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Increased Susceptibility of Juvenile Chinook Salmon to Vibriosis after Exposure to Chlorinated and Aromatic Compounds Found in Contaminated Urban Estuaries

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Abstract.—Saltwater-adapted juvenile chinook salmon *Oncorhynchus tshawytscha* exposed to aromatic and chlorinated compounds, representative of contaminants found in urban estuaries in Puget Sound, have a higher susceptibility to vibriosis than do fish exposed only to the solvent vehicle. Susceptibility to vibriosis was assessed by examining the percent cumulative mortality of the salmon after exposure to the bacterial pathogen *Vibrio anguillarum.* The aromatic and chlorinated compounds examined consisted of a sediment extract from the Hylebos Waterway that was enriched in butadienelike compounds (chlorinated-enriched Hylebos Waterway sediment extract [CHWSE]), a model mixture of polycyclic aromatic hydrocarbons (PAHs), a polychlorinated biphenyl mixture (Aroclor 1254), hexachlorobutadiene (HCBD), and 7,12-dimethylbenz[a]anthracene (DMBA). Two trials were conducted. In trial l, the percent cumulative mortality of juvenile chinook salmon exposed to *V. anguillarum* after receiving either CHWSE, HCBD, or the model mixture of PAHs ranged from 28% to 31% compared with the 16% observed in the acetone: emulphor control group at 7 d post-bacterial challenge. In trial 2, the net cumulative mortality of juvenile chinook salmon exposed to *V. anguillarum* after receiving either DMBA or Aroclor 1254 ranged from 46% to 49% compared with the 25% observed in the acetone:emulphor control group at 9 d postchallenge. The differences in mortality between groups of fish in the treated and control groups in both trials were significant at $P \leq 0.05$. These findings suggest that a higher predisposition to infection and subsequent disease can occur in salmon exposed to chemical contaminants found in urban estuaries of Puget Sound, Washington.

Estuaries are essential habitats for many commercially important fish species including a number of Pacific salmon (Levings and Bouillon 1997). Estuarine habitats provide refuge from predation, extensive foraging opportunities that support rapid growth, and an environment for juvenile salmon to facilitate the physiological transition from fresh to salt water (Thorpe 1994). It has been suggested that the greatest ecological differences between the various salmon species occur during their first year of life (Day et al. 1989), which, for some species, can be spent primarily within an estuary. The productivity of these nursery areas may be directly correlated to subsequent early ocean survival of salmonids (Parker 1962). Therefore, factors that affect the health of juveniles during this critical period may have subsequent negative effects during the estuarine transition period or later in their marine phase.

Estuaries are often impacted by detrimental land use practices and other human activities. Anthropogenic influences on estuaries include enrichment with excessive levels of organic materials and inorganic nutrients, heat, physical alterations, introduction of toxic materials, and changes in community structure through introduction of exotic species (Day et al. 1989). Specifically, toxic material from a variety of sources can accumulate in sediment as well as in salmonid prey species. Because juvenile salmon are undergoing numerous physiological adaptations during their residence in estuarine environments, there is concern that any additional stresses such as exposure to toxic chemicals, including pesticides, heavy metals, and pe-

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troleum products and by-products, may impair the fitness of out-migrant juvenile salmon.

Two waterways located in central Puget Sound in the state of Washington, the Hylebos Waterway of Commencement Bay and the Duwamish Waterway of Elliot Bay, are severely contaminated by a variety of organic and inorganic contaminants (Malins et al. 1984). Juvenile chinook salmon *Oncorhynchus tshawytscha* inhabit these waterways in the late spring and early summer. Previous studies demonstrated that juvenile chinook salmon sampled from these sites are exposed to a wide range of chemical contaminants (McCain et al. 1990; Stein et al. 1995; Collier et al. 1998), including high concentrations of aromatic and chlorinated compounds.

Laboratory studies with fish have demonstrated that their immune system is susceptible to specific contaminants such as aflatoxin B_1 (Arkoosh and Kaattari 1987), tributyltin (Rice and Weeks 1989), cadmium (Thuvander 1989), and pulp mill effluent (McLeay and Gordon 1977). The resulting immune dysfunction can cause serious sublethal effects. Subsequently, we found that juvenile chinook salmon from polluted environments such as the urban Duwamish Waterway are immunosuppressed whereas juvenile chinook salmon from the nonurban Nisqually estuary are not compromised (Arkoosh et al. 1991). To determine whether salmon from a contaminated environment are also more susceptible to an infectious disease, we collected juvenile fall chinook salmon from the same locations as in the previous study and exposed them in the laboratory to the bacterial pathogen *Vibrio anguillarum* (Arkoosh et al. 1998). We found that juvenile chinook salmon from the contaminated estuary were more susceptible to mortality induced by *V. anguillarum* than were fish from the nonurban estuary that were not exposed to contaminants. These disease challenge studies indicated that juvenile chinook salmon exhibiting contaminant-associated immunodysfunction also expressed an increased susceptibility to the pathogen *V. anguillarum* (Arkoosh et al. 1998). These previous field studies (Arkoosh et al. 1991, 1998) demonstrated that fish from the contaminated estuary were immunosuppressed and more susceptible to an infectious disease than were juveniles from noncontaminated environments.

Our aim in this current study was to determine whether contaminants, apart from other estuarine variables specifically associated with the Duwamish and Hylebos waterways, can independently increase disease susceptibility in juvenile chinook salmon exposed to *V. anguillarum.* These studies further substantiated a linkage between chemical contaminant exposure and increased disease susceptibility in juvenile fall chinook salmon.

Methods

Contaminants.—The following five contaminant solutions were prepared for the pathogen challenge study. (1) One solution is a mixture of organic contaminants extracted from contaminated sediment collected from the Hylebos Waterway (chlorinated-enriched Hylebos Waterway sediment extract [CHWSE]). We were particularly interested in the sediment from the Hylebos Waterway because it was part of a previous Natural Resource Damage Assessment (NRDA) study (Collier et al. 1998). The NRDA study demonstrated that chinook from the Hylebos Waterway had concentrations of hexachlorobutadiene (HCBD) in their livers that exceed those found in the Duwamish estuary. Accordingly, we used an extraction method that enriched for chlorinated butadiene compounds inclusive of HCBD. Other solutions included (2) a solution of HCBD that we determined to be a marker chemical for the Hylebos Waterway (Collier et al. 1998), (3) a model mixture of polycyclic aromatic hydrocarbons (PAHs) prepared to represent high-molecular-weight PAHs (three to five rings) found in the Hylebos Waterway sediment, (4) a PAH compound, 7,12-dimethylbenz[a]anthracene (DMBA), shown previously to suppress immune responses in juvenile chinook salmon (Arkoosh et al. 1994), and (5) a commercially acquired polychlorinated biphenyl (PCB) mixture (Aroclor 1254) similar to the mixture of PCBs found in the Duwamish and Hylebos waterways and shown previously to suppress immune responses in juvenile chinook salmon (Arkoosh et al. 1994).

Collection of sediment for extract preparation.—Hylebos Waterway sediment extract enriched for chlorinated compounds inclusive of butadiene compounds was developed from sediment collected at three sites near the mouth of the Hylebos Waterway. This sampling area was approximately 500 m from the mouth of the Hylebos Waterway and closest to the southern shore (Figure 1). This area was selected because of the presence of HCBD-like compounds in the sediment (Collier et al. 1998). Sediment used to prepare the extract was collected by the use of a Van Veen grab deployed from the Northwest Fisheries Science Center research vessel, the RV *Harold W. Streeter*. The grab was rinsed with isopropyl alcohol before sampling began to remove traces of contaminants that might

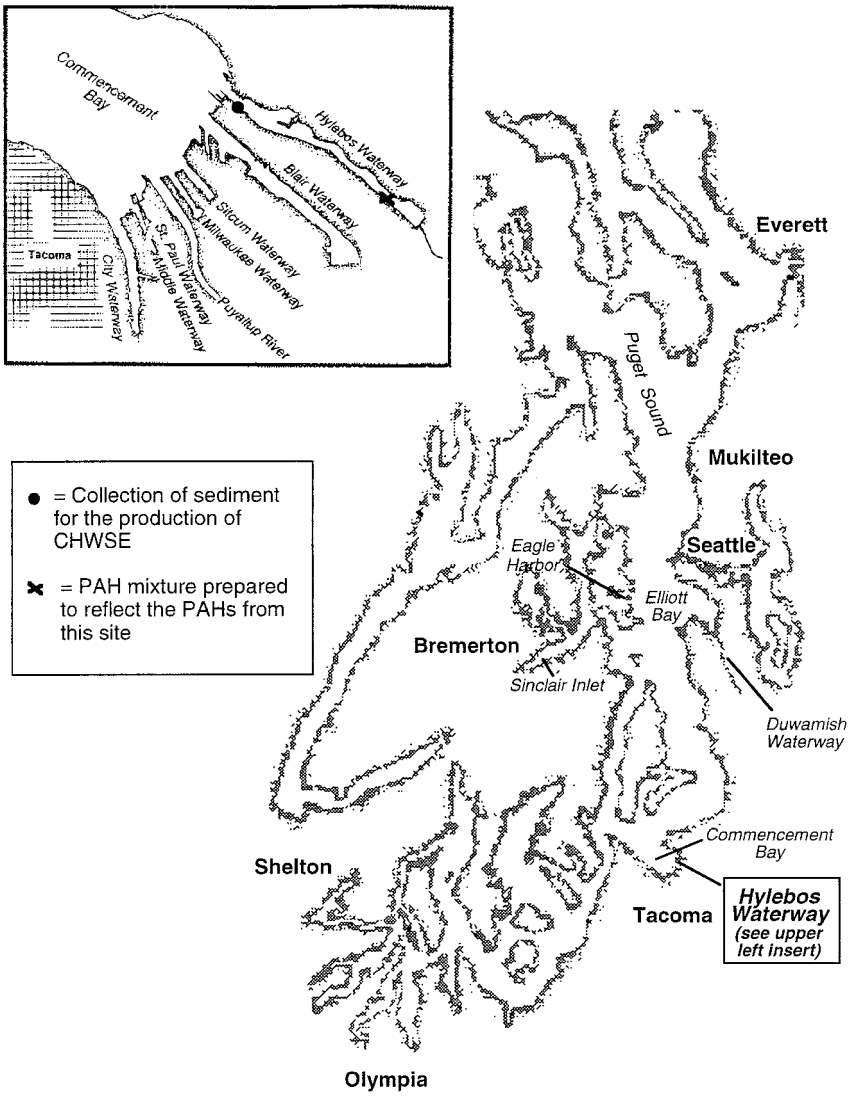

FIGURE 1.—A map of the Hylebos Waterway within Commencement Bay. Areas relevant to this study are marked on the map. Abbreviations are the following: CHWSE, chlorinated-enriched Hylebos Waterway sediment extract; and PAH, polycyclic aromatic compound.

be present from previous samplings. The top 2–3 cm of sediment was collected from each grab and placed in a 19-L container until approximately 15.6 L of sediment had been collected from each of the three sites within the sampling area. The three 19-L containers of sediment were returned to the laboratory and held at 4°C. The sediments from each sampling site were homogenized, and approximately equal amounts of sediment from each site were placed in a new 19-L container, homogenized, and stored for future use at 4°C.

Preparation of CHWSE.—The objective of preparing CHWSE was to obtain an extract of chemical contaminants that was primarily representative of the contaminant mixture in the Hylebos Waterway and also enriched in the less-polar chlorinated hydrocarbons such as HCBD. As stated previously, we found HCBD to be a marker chemical for the Hylebos Waterway (Collier et al. 1998). In a 4-L bottle, 330 g of sodium sulfate and 330 g of magnesium sulfate were mixed together. To this sulfate mixture, 500 g of sediment and 1.0 L of pentane were added, gently mixed, and vented as necessary. After the mixture reached room temperature,

TABLE 1.—Concentration of polycyclic aromatic hydrocarbons, chlorinated butadienes, pesticides, and polychlorinated biphenyls in the chlorinated-enriched fraction of a sediment extract from the Hylebos Waterway that was injected intraperitoneally into juvenile chinook salmon.

Analyte	ng/g of sediment[a]
Aromatic hydrocarbons	
Naphthalene	26
2-Methylnaphthalene	18
1-Methylnaphthalene	10
Biphenyl	6
2,6-Dimethylnaphthalene	10
Acenaphthylene	0.9
Acenaphthene	28
2,3,5-Trimethylnaphthalene	28
Fluorene	28
Phenanthrene	140
Anthracene	47
1-Methylphenanthrene	10
Fluoranthene	170
Pyrene	170
Benz[a]anthracene	23
Chrysene	33
Benzo[b]fluoranthene	6
Benzo[k]fluoranthene	5
Benzo[e]pyrene	9
Benzo[a]pyrene	10
Perylene	2
Indeno[1,2,3-cd]pyrene	0.3
Dibenz[a,h]anthracene	<0.02
Benzo[ghi]perylene	1
Dibenzothiophene	12
Chlorinated butadienes[b]	
Trichlorobutadiene	480
Tetrachlorobutadiene	450
Pentachlorobutadiene	160
Hexachlorobutadiene	150
Pesticides	
Hexachlorobenzene	110
Lindane (gamma-BHC)	2
Heptachlor	0.8
Aldrin	<0.06
Heptachlorepoxide	21
Oxychlordane	<0.07
Trans-chlordane	<0.06
Nonachlor-III	<0.06
Alpha-chlordane	2
Trans-nonachlor	5
Cis-nonachlor	0.4
Dieldrin	2
Mirex	2
o,p′-DDE	1
p,p′-DDE	0.4
o,p′-DDD	20
p,p′-DDD	1
o,p′-DDT	<0.1
p,p′-DDT	<0.1
Polychlorinated biphenyls	
Trichlorobiphenyl-18	17
Trichlorobiphenyl-28	6
Tetrachlorobiphenyl-44	1
Tetrachlorobiphenyl-52	3
Tetrachlorobiphenyl-66	<0.08
Pentachlorobiphenyl-101	3
Pentachlorobiphenyl-105	<0.06
Pentachlorobiphenyl-118	<0.07
Hexachlorobiphenyl-128	0.8
Hexachlorobiphenyl-138	2
Hexachlorobiphenyl-153	4
Heptachlorobiphenyl-170	1
Heptachlorobiphenyl-180	9
Heptachlorobiphenyl-187	2
Octachlorobiphenyl-195	2
Nonachlorobiphenyl-206	12
Decachlorobiphenyl-209	31

[a] Analyte concentrations were calculated on a sediment wet weight basis.

[b] Concentrations of butadienes were calculated using a response factor of 1 with gas chromatography–mass spectometry total ion current areas.

the volume of solvent was returned to 1 L with pentane. The bottle was capped and tumbled for a total of 4 h, stopping to degas as needed. The mixture was allowed to stand for 24 h at room temperature. After 24 h, the solvent extract was decanted and retained. Seven hundred milliliters of pentane were added to the 4-L bottle, tumbled for another 2 h, and allowed to stand for 2 h. The solvent was decanted and added to the solvent from the previous tumbling. Five hundred milliliters of pentane were added to the mixture and shaken, and the solids were allowed to settle. The solvent extract was poured off and added to the other solvent mixture. The solvent extract was then concentrated to approximately 50 mL. A chromatographic column containing 25 mL of silica gel was prepared and washed with pentane. The solvent extract was placed on the column, eluted with 80 mL of pentane, and collected in a 250-mL bottle. The collected extract was concentrated to 15 mL and treated with activated copper to remove sulfur. The extract was then exchanged into acetone by adding 2 mL of acetone and concentrated to 2 mL. The above procedure was repeated for a total of 6.0 kg of sediment. The extracts produced from each procedure were combined, and the volume of the acetone extract was adjusted to reflect a concentration of 0.4 g of sediment per microliter of acetone. An aliquot of the final extract was removed for chemical analysis and quantitation of chlorinated and aromatic hydrocarbons. The remainder was placed into 7-mL amber vials for storage until use. As stated above, the final concentration of chemical contaminants in 1 L of the CHWSE was equivalent to chemical contaminants in 0.4 g of sediment from the Hylebos Waterway

TABLE 2.—Sum of the concentration of polycyclic aromatic hydrocarbons, chlorinated butadienes, pesticides, and polychlorinated biphenyls in the chlorinated-enriched fraction of a sediment extract from the Hylebos Waterway that was injected intraperitoneally into juvenile chinook salmon. See Table 1 for the individual analytes.

Analyte	Total ng/g of sediment
Polycyclic aromatic hydrocarbons	790
Chlorinated butadienes	1,200
Pesticides	210
Polychlorinated biphenyls	94

in Commencement Bay. The concentrations of analytes in the extract are listed in Table 1. The extract is enriched in butadienelike compounds although it still retains appreciable quantities of PAHs. The sum concentration of PAHs, chlorinated butadienes, pesticides, and PCBs in the sediment extract is listed in Table 2.

Preparation of the PAH model mixture.—A model mixture of PAHs containing 10 high-molecular-weight compounds was prepared to reflect the same proportions of the analytes that were found in sediment from the Hylebos Waterway at a location adjacent to a ditch draining from an aluminum smelter site (Figure 1). Analytical grade compounds of 10 high-molecular weight hydrocarbons including fluoranthene, pyrene, benz[a]anthracene, chrysene, benz[b]fluoranthene, benz[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, and benzo[ghi]perylene were purchased from Sigma Chemical Co. (St. Louis, Missouri). The compounds were combined in acetone at the same percentages that were present at the sampling location with a concentration equivalent to 0.4 g of sediment extracted per microliter of acetone (Table 3). An aliquot of the PAH model mixture was analyzed to confirm the concentrations of the individual compounds. The final concentration of chemical contaminants in 1 L of the PAH model mixture was equivalent to that of chemical contaminants in 0.4 g of sediment from the Hylebos Waterway.

Preparation of the HCBD test solution.—Hexachlorobutadiene is considered a marker chemical for the lower Hylebos Waterway (Collier et al. 1998). Analytical grade HCBD (Sigma) was prepared in a 1:1 acetone:emulphor solution. See below for the HCBD concentrations used for determining the lethal dose (LD) response curve of HCBD.

Preparation of the PCB mixture (Aroclor 1254) and PAH test solution (DMBA).—Solutions of DMBA (Sigma) and Aroclor 1254 (Accustandard, Inc., New Haven, Connecticut) are representative of the types of PCBs and PAHs found in both Hylebos and Duwamish waterways. The chemicals were prepared in a 1:1 acetone:emulphor solution. The solutions were sonicated before use.

TABLE 3.—High-molecular-weight hydrocarbons that comprise the polycyclic aromatic hydrocarbon (PAH) model mixture. The compounds were combined in acetone at the same percentages in which they were found in sediment from the Hylebos Waterway to a concentration equivalent to 400 g of sediment extracted per milliliter of acetone.

PAH analyte	% of mixture
Fluoranthene	21
Pyrene	20
Benz[a]anthracene	7
Chrysene	13
Benz[b]fluoranthene	17
Benz[k]fluoranthene	6
Benzo[a]pyrene	6
Indeno[1,2,3-cd]pyrene	4
Dibenz[a,h]anthracene	1
Benzo[ghi]perylene	5

Fish.—Inspections performed by state fish health inspectors and observations by the hatchery managers revealed no evidence or signs of the principal salmonid diseases in salmon (9–12 g) used in these studies.

Juvenile chinook salmon were collected from the Kalama Creek Hatchery (Nisqually, Washington) for use in generating the LD response for CHWSE and HCBD solutions. The salmon were placed in 95-L coolers equipped with air stones to maintain proper oxygen levels during transport to the Mukilteo Field Station, a National Marine Fisheries Service facility located at Mukilteo, Washington. After arrival, the fish were immediately transferred to 1.83-m circular fiberglass tanks with fresh dechlorinated water. The juvenile salmon were slowly acclimated from freshwater to seawater over a 5-d period. Juvenile salmon were allowed to acclimate for a minimum 2-week period in seawater (9–12°C, 30–32‰ salinity) before the beginning of the LD tests. The juvenile salmon were fed 3% of their total body weight/d with Biodiet Grower Peletized Feed (2.5 mm; Bio-Oregon, Inc., Warrenton, Oregon).

The susceptibility of juvenile chinook salmon to *V. anguillarum* after exposure to toxicants was examined in two stages due to limited numbers of tanks. In trial 1, juvenile salmon were obtained from the Elk River Hatchery (Port Orford, Oregon), and for trial 2, juvenile chinook salmon were obtained from the Naselle (Washington)

Hatchery. Fish for both trials were transported in freshwater to the Salmon Disease Laboratory at the Hatfield Marine Science Center in Newport, Oregon. Fish were transported in 114-L coolers. The coolers were equipped with air stones to maintain proper oxygen levels and contained 0.3% NaCl (w/v) to decrease stress during transportation. Sealed containers of ice were also added to the coolers throughout transport to avoid elevation of water temperature. After arrival, the fish were immediately transferred to two 1.2-m circular tanks with aeration devices and flowing dechlorinated freshwater. Mortality was negligible after transport. After 1 week of acclimation, the fish were adjusted to a saltwater environment as described above. Fish were held in circular tanks (0.6 m diameter) in salt water (9–12°C, 30–32‰ salinity) and were fed as described above until the beginning of the experiment.

Generation of the LD response curves for CHWSE and HCBD solutions.—The LD response curves were determined by exposing 10 juvenile chinook salmon to each of the doses that consisted of 50, 200, 400, 600, and 800 g of sediment equivalent/kg of fish for CHWSE and 0.1, 1, 5, 30, 60, 100, 500, and 1,000 mg/kg of fish for HCBD. Ten fish were also exposed to the acetone : emulphor carrier solution. The fish were anesthetized with 1.5 mg of metomidate hydrochloride/L (Wildlife Laboratories, Fort Collins, Colorado), weighed, measured, and injected intraperitoneally with 1 μL per gram body weight of the contaminant. The salmon were held in 190-L tanks with running seawater and were observed for mortality over a 96-h period.

Generation of the LD response curves for the PAH model mixture, PCB mixture (Aroclor 1254), and the PAH test solution (DMBA).—The LD response curves of juvenile chinook salmon exposed to PAHs and PCBs have been determined previously by our laboratory (Arkoosh et al. 1994). Accordingly, this information was used to establish doses of the PAH model mixture, Aroclor 1254, and DMBA in the disease challenge study.

Contaminant exposure and pathogen challenge.—In the first trial, fish were exposed by intraperitoneal injection to sublethal doses of either CHWSE, HCBD, or the PAH model mixture. In the second trial, fish were injected with one dose of either Aroclor 1254 or DMBA. Doses of contaminants were chosen that would allow for a substantial but sublethal dose. Doses of the contaminants for the challenge with *V. anguillarum* were chosen on the basis of their LD response curve. In both trials, control fish were injected with the acetone : emulphor carrier solution. One week after contaminant injection, fish were exposed to either *V. anguillarum* or to the seawater diluent alone (see below).

In trial 1, the juvenile salmon were anesthetized with 1.5 mg of metomidate hydrochloride/L, individually weighed, and then injected intraperitoneally with 2 μL of inoculum per gram body weight of fish. Fish were injected with either HCBD, the PAH model mixture, CHWSE, or acetone : emulphor. Concentrations of the test solutions were 10% of the LD50 of HCBD (20 mg of HCBD/kg of fish), 10% of the LD50 of the PAH model mixture (6.3 mg of the model PAH mixture/kg of fish), or 41% of the LD30 of CHWSE (307 g of sediment equivalent/kg of fish). Because we were only able to achieve 30% mortality in the fish exposed to a very high concentration of CHWSE, we determined the LD30 for this contaminant instead of the LD50 (see Results). The groups of fish injected with each contaminant were kept separate in individual tanks. Thirty fish were randomly assigned to six tanks for each of the four treatments. Three tanks of each treatment were challenged with *V. anguillarum.* The remaining three tanks in the treatment received only the saltwater diluent. This latter unexposed group provided an estimate of background mortality.

In trial 2, Naselle Hatchery salmon were anesthetized as described above for trial l, weighed, and injected intraperitoneally with 2 μL of inoculum per gram body weight of fish. Fish were injected with either DMBA, Aroclor 1254, or acetone : emulphor. Concentrations of the DMBA and Aroclor 1254 solutions were 20% of the LD50. Therefore, the concentration of DMBA used was 13 mg of the PAH/kg of fish. The concentration of Aroclor 1254 used was 54 mg of the PCB mixture/kg of fish. The groups of fish injected with each contaminant were kept separate in individual tanks. Fifteen fish were randomly assigned to six tanks for each of the three treatments. Three tanks of each treatment were challenged with *V. anguillarum.* The remaining three tanks in the treatment received only the saltwater diluent. This latter unexposed group provided an estimate of background mortality.

Infection of salmon with V. anguillarum.—The bacterium *V. anguillarum* was grown at 25°C in trypticase soy broth to an optical density (OD) of approximately 1.7 before use. Use of the bacterial cultures at this OD insures that the culture is in an exponential growth phase. One week after ex-

posure to contaminants, juvenile chinook salmon were exposed to 6×10^{-5} mL of bacterial culture/mL of seawater (equivalent to 60,000 colony-forming units/mL of seawater) at this optimal OD. This dilution of bacteria was previously determined not to overwhelm any effects of the contaminants by killing the fish too rapidly (Arkoosh et al. 1998). Groups of fish that were exposed to the contaminants or to the seawater diluent and not challenged with *V. anguillarum* were also included. Accordingly, salmon from each treatment were placed in 7.6-L buckets with 4 L of seawater with or without the bacteria. The fish were held in the buckets for l h with constant aeration. After the l-h exposure, the salmon were immediately returned to their respective 0.6-m tanks with a flow rate of 7.6 L per minute. Mortalities were collected daily for the duration of the experiment.

Water treatment.—The water supply consisted of sand-filtered and ultraviolet light-treated seawater. Before the effluent was released into Yaquina Bay, Oregon, it was treated with charcoal and chlorine to prevent the introduction of chemicals and surviving bacteria, respectively, into the estuary.

Confirmation of pathogen-induced mortality.—Necropsies were performed on one randomly chosen mortality out of three mortalities to ensure that the majority of bacterial-exposed fish had been infected with *V. anguillarum* (Arkoosh et al. 1998). First, the dead fish were sprayed with 75% ethanol. A small incision was made into the ventral abdomen with a sterile scalpel blade, taking care not to damage any of the internal organs. In trial 1, a sterile loop was inserted into the kidney and then aseptically struck onto a trypticase soy agar (TSA) plate supplemented with 0.5% NaCl. Bacterial colonies from the TSA were examined for the presence of cytochrome oxidase activity with a dry slide cytochrome oxidase test (VWR, Seattle, Washington) and their ability to agglutinate with rabbit anti-*V. anguillarum* antiserum (Micrologix International, Ltd., Victoria, British Columbia, Canada). In trial 2, a sterile loop was inserted into the kidney and then aseptically struck onto a thiosulfate citrate bile salt sucrose (TCBS) agar. Colony growth on the TCBS agar was regarded as a *Vibrio* sp. (Manavalan Vanajakumar-Dhevendaran 1977).

Generalized linear models.—Generalized linear modeling (GLM) was used to determine the LD30 and the LD50 for CHWSE and HCBD solutions, respectively, and the confidence limits for these values (Kerr and Meador 1996). Statistical significance between treatments in the pathogen challenge studies was also assessed by the use of GLM. We assumed that the number of survivors and mortalities in both assays follow a binomial distribution (Hildén and Hirvi 1987; Kerr and Meador 1996). The analyses were performed with the GLMStat computer application (Beath 1995). To define a generalized linear model, it is important to identify the error structure and the link function that relates the linear predictor to the expected survival and/or mortality probabilities (Baker and Nedler 1978). The logistic generalized linear model was used for the analysis of data. For this model the error structure is binomial, and the linear predictor was related to the expected value of the datum by the logit link function.

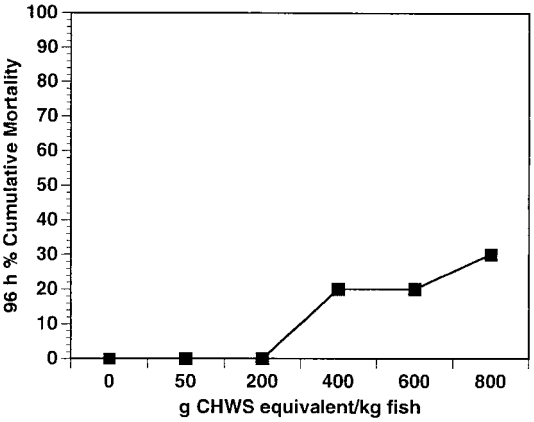

FIGURE 2.—The 96-h lethal dose curve of salmon given various doses of chlorinated-enriched Hylebos Waterway sediment extract (CHWSE).

Specifically for the pathogen challenge study, the logistic model was used to evaluate whether survival and mortality of fish treated with a contaminant and fish treated only with the acetone : emulphor carrier were significantly different ($P \leq 0.05$) beginning at 7 d postchallenge. This analysis was conducted after correction for background mortalities. To correct for background deaths, the number of mortalities of a particular treatment group not treated with *V. anguillarum* was subtracted from the mortalities of that treatment group exposed to the bacteria. If required, analyses were conducted beyond day 7. Experiments were continued until mortalities began to level off at an asymptote in at least one treatment group.

Results

Lethal Dose of CHWSE and HCBD Solutions

The cumulative 96-h LD curves of salmon given various doses of either CHWSE (Figure 2) or

TABLE 4.—Recovery and presumptive identification of *Vibrio* sp. among dead juvenile salmon after exposure to selected contaminants and subsequent challenge with *V. anguillarum* 7 and 9 d after challenge. Abbreviations are the following: Aroclor 1254, a polychlorinated biphenyl mixture; CHWSE, chlorinated-enriched Hylebos Waterway sediment extract; DMBA, 7,12-dimethylbenz[a]anthracene; HCBD, hexachlorobutadiene; PAH, polycyclic aromatic hydrocarbons; TCBS, thiosulfate citrate bile salt sucrose.

Contaminant	Positive agglutination[a]	Agglutination (%)	Oxidase positive[b]	Oxidase positive (%)	TCBS[c]	TCBS (%)
			Day 7 (trial 1)			
Acetone/emulphor	7/11	64%	11/11	100%		
HCBD	4/11	45%	11/11	100%		
CHWSE	12/12	100%	12/12	100%		
Model mixture PAH	7/11	64%	11/11	100%		
			Day 9 (trial 2)			
Acetone/emulphor					9/9	100%
DMBA					22/23	96%
Aroclor 1254					21/24	88%

[a] Number of dead fish examined that had bacteria that agglutinated with the anti-*V. anguillarum* antiserum/total number of fish examined.
[b] Number of fish examined that were oxidase positive/total number of fish examined.
[c] Number of fish examined that had colonies grow on TCBS.

HCBD (Figure 3) were determined. The 96-h LD50 and LD30 were determined for HCBD and CHWSE, respectively, to ensure the use of sublethal dosages of the two solutions in the pathogen challenge experiments. Because we were only able to achieve 30% mortality in the fish exposed to a very high concentration of CHWSE, we determined the LD30 for this contaminant. The LD30 determined for CHWSE was 741 g of CHWS equivalent/kg of fish. The upper and lower 95% confidence limits were 1500 and 540 g of CHWS (chlorinated-enriched Hylebos Waterway sediment) equivalent/kg of fish, respectively. The LD50 for HCBD was 200 mg of HCBD/kg of fish. The upper and lower 95% confidence limits were 450 and 130 mg of HCBD/kg of fish, respectively.

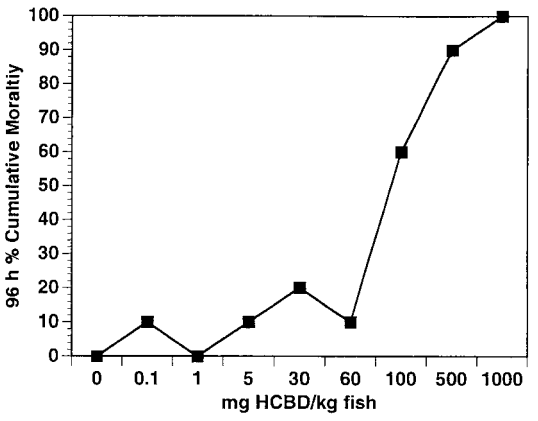

FIGURE 3.—The 96-h lethal dose curve of salmon given various doses of hexachlorobutadiene (HCBD).

Pathogen Challenge Studies

The percent cumulative mortality in trial 1 and trial 2 of the different groups of juvenile chinook salmon exposed to *V. anguillarum* is shown in Figures 4 and 5, respectively. These figures represent the net cumulative mortality attributed to exposure to the bacteria after subtraction of background mortality observed in juvenile chinook salmon that received chemical contaminants but were not exposed to bacteria. Background mortality at the end of the experiments was very low. Specifically, background mortality in trial 1 at day 7 for the various treatments was the following: acetone: emulphor (1.4%), CHWSE (1.2%), HCBD (2.7%), and the model mixture of PAH (0%). Background mortality in trial 2 at day 9 for the various treatments was the following: acetone:emulphor (6.6%), Aroclor 1254 (2.3%), and DMBA (6.7%). Statistical testing was performed beginning with 7-d postexposure data to determine treatment differences (Arkoosh et al. 1998). If statistical differences were not noted at 7 d postexposure, data obtained on the following days were also examined.

In trial 1, the net cumulative mortality of juvenile chinook salmon exposed to the bacteria after receiving either CHWSE, HCBD, or the model mixture of PAHs ranged from 28% to 31% compared with the 16% observed in the acetone:emulphor control group at 7 d post-bacterial challenge (Figure 4). Although in trial 2 a significant difference in the net cumulative mortality between juvenile chinook salmon exposed to Aroclor 1254 and acetone:emulphor was detected on day 8, a

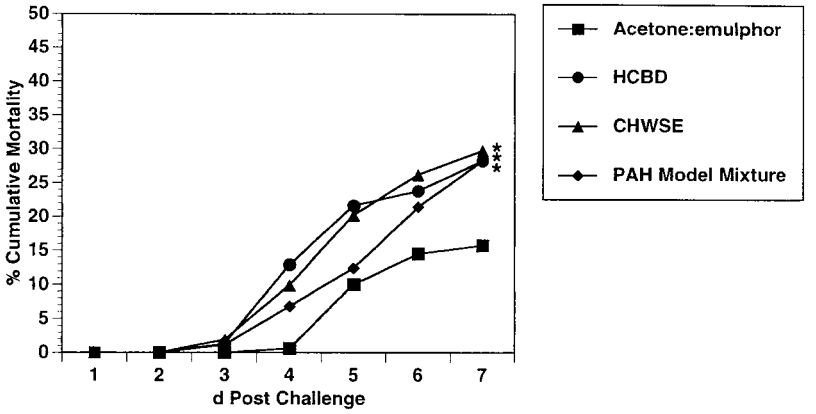

FIGURE 4.—Percent cumulative mortality of juvenile chinook salmon injected with either hexachlorobutadiene (HCBD), a model mixture of polycyclic aromatic hydrocarbons (PAHs), a chlorinated-enriched sediment extract from the Hylebos Waterway (CHWSE), or the carrier control (acetone:emulphor) after exposure to 6×10^{-5} mL of bacterial solution/mL of seawater. The cumulative mortalities are corrected for mortalities observed in juvenile chinook salmon injected with either HCBD, a model mixture of PAHs, a chlorinated-enriched sediment extract from the Hylebos Waterway, or the carrier control (acetone:emulphor) but not exposed to *Vibrio anguillarum*. The generalized linear modeling statistic was conducted beginning at 7 d postchallenge. Asterisks signify treatment groups that had a significantly greater percent cumulative mortality ($P \leq 0.05$) than that of the control fish injected with acetone:emulphor.

difference between DMBA and acetone:emulphor was not detected until day 9 after the challenge. The net cumulative mortality of juvenile chinook salmon exposed to the bacteria after receiving either DMBA or Aroclor 1254 ranged from 46% to 49% compared with the 25% observed in the acetone:emulphor control group at 9 d postchallenge (Figure 5). Therefore, the cumulative mortality was significantly higher in fish exposed to CHWSE, DMBA, Aroclor 1254, the PAH model mixture, or HCBD relative to fish receiving only the acetone:emulphor carrier.

Table 4 shows the percentage of salmon carcasses examined that were infected with *Vibrio* sp. by days 7 and 9 of the experiment during trials 1 and 2, respectively. Presumptive evidence of the

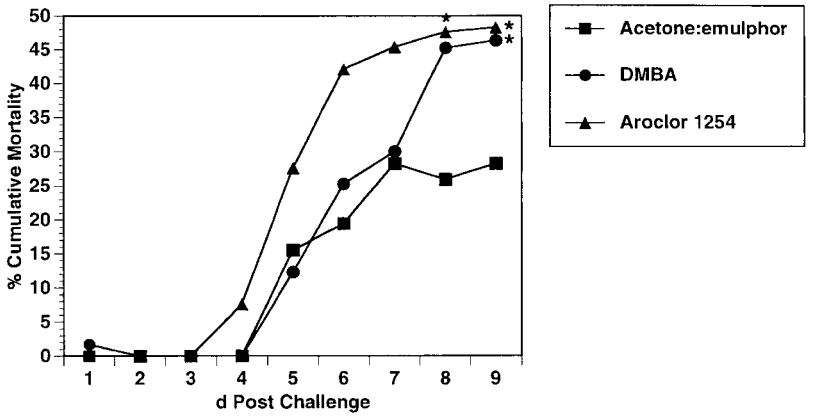

FIGURE 5.—Percent cumulative mortality of juvenile chinook salmon injected with either 7,12-dimethylbenz[a]anthracene (DMBA), a polychlorinated biphenyl mixture (Aroclor 1254), or the carrier control (acetone:emulphor) after exposure to 6×10^{-5} mL of bacterial solution/mL of seawater. The cumulative mortalities are corrected for mortalities observed in juvenile chinook salmon injected with either DMBA, Aroclor 1254, or the carrier control (acetone:emulphor) but not exposed to *Vibrio anguillarum*. The generalized linear modeling statistic was conducted beginning at 7 d postchallenge. Asterisks signify treatment groups that had a significantly greater percent cumulative mortality ($P \leq 0.05$) than that of the control fish injected with acetone:emulphor.

presence of *Vibrio* sp. ranged from 45% to 100% in the subsamples tested. The findings show that oxidase and TCBS were able to presumptively identify *Vibrio* sp. in a majority of the tested salmon (≥88%). However, the agglutination method could only confirm *V. anguillarum* in 45% of the mortalities in one group. Mortality was low among toxicant-exposed but unchallenged fish, thus suggesting that deaths in the challenged groups were indeed due to the pathogen and not due to other physiological characteristics. Subtracting the low-background mortalities from groups exposed to the pathogen takes into account those mortalities that were not specifically due to the pathogen and effectively corrects the numbers used in analyses.

Discussion

Juvenile chinook salmon exposed to contaminants associated with urban estuaries in Puget Sound, such as the Hylebos and Duwamish waterways, exhibited a higher susceptibility to mortality induced by the marine pathogen *V. anguillarum* than did the pathogen-exposed juvenile chinook salmon treated only with the carrier acetone:emulphor. The contaminants tested represent specific subsets of predominant estuarine chemical pollutants. The chlorinated hydrocarbons tested were characterized by HCBD, PCBs, and CHWSE (which is composed primarily of HCBD-like compounds), and the aromatic hydrocarbons were characterized by the model mixture of PAHs and DMBA. The present findings, together with our previous studies (Arkoosh et al. 1991, 1998), support the hypothesis that chemical contaminant exposure of juvenile salmon in contaminated waterways can influence their ability to produce a protective immune response such that their survival potential may be considerably reduced.

Two of our previous studies (Casillas et al. 1998; Collier et al. 1998) allow for comparisons of the contaminant burdens in the liver of salmon from the Hylebos Waterways with the liver contaminant burdens of fish injected with CHWSE in this study. We found that juvenile chinook salmon collected from the Hylebos Waterway have PCB and HCBD liver burdens of 125 and 2.2 μg wet weight/L, respectively (Collier et al. 1998), whereas juvenile salmon injected in another study with 400 g of CHWSE/kg of fish had liver contaminant burdens of 175 μg of PCBs/L and 2.4 μg of HCBD/L (Casillas et al. 1998). Thus fish in the current study, injected with 307 g of CHWSE/kg of fish, presumably received a dose that may have resulted in HCBD and PCB liver burdens equivalent to or slightly less than what is observed in salmon naturally exposed in the Hylebos Waterway.

The increased disease susceptibility of salmon exposed to estuarine-associated contaminants and then challenged with a marine bacterial pathogen is consistent with our previous findings demonstrating effects of chemical contaminants on immunocompetence and disease susceptibility of juvenile chinook salmon from the urban Duwamish Waterway (Arkoosh et al. 1991, 1998). In these previous studies, we found a suppressed secondary immune response in anterior kidney leukocytes to specific antigens and an increased percent cumulative mortality to *V. anguillarum* after natural exposure to chemical contaminants in this waterway. Juvenile salmon exposed to a model PAH, DMBA, or the PCB mixture Aroclor 1254 in the laboratory also exhibited a similarly suppressed secondary immune response in anterior kidney leukocytes to model antigens (Arkoosh et al. 1994). Both PAHs and PCBs are also known to induce immunosuppression in other species (Thomas and Hinsdill 1978; Ward et al. 1985).

Observations spanning the entire temporal scale of the experimental period in the present study provide useful data in evaluating differences in disease susceptibility of juvenile chinook salmon exposed to contaminants. This is especially apparent in trial 2. Differences between DMBA- and the control acetone:emulphor-injected groups were not significant until 9 d after exposure, whereas we were able to determine a difference between Aroclor 1254- and the control acetone:emulphor-injected fish at 8 d.

Our previous studies suggest that although juvenile chinook salmon are only briefly exposed to contaminants in an urban estuary as they migrate to sea, immune altering events may be persistent, and a consequent increase in disease susceptibility in chemically exposed juvenile salmon may extend into their early ocean life. Recruitment of fish to the adult stages is thought to be dependent on variables acting on the fish during their first year of life (Sissenwine 1984). Therefore, an increase in mortality rates within salmon populations from disease could potentially not only affect juvenile salmonids but also influence recruitment to adult stages.

Disease is considered to be one of the six major sources of natural mortality in fish (Möller and Anders 1986). Mortality in salmonid populations resulting from immunosuppression and infectious disease is difficult to determine. However, it has been suggested that both immune dysfunction and

infectious disease have an important role in population regulation (Sheldon and Verhulst 1996) by causing mass mortalities, impaired locomotion, delayed metamorphosis, and/or increased mortality from predation (Sindermann 1990). Also, fish that are immunosuppressed and are defending themselves against infection may have less energy for growth and reproduction (Sheldon and Verhulst 1996).

The potential for disease to influence population structure is also supported by studies with populations of coho salmon *O. kisutch*. It was found that a coho salmon population that had a large number of individuals infected with a trematode parasite (*Nanophyetus* sp.) as they left the estuary and entered the ocean environment had much lower adult survival rates than did a population with fewer individuals infected with the parasite (Schroder and Fresh 1992). Although our findings do not directly reflect cause and effect, the results from this previous study with coho salmon suggest that diseased fish are lost from the ocean population (Schroder and Fresh 1992). Together, the findings from our study and the previous study with coho salmon support an association between infection with a pathogen and lower survival potential.

In conclusion, our current findings suggest that juvenile chinook salmon exposed to chlorinated and aromatic compounds, characteristic of sediments from contaminated estuaries, are more susceptible to disease than are salmon not exposed to contaminants. Accordingly, these current findings together with our previous investigations suggest that the ecological risk for increased juvenile mortality and subsequent decreased adult recruitment is potentially greater for juveniles exposed to contaminants accumulated during their residence in a contaminated estuary. However, the magnitude of the potential increase in risk remains unknown.

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