

**HYLEBOS FISH INJURY STUDY**  
**Round II**

**Part 1: Effects of Chemical Contaminants from the Hylebos Waterway on Disease  
Resistance of Juvenile Chinook Salmon**

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## LIST OF ABBREVIATIONS

AHs	aromatic hydrocarbons
CHs	chlorinated hydrocarbons
CHWSE	chlorinated-enriched Hylebos Waterway extract
CYP1A	cytochrome P4501A
DMBA	dimethylbenzanthracene
DNA	deoxyribonucleic acid
GC/MS	gas chromatography/mass spectometry
GLM	generalized linear model
HCB	hexachlorobenzene
HCBD	hexachlorobutadiene
LC	lethal concentration
NOAA	National Oceanic and Atmospheric Administration
NMFS	National Marine Fisheries Service
NWFSC	Northwest Fisheries Science Center
PCBs	polychlorinated biphenyls
PFC	plaque-forming cell
PAHs	polycyclic aromatic hydrocarbons
SAP	Sampling and Analysis Plan
TSB	tryptocase soy broth

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## EXECUTIVE SUMMARY

The Hylebos Waterway of Commencement Bay, an urban estuary in central Puget Sound in the state of Washington, is severely contaminated with a variety of organic and inorganic contaminants. Juvenile salmon inhabit this waterway in the late spring and early summer. In 1994, studies (Round I of the Hylebos Fish Injury Study) were initiated to determine contaminant exposure in juvenile salmonids which migrate through this waterway. The findings showed that juvenile chinook and chum salmon sampled from this site are being exposed to a wide range of chemical contaminants (Collier et al. 1998), and the levels of exposure are comparable to levels which have previously been shown to cause impaired growth (Casillas et al. 1995a), immunosuppression, and increased mortality following pathogen exposure in juvenile salmon (Arkoosh et al 1998). Whether juvenile salmon exposed specifically to chemical contaminants characteristic of the Hylebos Waterway suffer injurious biological effects as a result of the exposure was not determined in the Round I study.

The objective of the present study (Part 1 of Round II of the Hylebos Fish Injury Study) was to determine if contaminants specifically associated with the Hylebos Waterway can alter (increase) the disease susceptibility of juvenile chinook salmon to the bacterial pathogen, *Vibrio anguillarum*. Specifically, juvenile salmon were exposed for a 7 day period in Oct, 1995 to either: 1) hexachlorobutadiene (HCB), a signature compound of the Hylebos Waterway, 2) to a chlorinated-enriched fraction of a sediment extract made from Hylebos Waterway sediment (CHWSE), or 3) a model mixture composed of 10 high-molecular weight polycyclic aromatic hydrocarbons (PAHs) in proportion to PAHs in a sediment sample from the Hylebos Waterway. The salmon were exposed to the chemicals by intraperitoneal injection to ensure consistent delivery of a specific dosage. Disease susceptibility was then assessed by exposing fish to *Vibrio anguillarum*, a known marine pathogen of salmon.

Significant ( $p \leq 0.05$ ) differences in mortality (increased disease susceptibility) between experimentally exposed juveniles and juveniles administered the carrier compound were observed. Exposure to chlorinated hydrocarbons, characterized by HCB and the CHWSE, which is composed primarily of polychlorobutadienes and certain aromatic hydrocarbons (e.g. phenanthrene, fluoranthene, pyrene), were associated with increased mortality induced by *Vibrio anguillarum*. These findings support the hypothesis that chemical contaminant exposure of juvenile salmon in the Hylebos Waterway influences their physiology such that their survival potential may be reduced. Since recruitment of salmon appears to be strongly influenced by factors acting on the first year of ocean life (Pearcy 1992), these results suggest that the risk for

increased juvenile mortality and subsequent decreased adult recruitment is potentially greater for juveniles exposed to contaminants accumulated during their residence in the Hylebos Waterway.



## INTRODUCTION

Estuaries are critical habitats for juveniles of several Pacific salmon species during their transition to life in the ocean (Levings and Bouillon 1997). Estuarine habitats provide refuge from predators, a rich food supply to support rapid growth, and are where juveniles make the transition from freshwater to marine conditions (Thorpe 1994). Urban estuaries, however, receive inputs of toxic anthropogenic substances from a variety of sources, and many of these chemicals can accumulate in sediments and thus can be retained in the estuary. There is concern that because juvenile salmon are undergoing numerous physiological adaptations during their residence in estuarine environments, any additional stresses such as exposure to toxic chemicals, may be injurious.

The Hylebos Waterway of Commencement Bay, in central Puget Sound in the state of Washington, is severely contaminated by a variety of organic and inorganic contaminants and juvenile chinook salmon inhabit this waterway in the late spring and early summer. In 1994, studies for Round I of the Hylebos Fish Injury Study were initiated to: 1) determine if contaminant source control had resulted in improvements in habitat quality, as determined by prevalences of liver disease in flatfish; 2) identify impaired reproductive function in flatfish; and 3) determine whether and to what degree there might be contaminant exposure of juvenile salmonids which migrate through this waterway. The findings showed (Collier et al. 1998) that there have been no appreciable changes in disease prevalences or levels of contaminant exposure in flatfish from this site since the 1970s. Moreover, female flatfish from the Hylebos Waterway are showing evidence of precocious sexual maturation in young animals and inhibited gonadal development in older fish. More importantly for the studies reported herein, the results also showed that two species chinook (*Oncorhynchus tshawytscha*) and chum (*Oncorhynchus keta*) of juvenile salmon sampled from this site are being exposed to a wide range of chemical contaminants and in particular showed increased exposure to chlorinated compounds (HCBD, HCB) that are elevated in Hylebos Waterway sediments compared to other sites in Puget Sound. The levels of exposure to CHs and PAHs are comparable to levels which have previously been shown to cause impaired growth, immunosuppression, and increased mortality following pathogen exposure in juvenile salmon from the contaminated Duwamish Waterway, Puget Sound. These studies provide the scientific rationale for determining if juvenile chinook salmon, exposed to chemical contaminants characteristic of the Hylebos Waterway, suffer injury from such exposure.

Immune dysfunction in mammals has been recognized as a serious sublethal effect of chemical contaminant exposure, affecting both cellular and humoral aspects of the immune

system (Dean et al., 1990). Laboratory studies have extended these findings to fish by demonstrating that their immune system is susceptible to specific contaminants, often resulting in a wide array of immunosuppressive effects (McLeay and Gordon 1977; Arkoosh and Kaattari 1987; Rice and Weeks 1989; Thuvander 1989). We have examined the immunocompetence of juvenile chinook salmon and found that juvenile chinook salmon from polluted environments are immunosuppressed (Arkoosh et al., 1991). In our studies with juvenile salmon from Puget Sound, WA, immunocompetence was evaluated by analyzing the functional ability of white blood cells to produce antibodies after either their first exposure to a new antigen (the primary response) or after re-exposure to the same antigen (the secondary or memory response) which is evaluated using a plaque-forming cell (PFC) assay. Such an approach has been shown to be sensitive in evaluating long-term chronic effects following short-term exposure to chemical contaminants (Arkoosh and Kaattari 1987). Previously, we showed that white blood cells (leukocytes) of juvenile chinook salmon collected from hatcheries and from a nonurban estuary (the Nisqually estuary in Puget Sound) were able to generate a significantly higher secondary PFC response to a foreign antigen than that produced during the primary PFC response, which is the normal and expected response (Arkoosh et al., 1991). In contrast, an enhanced secondary PFC response did not occur with leukocytes of juvenile chinook salmon exposed to pollution from the urban Duwamish Waterway estuary. Thus, this field study demonstrated that fish from the contaminated estuary were immunosuppressed and suggested that juvenile chinook salmon may be more susceptible to disease than juveniles from non-urban environments. This increased potential for disease increases the risk of mortality for juveniles in contaminant-exposed populations of chinook salmon.

In our previous study (Arkoosh et al. 1998), to determine if salmon from a contaminated environment may be more susceptible to disease, we collected juvenile fall chinook salmon from the urban Duwamish Waterway (Collier et al., 1998) estuary and from the nonurban Nisqually estuary and the respective releasing hatcheries, and exposed them in the laboratory to the marine pathogen, *Vibrio anguillarum*. *Vibrio anguillarum* is a fish pathogen known to cause disease when fish are under stress (Warren 1991). We found that juvenile chinook salmon from the contaminated estuary were more susceptible to *V. anguillarum* induced mortality than were fish from the hatchery on the river system. In contrast, juvenile fall chinook salmon from a nonurban estuary showed no increase in susceptibility to *V. anguillarum* induced mortality than did fish from the corresponding hatchery for this river system. These disease challenge studies indicate that juvenile chinook salmon exhibiting contaminant-associated immunodysfunction also exhibited increased susceptibility to pathogenesis by a virulent marine bacterium (Arkoosh et al., 1998). Further support for a linkage between contaminant exposure, immune function, and the potential for increased mortality is provided from recent laboratory studies. Two classes of

contaminants, PCBs and PAHs, found in the Hylebos Waterway are known to induce immunosuppression as well as to increase disease susceptibility in mammals (Ward et al., 1985; Dean et al., 1990). Our recent laboratory studies with juvenile chinook salmon administered sublethal doses of PCBs and PAHs showed suppression of their secondary PFC response (Arkoosh et al., 1994), as well as an increase in disease susceptibility (Arkoosh et al., 1998). These findings substantiate a linkage between immune dysfunction and increased disease susceptibility and support a probable causal relationship between impaired immunity, increased disease susceptibility and chemical contaminant exposure in juvenile fall chinook salmon from polluted urban estuaries. These findings also support the use of disease challenge experiments in linking contaminant exposure to a serious biological dysfunction. The ecotoxicological implications are for potentially increased mortality for juvenile salmon using contaminated estuaries.

Accordingly, having determined previously that juvenile fall chinook and chum salmon are exposed to contaminants during residence in the Hylebos Waterway (Collier et al. 1998), our aim in this study (Part 1 of Round II of the Hylebos Fish Injury Study) was to determine if contaminants specifically associated with the Hylebos Waterway can increase the disease susceptibility of juvenile chinook salmon to the bacterial marine pathogen, *Vibrio anguillarum*. Specifically, juvenile chinook salmon were exposed to one of the following for a 7-day period: 1) hexachlorobutadiene (HCB), a signature compound of the Hylebos Waterway, 2) to a chlorinated-enriched fraction of a sediment extract made from the Hylebos Waterway sediment, or 3) a model mixture composed of 10 high-molecular weight polycyclic aromatic hydrocarbons (PAHs) in proportion to the level of PAHs in sediment from the Hylebos Waterway. The chemical contaminants were administered 7 day prior to being challenged with *Vibrio anguillarum* after which mortality was monitored for a 14 day period. The hypothesis being addressed was whether differences in mortality between experimentally exposed juveniles and juveniles administered the carrier compound (acetone:emulphor) and exposed to *Vibrio anguillarum* were evident. In this study reference extract was not used as a control.

## METHODS

Details of the disease challenge study are described in detail in the SAP (Appendix 1). The chemicals identified in the CHWSE and their concentration are listed in Table 1. The composition of the PAH model mixture is listed in Table 2.

Table 1. Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon. Concentrations of selected aromatic hydrocarbons, chlorinated butadienes, pesticides, and polychlorinated biphenyls in sediment extract from the pentane extraction of sediment from the Hylebos Waterway (HWSE-P) that was used in the laboratory experiment. Values are reported as ng/g sediment.

Analyte	ng/g sediment <sup>a</sup>	Analyte	ng/g sediment
<b>Aromatic Hydrocarbons</b>		<b>Pesticides</b>	
naphthalene	26	hexachlorobenzene	110
2-methylnaphthalene	18	lindane (gamma-BHC)	2
1-methylnaphthalene	10	heptachlor	0.8
biphenyl	6	aldrin	< 0.06
2,6-dimethylnaphthalene	10	heptachlorepoxyde	21
acenaphthylene	0.9	oxychlorane	< 0.07
acenaphthene	28	trans-chlordane	< 0.06
2,3,5-trimethylnaphthalene	28	nonachlor-III	< 0.06
fluorene	28	alpha-chlordane	2
phenanthrene	140	trans-nonachlor	5
anthracene	47	cis-nonachlor	0.4
1-methylphenanthrene	10	dieldrin	2
fluoranthene	170	mirex	2
pyrene	170	o,p'-DDE	1
benz[a]anthracene	23	p,p'-DDE	0.4
chrysene	33	o,p'-DDD	20
benzo[b]fluoranthene	6	p,p'-DDD	1
benzo[k]fluoranthene	5	o,p'-DDT	< 0.1
benzo[e]pyrene	9	p,p'-DDT	< 0.1
benzo[a]pyrene	10		
perylene	2	<b>PCBs</b>	
indeno[1,2,3-cd]pyrene	0.3	trichlorobiphenyl - 18	17
dibenz[a,h]anthracene	< 0.02	trichlorobiphenyl - 28	6
benzo[ghi]perylene	1	tetrachlorobiphenyl - 44	1
dibenzothiophene	12	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
<b>Chlorinated Butadienes<sup>b</sup></b>			
Trichlorobutadiene	480		
Tetrachlorobutadiene	450		
Pentachlorobutadiene	160		
Hexachlorobutadiene	150		

<sup>a</sup> Analyte concentrations calculated on a sediment wet weight basis.

<sup>b</sup> Concentrations of the butadienes were calculated using a response factor of 1 with GC/MS total ion current areas.

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Table 2. Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon. High molecular weight hydrocarbons that comprise the PAH model mixture. The compounds were combined in acetone at the same ratios as they were present in the sediment at station 24 of the Hylebos Waterway to a concentration equivalent to 400 g sediment extracted per ml of acetone.

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**PAH Analytes**

Fluoranthene Pyrene Bcnz[a]anthracene Chrysene, Benz[b]fluoranthene	Benz[k]fluoranthene Benzo[a]pyrene Indeno[1,2,3-cd]pyrene Dibenz[a,h]anthracene Benzo[ghi]perylene
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**Exposure Assessment**

Information on the level of exposure for the treatments used here will be generated in subsequent experiments as described in the SAP (Appendix 1).

**Bacterial Lethal Concentration Curve**

Juvenile chinook salmon from the Salmon River Hatchery, OR, were obtained to conduct exposure and challenge studies which were conducted at our field station at Newport, OR. The concentration-response curve relating the percent of fish killed with increasing concentration of *Vibrio anguillarum* after a 7 day exposure period was conducted to determine the LC<sub>30</sub> and LC<sub>50</sub> for the challenge studies. The 168 h lethal concentration response curve of juvenile chinook salmon exposed to *V. anguillarum* was fitted using logit regression analysis using DeltaGraph™ (Wulf et al. 1991).

**Statistical methods - disease challenge**

Statistical significance of the challenge studies were assessed using generalized linear models (GLM). The number of survivors and mortalities in a disease challenge study follow a binomial distribution (Hildén and Hirvi 1987). Statistical analyses to assess significance of difference in mortality among the experimental treatment groups compared to the control group were assessed using GLM, a general linear model, 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 which relates the linear predictor derived from the GLM analysis to the expected survival/mortality probabilities (Baker and Nelder 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. This analysis was used to evaluate if survival/mortality of fish injected with contaminants was significantly different ( $\alpha = 0.05$ ) from salmon injected with the carrier 7d post challenge as described in Arkoosh et al. (1998) although data is reported for a 14 day period to more easily visualize differences in mortality among the treatment groups. These analyses were conducted after correction for mortalities in the group of juvenile salmon that served as the non-*Vibrio* treated control for these experiments. The background mortality was determined during the same period (Figure 3). The net mortality was determined by subtracting background mortality observed in juvenile chinook salmon that received chemical contaminants but were not exposed to bacteria. Statistical analyses were conducted separately for each exposure concentration of the bacteria.

## RESULTS

### Sediment Extract Chemistry

The objective of administering HCBD, a chlorinated-enriched fraction of sediment from the Hylebos Waterway (CHWSE), and a model mixture of PAHs was to expose juvenile salmon to compounds representative of chemical contaminants in the Hylebos Waterway. HCBD is considered a marker chemical for the Hylebos Waterway. HCBD was administered at a dose of 21 mg/kg. CHWSE was prepared from sediment collected near the mouth of the Hylebos Waterway. Specifically, these sediments were taken at Stations HY-07, -08, and -09. These sediment sites were designated and analyzed during the sediment injury studies conducted during Phase 1 of the Commencement Bay Damage Assessment investigations. The objective of preparing the sediment extract was to obtain an extract of chemical contaminants that was representative and enriched in the less polar CHs. The composition of analytes in the extract is listed in Table 1. The extract is enriched in butadiene-like compounds although it still retains significant quantities of AHs. The PCBs present in the sediment were largely removed in the preparation. The final concentration of chemical contaminants in 1  $\mu$ l of the CHWSE was equivalent to chemical contaminants in 0.2 gr of sediment. A model mixture of polycyclic aromatic hydrocarbons (PAHs) containing 10 high molecular weight PAHs was also prepared to reflect the same ratios of these analytes previously found in sediment during Phase I of the Commencement Bay Damage Assessment studies at Station HY-24. The final concentration of chemical contaminants in 1  $\mu$ l of the PAH model mixture was equivalent to chemical contaminants in 0.2 gr of sediment. The chemical contaminants along with the acetone:emulphor carrier were each administered at a volume equivalent to 1.5  $\mu$ l solution/g fish.

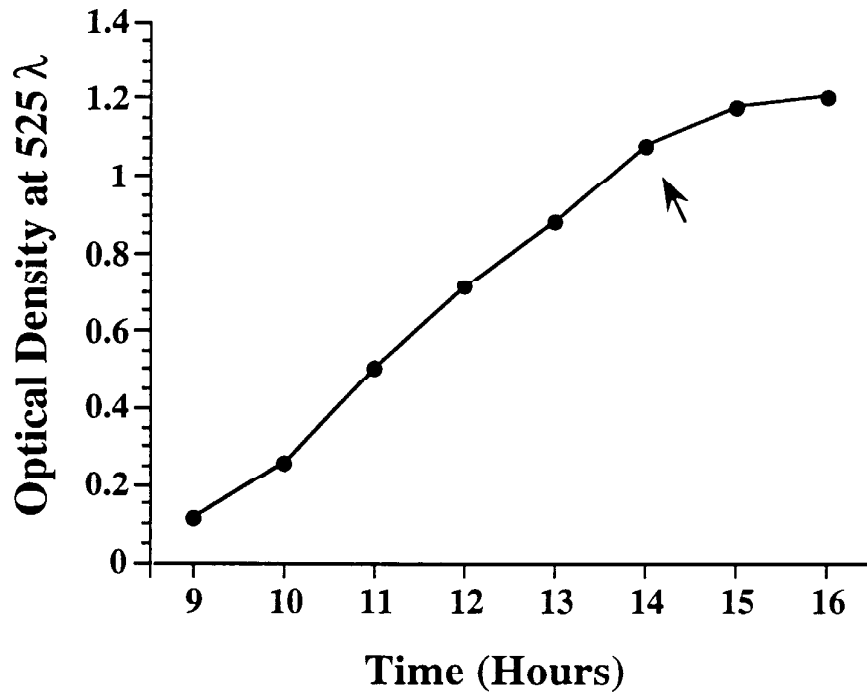


Figure 1. Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon. Growth curve of *Vibrio anguillarum*, expressed as the change in optical density of the culture media with incubation time. The arrow indicates the peak of the exponential growth phase of the bacteria.

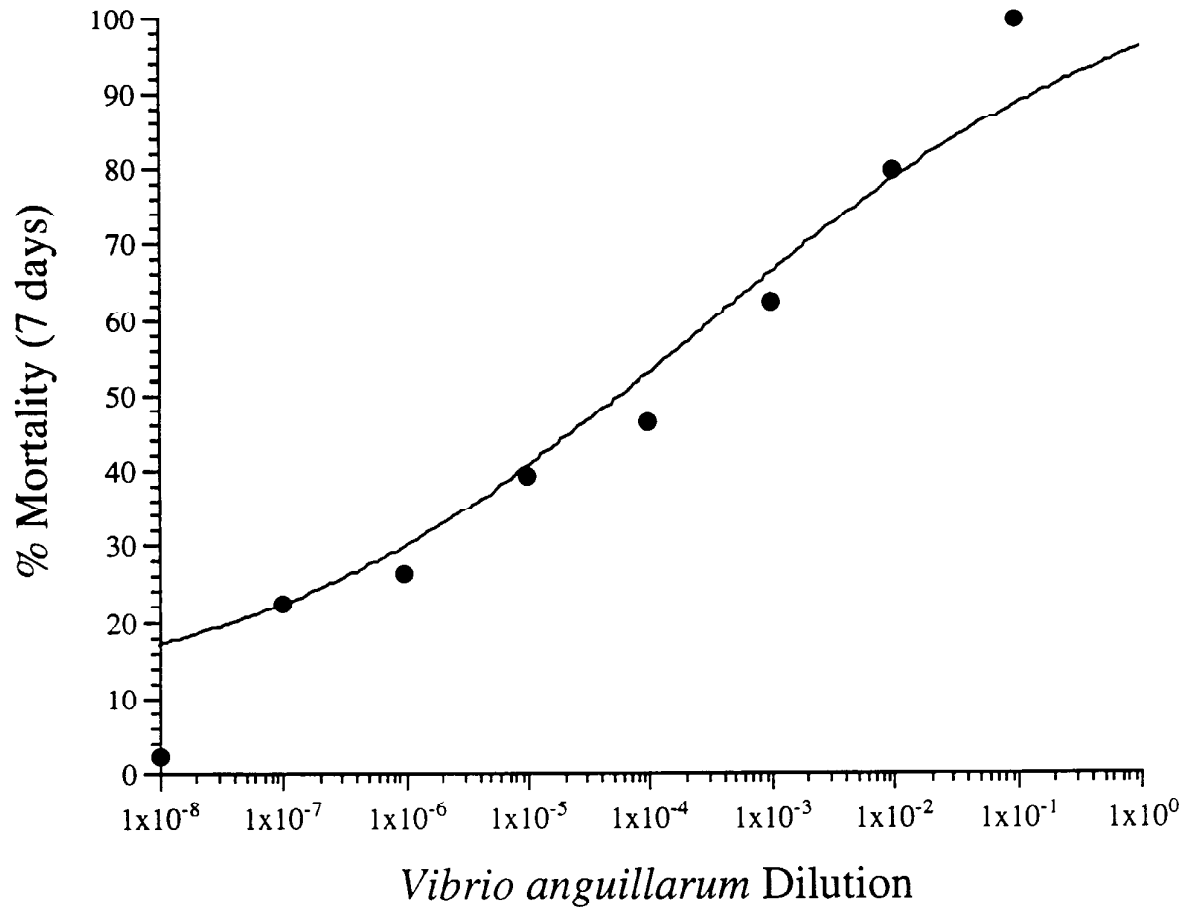


Figure 2. Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon. The 168 h (7 day) lethal response curve of juvenile chinook salmon exposed to *Vibrio anguillarum*. The *Vibrio anguillarum* dilution represents the volume (ml) of stock bacterial culture diluted per ml of sea water used for exposure of the pathogen to juvenile chinook salmon. The lethal response curve was used to select concentrations of *Vibrio anguillarum* for the disease challenge. See the Hylebos Phase II SAP for more details (Appendix 1).



#### Bacterial Growth and Concentration-Response Curve.

The peak of the exponential growth phase of *V. anguillarum* grown in 500 mL of TSB supplemented with 0.5% NaCl and slowly agitated at 25°C occurred at approximately 14 h with an optical density of 1.1 (Figure 1). In subsequent challenge experiments, *V. anguillarum* cultures were grown until an optical density of approximately 1.1 for preparation of the bacterial culture dilutions used for juvenile salmon exposures. The 168 h (7 d) lethal response curve for Salmon River Hatchery salmon exposed to *V. anguillarum* was determined by a logit regression analysis (Figure 2). The dilutions of bacteria inducing 50% mortality in the Salmon River fish over a 7 d period were  $6 \times 10^{-5}$  ml stock bacterial culture/mL sea water; this dilution was equivalent to approximately  $1 \times 10^7$  bacteria/mL sea water.

#### Disease Challenge Studies.

Background mortality and the percent cumulative mortality of the four different groups of juvenile chinook salmon exposed to *V. anguillarum* is shown in Figure 3 and 4, respectively. The cumulative mortality curves are reported for a 14 day period following exposure to the bacteria. Statistical testing was restricted to 7 days post exposure, as outlined in Arkoosh et al (1998). Recording mortality for a 14 day period allows observations for any unusual mortality events after the traditional 7 day observation period. The data in Figure 4 represent the net mortality attributed to exposure to the bacteria after subtracting background mortality (Figure 3) observed in juvenile chinook salmon that received chemical contaminants but were not exposed to bacteria. The net cumulative mortality of juvenile chinook salmon exposed to bacteria equivalent to an LC<sub>50</sub> dose after receiving either CHWSE, HCBBD, or the model mixture of PAHs ranged from 28 to 31% compared to the 17% observed in the acetone:emulphor control group at 7 days post bacterial challenge. The cumulative mortality was significantly higher in fish exposed to CHWSE, the PAH model mixture, or HCBBD relative to fish receiving only the carrier. There was essentially no change in the net cumulative mortality of juvenile salmon from

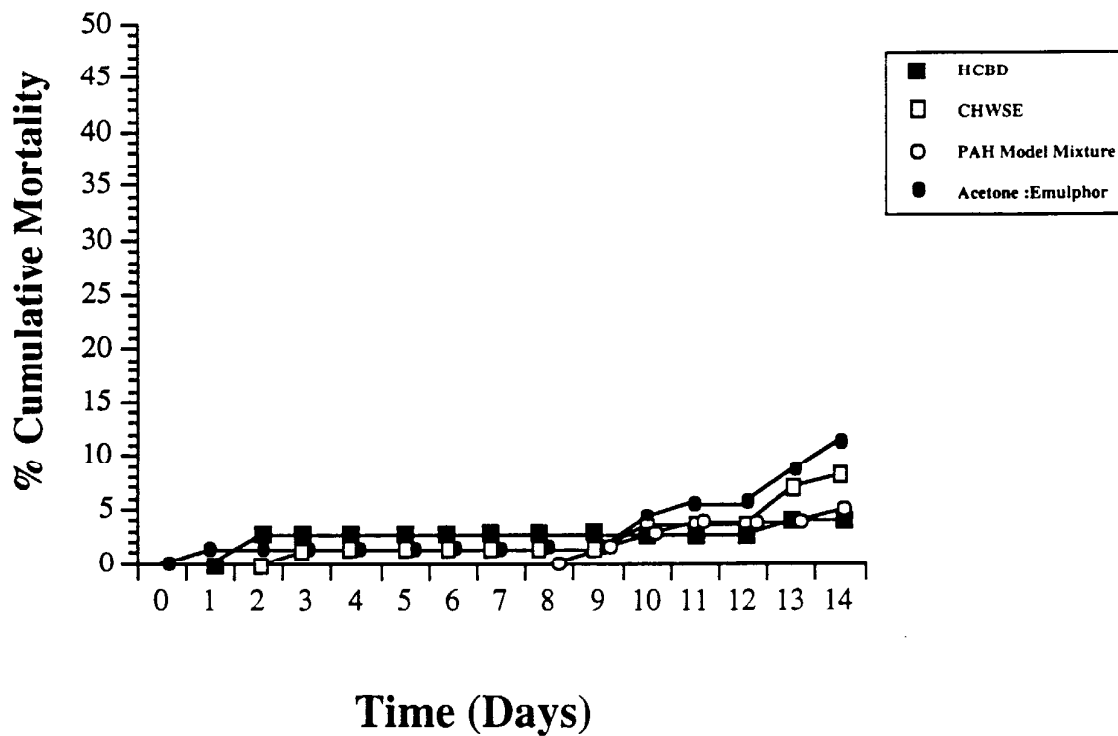


Figure 3. Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon. Background percent cumulative mortality of juvenile chinook salmon injected with either HCBD, a chlorinated-enriched sediment extract from the Hylebos Waterway (CHWSE), a model mixture of PAHs, or the carrier control (acetone:emulphor) but not exposed to *Vibrio anguillarum*.

## Lethal Concentration<sub>50</sub>

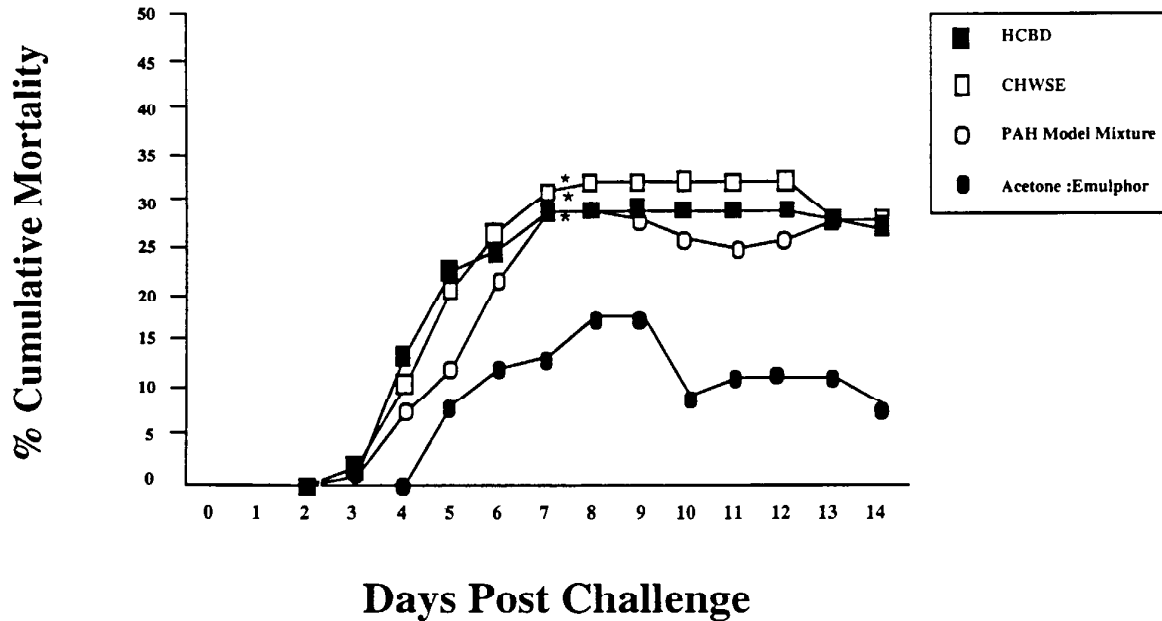


Figure 4. Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon. Percent cumulative mortality of juvenile chinook salmon injected with either HCBd, a chlorinated-enriched sediment extract from the Hylebos Waterway (CHWSE), a model mixture of PAHs, or the carrier control (acetone:emulphor) during a 14 day exposure to  $6 \times 10^{-5}$  ml bacterial solution/ml seawater (LC<sub>50</sub>). The net cumulative mortalities due to the bacteria are corrected by subtracting mortalities observed in juvenile chinook salmon injected with either HCBd, CHWSE, the model mixture of PAHs, or the carrier control (acetone:emulphor) but not exposed to *Vibrio anguillarum*. NA signifies data not available. \* indicates significantly ( $p \leq 0.05$ ) different from acetone:emulphor control group.

the 7 to 14 day observational period and the magnitude of the differences in mortality between the carrier control and the three treatment groups remained relatively unchanged. Because the acetone: emulphor control group for the LC<sub>30</sub> treatment group was lost, due to a procedural error (loss of air to the tanks), the significance of mortality due to exposure to chemical contaminants could not be determined for this treatment group; the data for LC<sub>30</sub> - dose treatments are in the Appendix.

Table 3 shows the percentage of salmon carcasses examined that were infected with *V. anguillarum* on days 7 and 14 of the experiment using an LC<sub>50</sub> exposure dose. Confirmation of the presence of *V. anguillarum* ranged from 44% to 100% in the subsamples tested. The agglutination test identifies bacteria subsampled from each fish that are able to agglutinate anti-*V. anguillarum* antiserum whereas the oxidase positive test identifies fish with bacteria which have cytochrome oxidase enzyme activity which is characteristic of *Vibrio* spp. *Pseudomonas* spp. and *Aeromonas* spp. bacteria, and includes *V. anguillarum*. The findings show that we were able to attribute a majority of the mortalities to *V. anguillarum*.

Table 3. Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon. Ratio and proportion of juvenile salmon identified to be infected with *V. anguillarum* after exposure to waterborne *V. anguillarum* equivalent to the LC<sub>50</sub> concentration.

	Positive Agglutination <sup>a</sup>	Agglutination (%)	Oxidase Positive <sup>b</sup>	Oxidase Positive (%)	Combined <sup>c</sup>
<b>Day 7<sup>d</sup></b>					
acetone/emulphor	7/11	64%	11/11	100%	64%
HCBd	4/9	44%	9/10	90%	44%
CHWSE	12/12	100%	12/12	100%	100%
Model Mixture PAH	7/11	64%	11/11	100%	64%
<b>Day 14</b>					
acetone/emulphor	8/16	50%	12/16	75%	50%
HCBd	4/9	44%	9/10	90%	44%
CHWSE	14/14	100%	14/14	100%	100%
Model Mixture PAH	7/13	54%	13/13	100%	54%

<sup>a</sup> Number of fish examined which had bacteria that agglutinated with the anti-*V. anguillarum* antiserum/total number of fish examined

<sup>b</sup> Number of fish examined which were oxidase positive/ total number of fish examined.

<sup>c</sup> Percentage of fish that had bacteria that were both oxidase positive and agglutinated anti-*V. anguillarum* antiserum.

<sup>d</sup> The number of days after *V. anguillarum* challenge.

## DISCUSSION OF MAJOR FINDINGS

### **Depressed disease resistance results from exposure of juvenile chinook salmon to contaminants specific to the Hylebos Waterway.**

Juvenile chinook salmon exposed to contaminants associated with the Hylebos Waterway exhibited a higher susceptibility to mortality induced by the marine pathogen *V. anguillarum*, than did juvenile chinook salmon treated only with the carrier. These findings suggest that a higher predisposition to infection/disease may occur in salmon exposed to chemical contaminants specific to the Hylebos Waterway. At the doses tested, each of the treatment groups, which represent specific subsets of chemical contaminants characteristic of the Hylebos Waterway, increased the disease susceptibility of juvenile chinook salmon. Chlorinated hydrocarbons, characterized by HCBD and the CHWSE, which is composed primarily of HCBD-like compounds, and aromatic hydrocarbons, characterized by the model mixture of PAHs, affected the susceptibility of juvenile chinook salmon to mortality induced by a marine bacterial pathogen. The present findings support the hypothesis that chemical contaminant exposure of juvenile salmon in the Hylebos Waterway influences their physiology such that their survival potential may be reduced. This conclusion is supported by studies in mammals, which have also demonstrated that immunoaltering chemicals can increase disease susceptibility (Friend and Trainer 1970; Thigpen et al. 1975; Loose et al. 1978).

The increased susceptibility of salmon first exposed to contaminants associated with the Hylebos Waterway and then challenged with a marine bacterial pathogen is consistent with our previous findings showing effects of chemical contaminants on immunocompetence and disease susceptibility of juvenile chinook salmon from the Duwamish Waterway in Elliott Bay also located in Puget Sound, WA. Arkoosh et al. (1991, 1998) showed a suppressed secondary immune response in anterior kidney white blood cells to specific model antigens and a greater percent cumulative mortality to *V. anguillarum* after natural exposure to chemical contaminants in the waterway, including PAHs and PCBs. Juvenile salmon exposed to a model PAH, dimethylbenzanthracene (DMBA) or the PCB mixture, Aroclor 1254, in the laboratory also exhibited a similar suppressed secondary immune response in anterior kidney white blood cells to model antigens (Arkoosh et al. 1994). Both PAHs and PCBs are known to induce immunosuppression in other species (Thomas and Hinsdill 1978; Ward et al. 1985).

**The overall effects of exposure to chemical contaminants specific to the Hylebos Waterway on disease resistance in juvenile salmon could affect their survival potential in the estuary and early ocean environment.**

Although the salmon in the present study were found to have an altered disease resistance shortly after exposure to toxic chemicals, previous data suggests that juvenile salmon may experience continued altered health for an extended period of time after exposure. We found that fish from the contaminated Duwamish estuary held for two months in noncontaminated sea water still exhibited increased mortality associated with the earlier exposure to chemical contaminants (Arkoosh et al. 1998). This finding is also supported by laboratory studies evaluating the duration of immunosuppression in salmonids after an initial exposure to other immunosuppressive chemical contaminants. A study (Arkoosh and Kaattari 1987) examining the effects of aflatoxin B<sub>1</sub>, a mycotoxin, on the immune response of rainbow trout demonstrated that a brief exposure of embryos to this immunotoxin, at sublethal concentrations, can manifest its effects months later. The trout experienced immune dysfunction 5-6 months after a single exposure to aflatoxin B<sub>1</sub> (Arkoosh and Kaattari 1987). Our previous studies in conjunction with the study reported by Arkoosh and Kaattari (1987) 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 exposed juvenile salmon may extend into their early ocean life. Because recruitment of fish to the adult stages is considered to be dependent on factors acting during the first year of life (Sissenwine 1984), the potential for contaminants to potentiate disease outbreaks and influence mortality rates within the population is a possibility. The potential for disease to influence population structure is supported by studies showing that coho salmon infected with a parasite (*Nanophytes* sp.) as they leave freshwater and enter the ocean environment had much lower adult survival rates than coho with lower infection rates (Schroder and Fresh 1992). Although these findings do not directly reflect cause-and-effect, the results from other previous studies of coho on the Washington coast suggested that diseased fish were lost from the ocean population (Schroder and Fresh 1992), supporting an association between infection with a pathogen and lower survival potential.

The consequences of immunosuppression and an increase in disease susceptibility may be greater than are currently recognized. Immunosuppression has been suggested to play a major role in population regulation and disease itself has been shown to be an important factor in the extinction of several species (Sheldon and Verhulst 1996). The ramifications of disease on marine fish populations can be manifold (Sindermann 1990) including mass mortalities, impaired locomotion, delayed metamorphosis, and increased mortality from predation. In addition,

immunosuppressed animals may allocate greater energy and resources for defending themselves against infection, reducing resources for other vital physiologic functions, such as growth and reproduction (Sheldon and Verhulst 1996). In summary, the findings from Round I of the Hylebos Fish Injury Study (Collier et al 1998) show that the relatively brief residence of juvenile chinook and chum salmon in the Hylebos Waterway increased exposure to chemical contaminants and induced early biological alterations (elevated hepatic CYP1A and DNA damage). In the present study we demonstrated that a model suite of PAHs characteristic of sediments from the Hylebos Waterway, as well as specific Hylebos-associated chemicals (organic solvent extracts of Hylebos sediment), increased disease susceptibility. Because recruitment of salmon appears to be strongly influenced by factors acting on the first year of ocean life (Pearcy 1992), alterations in their overall fitness during this period could be detrimental. Although there is uncertainty in delineating the contribution of the many individual factors that affect survival of juvenile salmon in the estuarine and marine environment, disease susceptibility has consistently been linked to an increase in mortality (Grenfell and Dobson 1995) in natural populations. The findings from the present study showed that exposure to chemical contaminants associated with the Hylebos Waterway increases the susceptibility of juvenile chinook salmon to disease. Quantitating the level of increased risk of subsequent mortality, however, cannot be assessed from the current information. Additional studies to determine the relationship between level of exposure to chemical contaminants and the extent of disease susceptibility are needed to assess further the level of risk to juvenile salmon of increased disease susceptibility from exposure to toxic chemicals present in the Hylebos Waterway.

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**Hylebos Fish Injury Study - Round II, Part I  
Individual Data and Quality Assurance Results  
CASE NARRATIVE**

**Effects of Chemical Contaminants from the Hylebos Waterway on Disease Resistance of  
Juvenile Chinook Salmon**

*Calibrations*

Prior to initiation of the study, balances were calibrated to insure their accuracy. Balances were re-zeroed between weight determinations.

*Quality control*

A growth curve for *Vibrio anguillarum* was generated to identify the peak of exponential growth for the culture of *V. anguillarum* used in these studies. The optical density of the culture over time was the measure of growth of *V. anguillarum*. The optical density that replicated the peak of exponential growth of *V. anguillarum* identified previously was used on the day of the challenge studies since the culture may be growing at a different rate of speed than previously observed. The *V. anguillarum* growth curve is reported in the Interpretive Report.

A lethal response curve was generated to identify the concentrations of *V. anguillarum* that would likely allow for a protracted death of juvenile salmon over a seven day period. The lethal concentration whereby 30% and 50% of the fish died after a 7 day challenge to *V. anguillarum* was calculated from the lethal response curve. These concentrations of *V. anguillarum* were not likely to overwhelm the fish to the point a difference in treatment mortality as a result of the disease challenge could not be detected. The lethal response curve is reported in the Interpretive Report.

Mortalities from each tank were collected on a daily basis for two weeks and recorded by the sampler. Data from each of the tanks are shown in the following tables. The data from the necropsy are also presented in table form. As stated in the Sampling and Analysis Plan two controls groups were used. These include fish injected only with the carrier (acetone:emulphor) and exposed to one of two concentrations of *V. anguillarum* (LC<sub>30</sub>, LC<sub>50</sub>) and fish injected with either acetone:emulphor, HCBd, the model mixture of PAHs, or CHWSE, but not subject to *V. anguillarum* challenge. The acetone:emulphor control groups exposed to the various concentrations of *V. anguillarum* were used for comparison to the contaminant-injected groups that were exposed to *V. anguillarum* to identify treatment differences in susceptibility to a disease challenge whereas fish injected with either acetone:emulphor, HCBd, the model mixture of PAHs, or CHWSE, but not subject to *V. anguillarum* challenge were conducted to identify the average number of fish that were dying directly from toxic exposure. Mortalities derived from this latter case were used to correct for contaminant derived mortalities in the treatment groups exposed to contaminants and challenged to *V. anguillarum*. The data summarizing the correction for mortalities attributable solely to exposure to each treatment are also included. This data is presented in two tables, one describing the average mortality for juvenile salmon over the same 7 day period that had received acetone:emulphor (the carrier), HCBd, the model mixture of PAHs, or CHWSE, but were exposed to *V. anguillarum*, the other table summarizes the uncorrected and corrected mortalities attributable to each treatment in each tank. The corrected mortalities were achieved by using the average mortality observed solely due to acetone:emulphor (the carrier), HCBd, the model mixture of PAHs, or CHWSE exposure and converting mortalities as survivors to an equivalent level in the groups receiving acetone:emulphor (the carrier), HCBd, the model mixture of PAHs, or CHWSE and then exposed to *V. anguillarum*. For example, if the average mortality in fish in all tanks receiving acetone:emulphor (the carrier) only was one and the mortality in a tank of fish receiving

acetone:emulphor (the carrier) and exposed to *V. anguillarum* over the same 7 day period was 5, then the corrected mortality due to exposure to *V. anguillarum* exposure for this particular tank of fish was recorded as 4, achieved by subtracting 1 from 5. Also, fish which died on the day of challenge were considered to have died from handling stresses due to the act of challenging and were not considered as mortalities due to the bacterial challenge.

Effects of chemical contaminants from the Hylebos Waterway on disease resistance of juvenile chinook salmon.

Data tables of daily mortality and cumulative survival of juvenile salmon exposed to acetone:emulphor, HCBd, A PAH model mixture, or CHWSE for a 8 day period and then challenged to *Vibrio anguillarum* via waterborne exposure.

Tank #: 49

Injection date: 10/12/95

Injected with: Acetone:Emulphor

Challenge date: 10/20/95

Date	Mortalities	Cumulative Survival	Sampler
12-Oct	-	30	
13-Oct	0	30	PH
14-Oct	0	30	PH
15-Oct	1	29	PH
16-Oct	0	29	ANK
17-Oct	0	29	PH
18-Oct	0	29	ANK
19-Oct	0	29	ANK

**Vibrio Treatment: LC0**

Date	Mortalities	Cumulative Survival	Sampler
20-Oct	20 <sup>a</sup>	10	ERC
21-Oct	1	9	ERC
22-Oct	0	9	ANK
23-Oct	0	9	PH
24-Oct	0	9	ERC
25-Oct	0	9	ANK
26-Oct	0	9	ANK
27-Oct	0	9	PH
28-Oct	0	9	ERC
29-Oct	0	9	ERC
30-Oct	2	7	ERC
31-Oct	0	7	PH
1-Nov	0	7	ANK
2-Nov	0	7	PH
3-Nov	1	6	ANK

<sup>a</sup> Fish which died on the day of challenge were considered to have died from handling stresses due to the act of challenging and were not considered as mortalities due to the bacterial challenge.