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Effects of field-contaminated sediments and related water soluble components on haemocyte function and *Perkinsus marinus* susceptibility and expression in oysters

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This paper reviews and discusses our recent findings on the effects of contaminated sediments (CSs) and related water-soluble fractions (WSFs) on haemocyte function/activity and the onset and progression of an infectious disease caused by the protozoan parasite, *Perkinsus marinus* (Dermo) in the eastern oyster, *Crassostrea virginica*. Sediments used to generate WSFs and sediments used for the whole CS exposure experiments were collected in different areas of the southern branch of the Elizabeth River, a heavily polluted sub-estuary of the Chesapeake Bay, USA. The WSFs were dominated by low molecular weight polycyclic aromatic hydrocarbons (PAHs). The CSs used for whole CS exposure experiment had elevated concentrations of high molecular weight PAHs. Polychlorinated biphenyls (PCBs) and metals were also present in the CSs. No PCBs were detected in the WSFs. *In vitro* exposure of haemocytes to WSFs derived from CSs reduced to haemocytes' chemotaxic, phagocytic, and chemiluminescent responses to some extent. Exposure of oysters to suspended CSs stimulated neutral red uptake, mitochondrial dehydrogenase production and ^{3}H-leucine incorporation in haemocytes. Exposure of oysters to 0, 15, 30% WSFs increased the oysters' susceptibility to laboratory-induced infection caused by *P. marinus*. Exposure of oysters to 15, and 30% dilutions of WSFs for 33 days or to 1.0, 1.5, and 2.0 g CSs for 30 days significantly elevated the expression/progression of latent *P. marinus* infection in oysters in a dose-dependent manner.

Keywords: pollutants, polycyclic aromatic hydrocarbons (PAHs), metals, haemocytes, disease, oyster, *Perkinsus marinus*.

Abbreviations: CL, chemiluminescence; CSs, contaminated sediments; LDH, lactate dehydrogenase; MTT reduction, reduction of the tetrazolium dye (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazon; NR, neutral red; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; TBT, tributyltin; WSFs, water-soluble fractions.

Introduction

Environmental contaminants may pose a severe threat to the health of aquatic organisms. Mass mortalities of organisms have been observed in connection with episodic events, such as chemical spills and waste outfalls. Sublethal effects are less well known and may affect the immunocompetence of the organism, resulting in reduced disease resistance (Sinderman 1983, 1993, Mix 1988, Vethaak and Rheinalt 1992). Alternatively, stress may result in increased energy expenditure, thus affecting growth and reproduction (e.g. Lugo 1978, Bridgham 1988, Cross and Hose 1988). There are several reviews of pollution-associated diseases in fish and shellfish (e.g. Sinderman 1983, 1993, Moller 1985, Mix 1988). Although there is increasing evidence suggesting a role of pollution in initiating and activating infectious diseases in aquatic animals in the field, a pollution/infectious disease

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relationship has only recently been demonstrated in the laboratory (Chu and Hale 1994, Kagley *et al.* 1997, Chu *et al.* submitted). This paper reviews and discusses the recent investigations in our laboratory of the relationship between pollutant exposure and the onset and progression of the infectious disease caused by the protozoan parasite, *Perkinsus marinus* (Dermo). Observed effects of environmental pollutants on oyster haemocyte functions/activities and their relationship to the increased *P. marinus* susceptibility and expression in oysters are also discussed.

Environmental contaminants

Sediments are a significant potential source of pollutants to aquatic organisms. Contaminated sediments (CSs) collected from the Elizabeth River, a subestuary of lower Chesapeake Bay, Virginia, USA, were used in the exposure experiments. Sediments used to generate WSFs and for whole CS exposure were collected from different areas of the southern branch of the Elizabeth River. Sediments from this branch of the river have been previously reported to contain elevated concentrations of pollutants, principally PAHs, derived from creosote releases over the last century (Bieri *et al.* 1986). High incidences of pathological lesions (Hargis and Zwerner 1988, Thiyagarajah *et al.* 1989), neoplasms (Hargis *et al.* 1989, Vogelbein *et al.* 1990), reduced immunocompetence in fish (Weeks *et al.* 1986), and modulated haemocyte function in eastern oysters (Faisal and Demmerle-Sami 1994) due to exposure to Elizabeth River sediments have also been reported. The bioavailability of sediment-associated pollutants is a critical factor mediating their toxicity (US EPA 1988, Adams *et al.* 1992). In an aquatic environment, organisms are continuously exposed to both water- and sediment-borne contaminants, particularly benthic sedentary filter-feeding bivalves, such as oysters. We therefore examined the effects of CSs, as well as the water-soluble fractions (WSFs) derived from CSs, on oyster haemocyte activities/functions and *P. marinus* susceptibility and progression in oysters.

The sediments used to generate WSFs were collected near a creosote plant in the southern branch of the Elizabeth River and were more heavily contaminated, and contained higher percentages of lower molecular weight compounds, e.g. naphthalene, than the sediments used for the whole CS exposure experiment. The WSFs were prepared, as described by Chu and Hale (1994), by mixing CSs in 1 μm filtered York River estuarine water (2.5 kg CS per 40 l water) for 1.0 h. The CSs–water mixture was allowed to settle overnight before filtration through a 6.0 μm absolute removal cartridge filter and a 1 μm filter. The WSFs contained low molecular weight PAHs (table 1). No PCBs were detected in the WSFs.

Surficial sediments collected from Scuffletown Creek, a small tidal creek on the eastern bank of the southern branch of the Elizabeth River, were used for whole CS exposure experiments. These sediments contained elevated concentrations of high molecular weight PAHs (table 2). PCBs and metals were also present in the sediments. The lower concentrations of lower molecular weight, more water-soluble compounds in sediments may be due to weathering processes. Scuffletown Creek is located directly across the river from the above-mentioned creosote plant. To test the effects of surficial CSs on haemocyte activities/functions and subsequent *P. marinus* progression, sediments were collected from the field at two different times, but from approximately the same location in Scuffletown Creek. They differed somewhat in the total contaminant content due to spatial

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Table 1. Concentrations (SD) in mg l^{-1}, of the major organic contaminants detected in representative WSFs, generated from Elizabeth River Sediments, and control water (filtered York River Water); $n = 3$ (Chu and Hale 1994).

PAHs	Control	WSFs
Naphthalene	<0.001	1.51 (0.52)
Acenaphthalene	<0.001	0.424 (0.033)
2-Methylnaphthalene	<0.001	0.224 (0.018)
Phenanthrene	<0.001	0.210 (0.026)
Fluorene	<0.001	0.201 (0.031)
Dibenzofuran	<0.001	0.195 (0.012)
1-Methylnaphthalene	<0.001	0.151 (0.022)
Carbazole	<0.001	0.148 (0.013)

Table 2. Concentrations (mg kg^{-1} dry sediment; mean ± SD) of organic contaminants and metals in sediments; Expt 1 = effects of contaminant sediment (CS) on oyster haemocyte function; Expt 2 = effect of CS on *Perkinsus marinus* expression.

Analytes metals	Expt 1	Expt 2	Analytes, PAHs	Expt 1 $n = 4$	Expt 2 $n = 5$
Ag	1.90	2.20	Phen	3.49 ± 0.46	3.51 ± 1.58
As	13.00	27.00	Anthr	1.50 ± 0.35	1.19 ± 0.50
Cd	2.70	2.60	Fluor	10.40 ± 1.17	9.50 ± 3.62
Cr	43.00	54.00	Pyrene	8.95 ± 0.69	8.94 ± 3.64
Cu	95.00	164.00	BaF	8.40 ± 0.28	6.48 ± 0.55
Ni	2.20	2.40	BaA	4.99 ± 0.85	4.47 ± 0.90
Pb	107.00	148.00	Chr	7.48 ± 1.59	6.31 ± 1.50
Sb	<0.50	<0.50	BaFa	11.20 ± 0.95	12.90 ± 0.95
Se	<0.50	<0.50	BeP	5.50 ± 0.71	5.01 ± 1.03
Tl	<0.30	<0.30	BaP	4.40 ± 0.83	5.05 ± 1.12
Zn	360.00	450.00	IP	3.18 ± 0.59	3.29 ± 0.74
Hg	4.50	4.70	BghiP	2.72 ± 0.45	3.04 ± 0.70
			Total PAHs	187.00 ± 32.00	156.00 ± 34.00
			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

Phen, phenanthrene; Anthr, anthracene; Fluor, fluoranthene; BaF, benzofluorenes; BaA, benzo(*a*)anthracene; Chr, chrysene; BaFa, benzofluoranthenes; BeP, benzo(*e*)pyrene; BaP, benzo(*a*)pyrene; IP, indeno(1,2,3-*cd*)pyrene; BghiP, benzo(*ghi*)perylene.

heterogeneity of pollutants. The CSs used for the haemocyte activities/functions experiment contained a higher total PAH content; whereas, the CSs for the *P. marinus* expression/progression experiment had higher metal and PCB concentrations (table 2).

Effects of contaminated sediments (CSs) derived-water soluble fractions (WSFs) and CSs on haemocyte activities and functions

The *in vitro* effect of WSF on haemocyte activities, such as phagocytosis, chemotaxis, and chemiluminescence was tested by exposure of haemocytes to 0, 30 and 50% dilutions of the WSFs ($n = 3$–4, each replicate contained pooled

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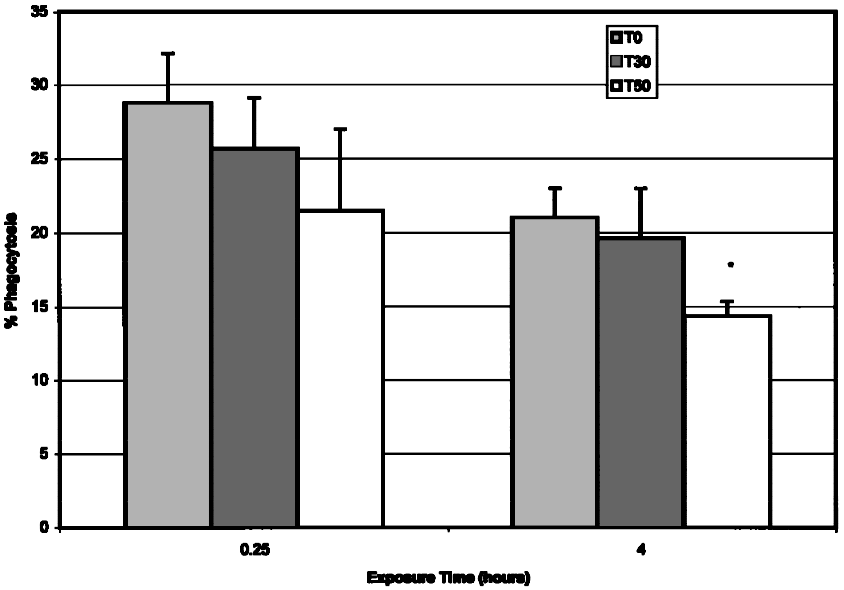

Figure 1. Mean (± SE) phagocytic index (no. of haemocytes that phagocytosed at least 1 zymosan particle/total no. of haemocytes) in haemocytes exposed to 0, 30, and 50% WSFs. $n = 3–4$ replicates, each replicate contained pooled haemocytes from 10–20 oysters. Zymosan (yeast extract) was used as stimulant.

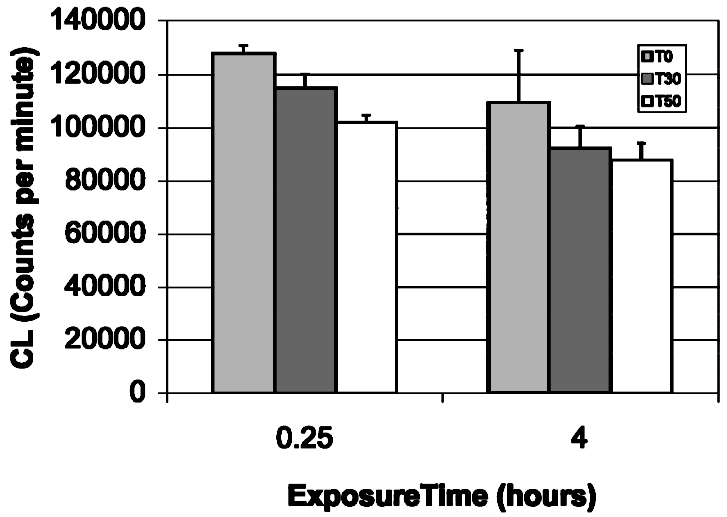

Figure 2. Mean (± SE) chemiluminescence response (counts min^{-1}) in haemocytes exposed to 0, 30, and 50% WSFs. $n = 3–4$ replicates, each replicate contained pooled haemocytes from 10–20 oysters. Zymosan (yeast extract) was used as stimulant.

haemocytes from 10–20 oysters). Samples were taken at 0.25, or 4 h. Decreases of mean phagocytosis, chemotaxis, and chemiluminescence in WSF-exposed haemocytes were noted (figures 1, 2 and 3; Chu *et al*. unpublished data). However, only the reduction of phagocytosis was statistically significant in haemocytes exposed to 50% WSFs for 4 h. The viability of WSF-exposed haemocytes was evaluated using the trypan blue exclusion assay. Mean viability decreased in WSF-

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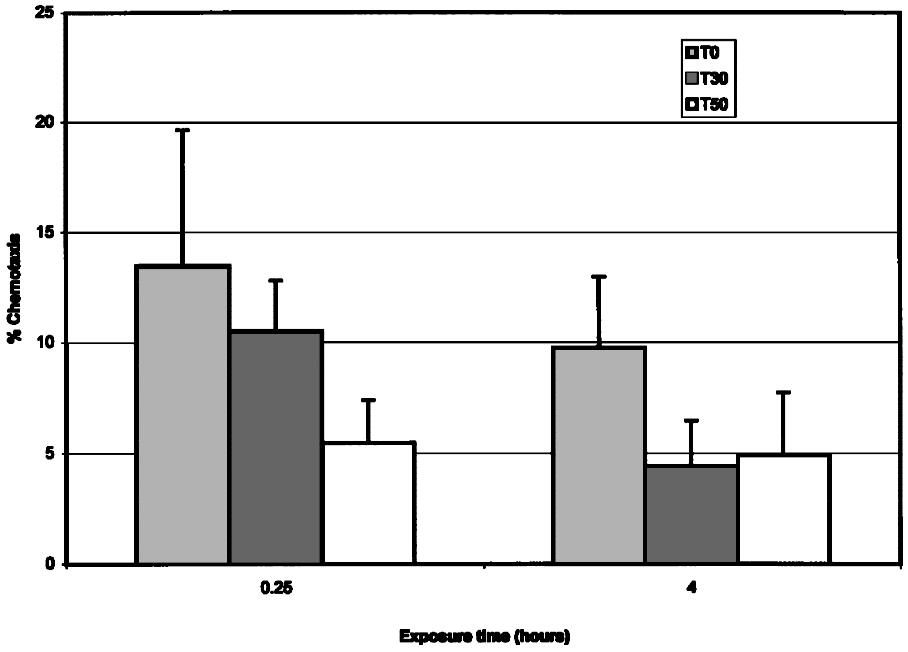

Figure 3. Mean (± SE) chemotaxis (% of migrated haemocytes) of haemocytes exposed to 0, 30, and 50% WSFs. $n = 3$–4 replicates, each replicate contained pooled haemocytes from 10–20 oysters. Zymosan (yeast extract) was used as stimulant.

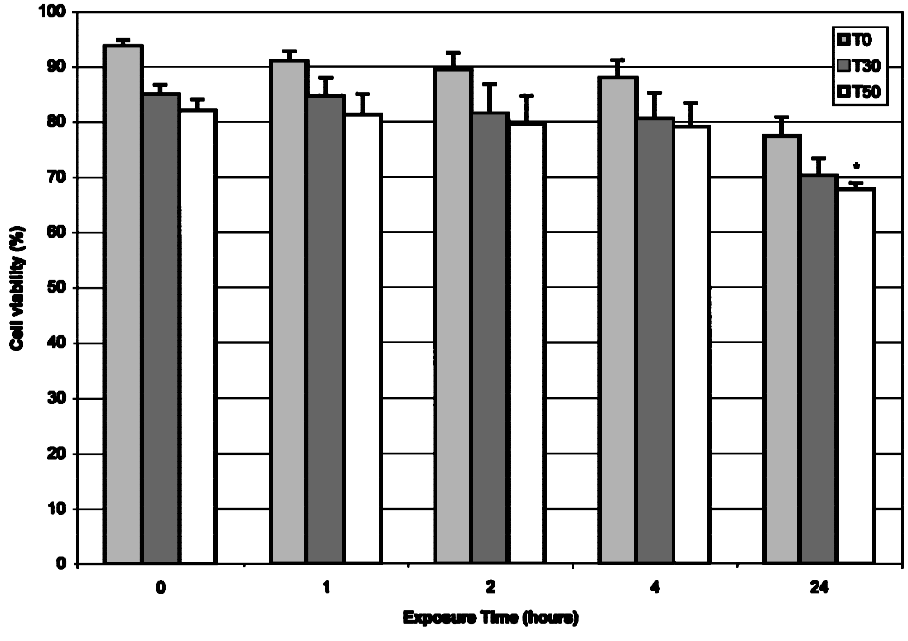

Figure 4. Mean (± SE) viability of haemocytes exposed to 0, 30 and 50% WSFs. $N = 3$–4 replicates, each replicate contained pooled haemocytes from 10–20 oysters. Trypan blue exclusion assay was used to assess cell viability.

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exposed haemocytes. Viability declined significantly in haemocytes exposed to 50% WSFs for 24 h (figure 4, Chu *et al*. unpublished data).

The *in vivo* effect of CSs on the haemocyte functions, neutral red (NR) uptake, production of dehydrogenase (MTT reduction), and ^{3}H-leucine incorporation was tested by exposing oysters daily to 0, 1.0, 1.5, or 2.0 g of suspended CSs (Chu *et al*. 1997, Chu *et al*. submitted). In order to avoid possible pathological effects caused by *P. marinus* infection, oysters were obtained from an area outside the normal geographic range of Dermo (Damariscotta River, Maine, about 25 miles from the Atlantic coast). After 5, 10, 20 and 40 days of exposure, NR uptake, MTT reduction and ^{3}H-leucine incorporation in oyster haemocytes and levels of plasma lipid, protein and lactate dehydrogenase (LDH) were measured. ^{3}H-leucine incorporation, MTT reduction, and NR uptake assays do not directly measure immune responses, but provide an insight into the physiological function of the tested haemocytes. It was found that MTT reduction increased after 10 days of CS exposure; exposure to daily doses of 2.0 g CSs stimulated NR uptake at 40 days; and dosing 1.5 g CSs daily for 20 days augmented ^{3}H-leucine incorporation (Chu *et al*. 1997, Chu *et al*. submitted). CS exposure, however, did not affect the oysters' plasma protein and lipid concentrations, plasma lipid class composition, or the plasma LDH level.

Like other invertebrates, the oyster does not have immunoglobulins and lacks specific immune responses. Oyster haemocytes form the primary line of defence against infectious agents. The effects of environmental pollutants on their function and activities are of great interest. Any impact on haemocyte function arising from pollutant exposure could compromise the oysters' immunocompetence. *In vitro* exposure of haemocytes to WSFs and exposure of oysters to whole CSs modulate certain haemocyte functions/activities. While the former reduced phagocytic, chemotactic, and chemiluminescent activities to some extent, the latter stimulated MTT reduction, NR uptake, and ^{3}H-leucine incorporation in haemocytes. Nevertheless, oysters in the whole CS exposure experiment appeared to acclimatize physiologically to sublethal pollutant exposure, i.e. after 30 days of CS exposure there was no significant difference in MTT reduction or ^{3}H-leucine incorporation, with the exception of NR uptake, between control and exposed oysters.

Immunotoxicity of PAHs on certain defence-related cellular activities has been documented in various organisms, including oysters (e.g. Weeks and Warinner 1984, Anderson 1988, Sami *et al*. 1992, 1993, Faisal and Demmerle-Sami 1994, Grundy *et al*. 1996a, b, Moore *et al*. 1996). PAH exposure caused either suppression or stimulation of certain haemocyte functions/activities in oysters (Sami *et al*. 1993, Faisal and Demmerle-Sami 1994) and in the mussel, *Mytilus edulis* (Grundy *et al*. 1996a) depending on exposure concentration and duration. Suppression and/or enhancement of haemocyte activities due to heavy metal exposure were also noted in eastern oysters (Cheng and Sullivan 1984, Larson *et al*. 1989, Anderson *et al*. 1992, Dorange *et al*. 1995, Oliver and Fisher 1997). The mechanism(s) behind the stimulation of haemocyte activities and/or functions is complex and not completely understood. Disruption of membrane stability could be one of the causes. The observed stimulated MTT reduction, NR uptake, and ^{3}H-leucine incorporation in haemocytes of CS-exposed oysters were probably induced by multiple contaminants present in the CSs.

The blood is the major system for transporting energy-related biomolecules between storage and utilization sites in the haemolymph of invertebrates. Animals

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may need to mobilize energy reserves and transport them via serum/plasma for increased energy expenditure due to stress. Thus, changes in concentrations of certain plasma or serum components (e.g. lipids) could be an indication of stress. Serum and plasma enzymes, such as LDH, can be used as markers of tissue or cell damage. However, no change was detected in the above components following CS exposure.

Effects of contaminated sediments (CSs) and CSs-derived water soluble fractions (WSFs) on *P. marinus* susceptibility and progression in oysters

The eastern oyster, *C. virginica*, has historically supported a major fishery on the east coast of the United States. *P. marinus* is one of the two parasites causing severe mortality in eastern oyster populations from the mid-Atlantic to the Gulf of Mexico since the 1950s. Three life stages of this parasite, meront, prezoosporangia, and biflagellate zoospores, have been identified and described (Perkins 1988). These three life stages are infective and the meront stage is believed to be mostly responsible for transmitting the disease in the field. The abundance and distribution of *P. marinus* in the field are positively correlated with temperature and salinity (Andrews 1988, Andrews and Ray 1988). Results of recent studies in our laboratory suggest that, in addition to warm temperatures and high salinity, environmental pollution could be a potential stressor contributing to *P. marinus* susceptibility and progression in oysters (Chu and Hale 1994, Chu *et al*. 1997, Chu *et al*. submitted). We exposed oysters from the Rappahannock River, VA (a tributary of the southern Chesapeake Bay affected by *P. marinus*) with low prevalence of *P. marinus* infection to 0, 15, and 30% dilutions of CSs-derived WSFs daily for 35 days. Oysters were then challenged with *P. marinus* by individual inoculation into the shell cavity with 0.1 ml 1 μm filtered estuarine (York River, VA) water containing 1000 *P. marinus* meronts. After *P. marinus* challenge, WSF exposure was continued for an additional 21 days. The thioglycollate assay described by Ray (1952, 1966) was used for *P. marinus* diagnosis. Intensity of infection was ranked negative, light, moderate, and heavy based on the number of stained *P. marinus* particles contained in the oyster tissue smear (Ray 1952, Andrews 1988). *P. marinus* prevalence (% of infected oysters) in each treatment group was calculated. Prevalence of *P. marinus* infection was found to increase significantly as WSF concentration increased in both *P. marinus* challenged and non-challenged oysters (figure 5, logistic regression, $P = 0.018$; Chu and Hale 1994). Increased susceptibility to laboratory-induced infection was noted when the experiment was repeated using oysters from an area (Damariscotta River, ME) outside the normal geographic range of *P. marinus*, while no infection was noted in control oysters (figure 6, logistic regression, $P = 0.023$; Chu and Hale 1994). In summary, these results indicate that WSF exposure enhanced preexisting *P. marinus* infection and increased the oysters' susceptibility in experimental-induced infection, in a dose-dependent manner.

To further explore the role of pollution on the onset and progression of infectious disease caused by *P. marinus*, we also tested the effect of CSs on *P. marinus* expression in oysters from a *P. marinus* enzootic area (Point of Shoals, James River, VA) with an initial infection prevalence of 39%. Oysters were exposed daily to 0, 1.0, 1.5, or 2.0 g of suspended CSs (corresponding to 0, 156 μg, 234 μg, and 312 μg PAHs daily) for 30 days. Cessation of exposure of oysters for 3, 7, and

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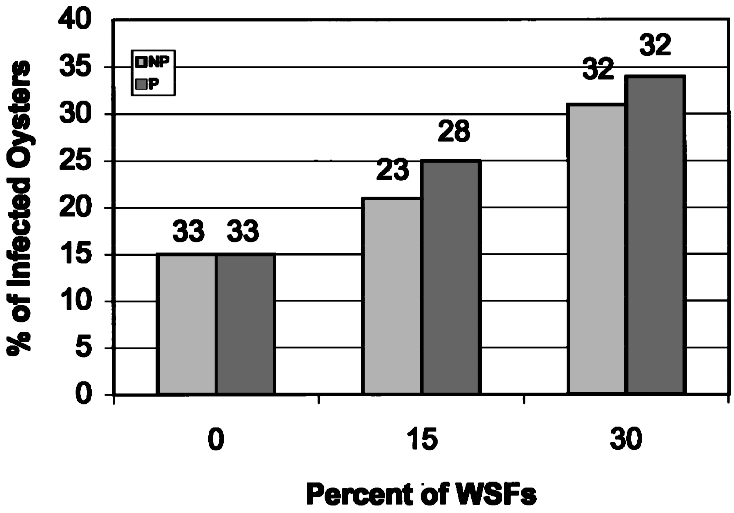

Figure 5. (modified from Chu and Hale 1994). Prevalence (% of infected oysters) in *Perkinsus marinus* challenged (P) and non-challenged (NP) oysters after exposure to 0, 15, and 30% WSFs. Oysters were exposed to WSF dilutions for 35 days, and then were challenged with *P. marinus*. WSF exposure was continued for an additional 21 days. The number of oysters in each treatment is indicated on the top of each bar.

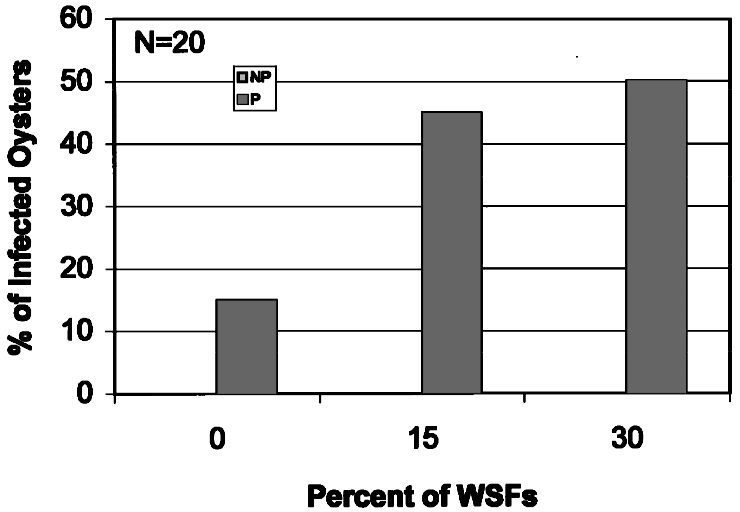

Figure 6. (modified from Chu and Hale 1994). Prevalence (% of infected oysters) in *Perkinsus marinus* challenged (P) and non-challenged (NP) oysters after exposure to 0, 15, and 30% WSFs. Oysters were exposed to WSF dilution for 35 days, and thereafter challenged with *P. marinus*. WSF exposure was continued for an additional 35 days.

14 days did not moderate *P. marinus* progression. At the end of the experiment, cumulative prevalences were 47, 65, 50, and 74% in oysters provided with 0, 1.0, 1.5, and 2.0 g of CSs, respectively. Treatment with CSs appeared to increase disease expression in oysters (figure 7, logistic regression, $P = 0.0334$; Chu *et al.* 1997, Chu *et al.* submitted). Most of the infections were light. Only a few oysters were moderately or heavily infected by the parasite. No significant effect was found

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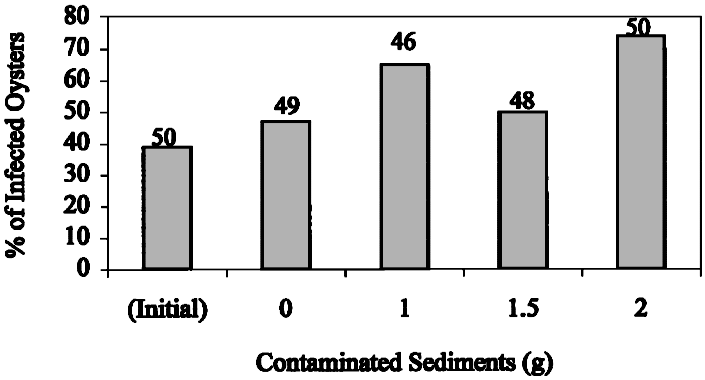

Figure 7. *Perkinsus marinus* prevalence (% of infected oysters) in control and CS-exposed oysters after 30 days of exposure. Control, Dose 1, 2, and 3 = 0, 1.0, 1.5, and 2.0 g CSs, respectively (corresponding to 0 µg, 156 µg, 234 µg, 312 µg PAHs). The number of oysters in each treatment is indicated on the top of each bar.

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in any measured cellular or hormonal parameters (i.e. total circulating haemocytes, % of granulocytes, NR uptake, MTT reduction, and ^{3}H-leucine incorporation) in oysters after 30 days of CS exposure. A preliminary experiment examining the impact of sediment dosage (unspiked clay particles, 5–50 µm) on haemocyte activities (phagocytosis, intracellular killing, ^{3}H-leucine, ^{3}H-thymine, and ^{3}H-uridine incorporation), plasma protein, plasma lipid, and condition index suggested that sediment amounts from 0 to 2 g day^{-1} for 30 days had no impact on these endpoints.

Relationship between haemocyte functions/activities and disease susceptibility and expression

Impaired immune function, due to pollutant stress, leading to infectious disease outbreaks in aquatic organisms has long been suggested (Sinderman 1983, 1993). Immunocompetent cells such as haemocytes in oysters are primarily responsible for host defence. Quantifying disease susceptibility and expression offers an assessment of the physiological/immunological significance of changes in haemocyte functions/activities due to pollutant stress. Such a direct relationship, however, was not observed in our study. Although modulation of certain haemocyte activities/functions was noted in the *in vitro* WSF and *in vivo* CS exposure studies, no statistically significant changes were observed in any cellular parameters in oysters in the experiment examining the CS effects on *P. marinus* expression in oysters, even though the sediments contained similar contaminant burdens as those used to test the CS effect on haemocyte functions/activities (table 2). Similarly, while tributyltin (TBT) exposure augmented *P. marinus* infection and disease related mortality in eastern oysters, no significant effect was noted in haemocyte activities, such as phagocytosis and chemiluminescence due to TBT exposure (Fisher *et al.* 1995, Anderson *et al.* 1996). Anderson *et al.* (1996) observed increased chemiluminescence and number of circulating haemocytes associated with advanced *P. marinus* infection in their study. Pathological effects could have masked the sublethal toxic responses. However, no significant effect was found in

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oysters due to infection in our study, perhaps because most infections were light. The mechanisms triggering the elevated disease susceptibility and expression in oysters exposed to contaminants are unknown and remain to be elucidated.

Cellular and biochemical responses, and disease susceptibility and expression as pollutant exposure biomarkers

Various assays are now available to test toxic effects at subcellular and cellular levels. Effect or damage due to pollutant exposure most probably begins at the subcellular level and continues with changes in cell function until a distinct observable endpoint is reached (Bayne *et al*. 1988). Measurement of cellular and subcellular responses should provide an insight into the physiological fitness of the tested organisms. Alteration in any subcellular and/or cellular parameter could be a warning signal of potential adverse toxic effects. Thus, cellular responses could be useful indicators of sublethal stress. It was shown in our study that *in vitro* exposure of oyster haemocytes to CSS-derived WSFs reduced to some extent the cellular responses, such as phagocytosis, chemiluminescence and chemotaxis. It should be noted that the impact of short-term exposure to a sublethal level of stress may be difficult to detect due to high individual variation in response, thereby masking toxicological significance. Thus, increasing the replication of animals or pools of animals is desirable. However, high individual and population variability is a reality. In some cases, if results obtained in repeated trials are consistent, it may be unnecessary to reject the biological significance of the data, although they are statistically insignificant. Also, physiological and immunological responses vary not only by species, but also as a function of exposure dose, duration, and mode; and the population involved and their environmental history. This could be seen in our study; i.e. *in vivo* exposure of oysters to CS altered the haemocyte functions such as MTT reduction, NR uptake, and ^{3}H-leucine incorporation in the experiment using oysters from Maine, but not in the experiment using test organisms from a Dermo enzootic area of the Chesapeake Bay. Hence, it is difficult to directly compare data between various exposure times and different laboratories. Disease susceptibility and expression may be one of the endpoints induced by pollutant stress that can be quantified. Although no linkage could be found between cellular effect and accelerated disease susceptibility/expression, our studies provide further evidence that pollution is a potential factor in infectious disease outbreaks and progression in aquatic animals. Blood biochemical alteration could originate from effects at cellular, subcellular and/or molecular levels. Nevertheless, no significant change was noted in plasma chemistry due to CS exposure. In nature, however, both acute and chronic exposures are of concern. It also may be difficult to distinguish the toxic effect from an adaptive response (acclimatizion) of organisms exposed to sublethal stress (Mayer *et al*. 1992). Application of these parameters to assess pollutant effects in the field is feasible. However, a large database is required to establish the 'normal range' of these parameters in relation to the physiological status of the test organisms and the seasonal changes of environmental variables, such as temperature, salinity, food availability, and dissolved oxygen of the test sites.

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