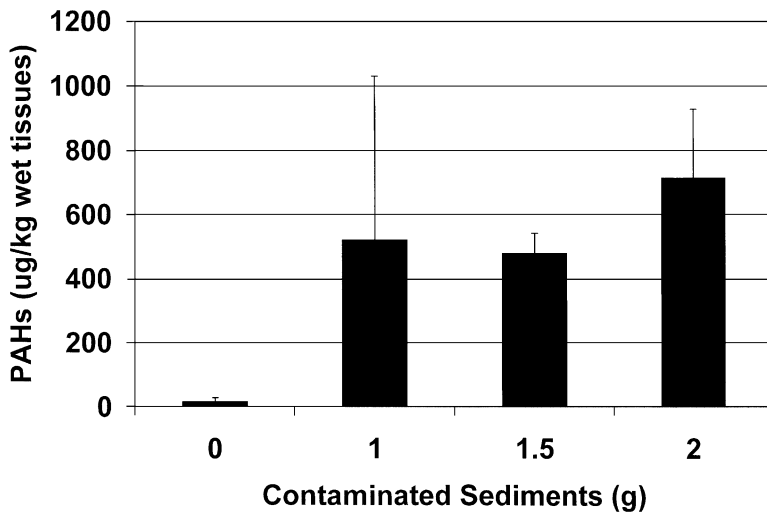


Fig. 5. PAHs concentrations in control and field contaminated sediment (FCS) exposed oysters of Experiment 2 ($n=3$, after 30 days of exposure). Daily doses of 0, 1.0, 1.5, and 2.0 g FCS.

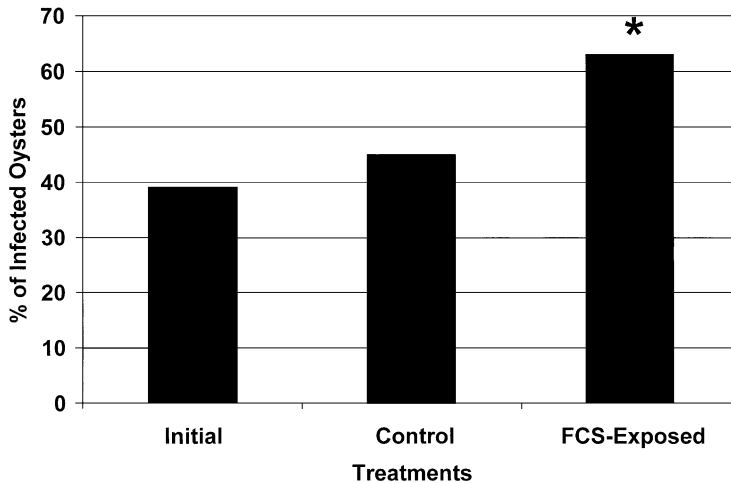


Fig. 6. *P. marinus* prevalences (percent of infection) in initial ($n=49$), and in control (0 g FCS, $n=39$) and field contaminated sediment (FCS) exposed oysters (daily doses of 1.0, 1.5, and 2.0 g FCS, $n=114$) after 30 days of exposure. * Indicates significantly different from the initial and control (0 g FCS) groups.

infected. Most of the infections in oysters in all treatments were light and depuration of oysters did not eliminate the infection.

4.1.2.3. Hemocyte number and activities; plasma protein and lipid; condition index (CI). No significant effect of suspended FCS exposure was found on the concentration of total circulating hemocytes, percent of granulocytes or hyalinocytes, MTT reduction, ^3H -leucine incorporation, or NR uptake. Similarly, particulate exposure did not induce any change in plasma protein or lipid concentrations; nor did these parameters change over time. When data were pooled from all infected and uninfected oysters, regardless of treatment, to test for any pathological effects, no significant differences were noted in the above parameters between infected and uninfected oysters. No apparent differences were found in oyster CI between treatments or over time.

5. Discussion

Bioavailability of sediment- or particle-associated pollutants plays a decisive role in toxicity (Weltens, Goossens, & Van Puymbroeck, 2000). The sediments used in the present study contained a suite of contaminants, dominated by high molecular weight PAHs with contributions of other contaminants, such as heavy metals and organochlorines. While this makes it more difficult to ascribe any subsequent effects solely to a single contaminant class, it is more representative of what organisms experience in the field. Scuffletown Creek sediments differed with respect to PAH content from sediments previously used to generate water soluble fractions (WSFs;

Chu & Hale, 1994). The latter sediments, collected in closer proximity to a creosote plant, were more heavily contaminated and contained higher percentages of lower molecular weight compounds. In contrast, the major contaminants in the Scuffletown Creek sediments were higher molecular weight PAHs (≥ 178 amu, Table 1). Sediment PCB and DDT metabolite (4,4'-DDD (DDD = 1,1-bis(p-chlorophenyl)-2,2-dichloroethane) and DDE (DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene)) burdens were much lower than the PAHs (Table 1). Metal concentrations were elevated relative to pristine areas. However, with the exception of Hg and of Zn, these concentrations were below the median effects levels for sediment associated metals proposed by Long, MacDonald, Smith, and Calder (1995). Accumulation of metals, PCBs and DDT metabolites in oysters were not examined in the present study. The bioavailability of PAHs is evidenced by their accumulation in suspended FCS-exposed oysters (Figs. 1 and 2). The lower uptake of PAHs in Experiment 2 is interesting. While the sediments used were collected from the same area and were similar in terms of metals and organic pollutant contents, the oysters were obtained from two different areas. Paralleling this, PAH burdens in oysters from the relatively pristine Rappahannock River in Virginia were reported to be approximately twice those of Elizabeth River oysters when both were transplanted to a contaminated Elizabeth River location (Bender & Huggett, 1989).

The oyster lacks a specific immune response, but possesses various humoral and cellular factors important in defense against pathogenic and nonpathogenic organisms. Hemocytes are crucial in its defense system. Any impact on their function (stimulation or suppression) arising from contaminated particulate exposure could compromise the oysters' immune competence. Results from the preliminary experiment examining the impact of varying dosages of clay particles alone on hemocyte activities (phagocytosis of *P. marinus*, ^3H -leucine incorporation) suggested that amounts from 0–2 g day $^{-1}$ had no impact on these endpoints (Table 2). Therefore, the stimulation of hemocyte NR uptake, MTT reduction, and ^3H -leucine incorporation in suspended FCS-exposed oysters in Experiment 1 appeared to be related to sediment-associated contaminants. As noted, high molecular weight PAHs were the dominant contaminants observed, but contributions from other sediment constituents can not be ruled out. Cytotoxicities of organic pollutants on certain cellular activities have been documented in various organisms including oysters. Either suppression or stimulation of cellular activities were reported in these studies. Exposure of oysters (*C. virginica*) and mussels (*Mytilus edulis*) to PAHs reduced uridine uptake, an indication of RNA synthesis, disrupted hemocyte lysosomal integrity in terms of NR uptake and retention, and hemocyte phagocytic and chemotactic ability (Chu, 2000; Faisal & Sami, 1994; Grundy, Moore, Howell, & Ratcliffe, 1996a; Grundy, Ratcliffe, & Moore, 1996b). While in vitro exposure to high concentrations of dieldrin (1–10 mg l $^{-1}$) and naphthalene (10–100 mg l $^{-1}$) depressed the oyster hemocytes' chemiluminescence, in vivo exposure to naphthalene (1.0 mg l $^{-1}$) and dieldrin (0.1–1.0 mg l $^{-1}$) stimulated hemocyte CL response (Larson, Robertsom, & Hetrick, 1989). Similarly, we found that exposure of oysters for 14 weeks to 0.1 μg l $^{-1}$ tributyltin (TBT, an additive to antifouling paint) elicited a greater CL response than that observed in unexposed control oysters (Chu et al.,

unpublished results). The above observations indicate that, while certain aspects of the defense system may be suppressed by pollutant stress, others could be enhanced.

In our study, stimulation of NR uptake, MTT reduction and ^3H -leucine incorporation in hemocytes generally occurred after suspended FCS exposure, although stimulation was only significant at some exposure times, due in part to high individual variability. The enhanced NR uptake, MTT reduction and ^3H -leucine incorporation in hemocytes of exposed oysters suggest a shift in hemocyte function from their “normal range”. However, the mechanism(s) behind stimulation are poorly understood. Disruption of membrane stability could be one of the factors responsible for the observed stimulated NR uptake in hemocytes of suspended FCS-exposed oysters. The increase in NR uptake may be the result of contaminant penetration into the phospholipid monolayer of the hemocyte’s membrane, thus increasing membrane permeability. Reduced NR lysosomal retention time in hemocytes of PAH-exposed *Mytilus edulis* was noted by Grundy et al. (1996a, 1996b). Lysosomal integrity, however, was not examined in the present study, and therefore, the degree of lysosomal membrane damage and loss of NR over time to the cytosol from the lysosomal compartment are unknown. The increases in MTT may indicate stimulation of mitochondrial enzyme production (e.g. mitochondrial dehydrogenase) and/or generation of superoxide anion because of membrane-excitation of the hemocyte. The decline of MTT reduction in all treatment groups at the end of the experiment suggests possible confinement stress (oysters maintained in 2-l jars). It is not known if the increased ^3H -leucine incorporation observed is due to induction of stress protein synthesis. The enhanced hemocyte responses observed in suspended FCS-exposed oysters were not directly correlated with particle dosages, length of exposure, or PAH body burdens of the oysters. Thus, the stimulated responses may have been induced by other contaminants present in the FCS and/or the combined effects of multiple contaminants therein. Overall, the Maine oysters may have adapted physiologically to pollutant exposure, since after 40 days of suspended FCS exposure there was no significant difference in MTT reduction or ^3H -leucine incorporation between control and exposed oysters.

Our previous study revealed that Elizabeth River sediment-derived WSFs, containing predominantly low molecular weight aromatics not only advanced latent *P. marinus* infection in oysters, but also increased their susceptibility to novel infection (Chu & Hale, 1994). Studies by other investigators have also shown that exposure of oysters to water-borne TBT intensified *P. marinus* infections and resulted in oyster mortality (Anderson et al., 1996; Fisher et al., 1999). However, no published research has examined interactions of sediment-associated contaminants and infectious disease. In the current study, suspended FCS exposure appeared to increase latent *P. marinus* infection in oysters (Fig. 6). Infection in oysters exposed to varying doses of nontoxic clay particles did not intensify (Table 2) and no change in disease progression was observed in unexposed oysters (Fig. 6). Thus, it is believed that the increased disease prevalence in Experiment 2 was due to contaminants associated with the field collected sediment particles, not the particles per se.

In contrast to Experiment 1, no modulation of hemocyte activities was seen in Experiment 2 using oysters from a Dermo enzootic area of the Chesapeake Bay,

despite exposure to suspended sediment particles containing similar contaminant burdens. Interestingly, PAH accumulation in the Virginia oysters also was lower than in the Maine oysters used in Experiment 1. It is not certain whether the lack of effect on cellular response was related. The difference in the physiological states of these two populations may have contributed to the varied observations in cellular responses and PAH bioaccumulation, although they were acclimated to similar experimental conditions before exposure. Chesapeake Bay oysters were obtained from a Dermo enzootic area with an initial infection prevalence of 39%, compared to Maine oysters which had no detectable *P. marinus* infections.

To compensate for stress, animals may mobilize energy reserves and transport them via serum/plasma to fuel increased energy expenditures. Thus, stress may be reflected in changes in plasma or serum chemistry. However, no change was detected in the plasma protein, LDH or lipid concentrations due to suspended FCS exposure in the current study. Also, no apparent pathological effect was observed in oysters due to infection, perhaps because most infections observed were light.

Impaired immunofunction due to pollutant stress may lead to infectious disease outbreaks in aquatic organisms (Sinderman, 1983, 1993). However, such a relationship was not observed in the present study. No statistically significant change was noted in any measured humoral or cellular parameters in suspended FCS-exposed oysters in Experiment 2, although increased Dermo prevalence occurred. Similarly, while in vivo TBT exposure augmented the progression of Dermo infection, no significant effects were observed by Anderson et al. (1996) in defense-related activities measured in oysters. Further study is needed to elucidate the mechanisms responsible for elevated *P. marinus* expression in suspended FCS-exposed oysters.

In summary, the present study examined the relationship between pollution and infectious disease by exposing oysters to complex mixtures of contaminants present in the field via a natural exposure route (filtration and ingestion of suspended sediment particles). It provides further evidence that pollution is a potential factor activating latent infection in aquatic organisms. Physiological responses and contaminant (PAH) bioaccumulation appeared to vary between oyster populations. The mechanisms triggering the elevated *P. marinus* expression in suspended FCS-exposed oysters requires further investigation.

Acknowledgements

This work was supported by the Chesapeake Bay Environmental Effects Studies Toxic Research Program sponsored by the United States National Oceanographic and Atmospheric Administration-Sea Grant Program. Metal analyses were performed by Dr. Gary Rice of the Chemistry Department at the College of William Mary. The authors would like to thank Elizabeth Bush, Georgeta Constantin and Daniel Marcellino for invaluable technical assistance, Vincent Encomio and Luis Cruz-Rodriguez for assistance of part of the statistical analysis and graphic work, and Dr. Kenneth Webb for critical review of the first draft of the manuscript and the three anonymous reviewers of the submitted manuscript. VIMS Contribution no. 2358.

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