

**COMPARISON OF SHORT-TERM CHANGES IN CONDITION  
OF HATCHERY-RAISED CHINOOK SALMON  
BETWEEN POLLUTED AND REFERENCE ESTUARIES**

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**Abstract**

To assess the bioavailability and effects of polycyclic aromatic hydrocarbons (PAHs) to organisms residing near an aluminium smelter, hatchery raised, outmigrating juvenile chinook salmon were analysed histopathologically, biochemically and chemically. Salmon stocks were first sampled from the Kitimat hatchery prior to release into the Kitimat River or the Dala and Kildala Rivers. Two weeks after release, fish were collected in the inner harbour of Kitimat estuary, near an aluminum smelter and the Kildala estuary, which is remote from heavy industry. The chinook salmon showed differences in short term physiological stress responses related to sampling site. The Kitimat estuary is heavily contaminated with PAHs and uptake of these compounds was indicated by pyrenol-conjugate concentrations measured by synchronous scan fluorescence spectrometry. PAHs were about 100-fold greater in bile of fish from the smelter harbour than from the Kildala estuary or the hatchery. Observed physiological differences between the hatchery and field fish are attributed to crowded hatchery conditions and changes related to a fresh to salt water transition. The superior immune status of the Kildala fish, measured by phagocytic respiratory burst activity, and decreased interrenal cell activity,

determined by nuclear diameter measurements, suggest that these fish have adapted to their environment better than the Kitimat estuary fish.

## **Introduction**

Marine environments near industrial and urban centers are exposed to a wide range of chemicals that may be transformed, either biologically or chemically, into new potentially toxic compounds (Malins and Hodgins 1981). Research has shown a positive link (by association) between certain xenobiotic chemicals present in sediments, seawater or food organisms, with histological and biochemical changes in demersal fish species. For instance, differences in overall health (condition factor measurements and various hematological variables), immune status (respiratory burst activity of phagocytes (Lemaire-Gony et al., 1995) and plasma lysozyme), stress responses involving the endocrine system (increase in interrenal cell nuclear diameters (MacDonald et al., 1988)) plus incidences of liver (Myers et al., 1987) and/or gill lesions (Haensly et al., 1982), have all been correlated with exposure to xenobiotics.

The Kitimat hatchery produces between one and three million chinook salmon, *Oncorhynchus tshawytscha*, annually for release into the Kitimat River, its tributary Hirsch Creek, and the Kildala and Dala Rivers. After release from the hatcheries the juvenile salmon reside in and are dependent on the neighboring estuaries for their food and shelter. During the time of this study, total polycyclic aromatic hydrocarbon (PAH) concentrations in Kitimat Estuary and Arm sediments varied. Mean PAH values exceeded 200 mg/kg near the aluminum smelter to less than 3 mg/kg at a distance of 3 km across or down the Arm (Payne et al., 1996). In order to assess the bioavailability of PAHs to organisms residing in the Kitimat estuary, a study was conducted using hatchery-raised juvenile chinook salmon following the lead of researchers at the Northwest Fisheries Science Center in Seattle, Washington (Stein et al., 1995). These researchers measured various biomarkers of exposure to PAH of hatchery-raised juvenile chinook salmon that were caught 2-3 weeks after release from hatcheries in various marine sites in Puget sound. In this study, we examined whether outmigrating juvenile chinook salmon assimilate contaminants that produce histopathological, biochemical and chemical changes that compromise their ability to survive.

## Materials and Methods

### Field Collection

Juvenile chinook salmon, *Oncorhynchus tshawytscha*, were obtained from the Kitimat Hatchery at peak emergence (May 2, 1994) and from the Kildala estuary (May 24, 1994) and Kitimat Harbour (May 30, 1994) two weeks after release. Fish were collected by enticing them to the surface with fish feed followed by capturing them with a dip net. Fish were housed in coolers with aerated seawater. Meristic data were recorded and dissections done in a temporary field laboratory or at the hatchery.

### Histology

Gill, liver and interrenal tissues were examined from a total of 25 fish from each site. The salmon were euthanized by spinal severance and fixed whole in Deitrich's formalin (Gray 1954). Samples were decalcified in Cal-EX (Fisher Scientific) overnight at room temperature, washed in running water, dehydrated in graded ethanol, cleared in toluene, and embedded in paraffin (Humason 1979). Serial sections were cut at 6  $\mu\text{m}$  and stained with Gill's hematoxylin and eosin for general histology. Findings were classified, respectively, using nomenclature consistent with Myers et al. (1987) for liver lesions or Mallatt (1985) for gill changes. Alterations in interrenal cell nuclear diameters as defined by Donaldson (1984), were measured using a computer program for video microscopy (Cohu Solid State Video Camera) and measurements (BioScan Optimas version 3.14, Edmonds, WA, 1992). Twenty five cells were counted in one section from each of the 25 fish per group.

Statistical analyses were performed using the PC-based program, SPSS standard version for Windows (version 10.0.5, 1999). Coding schemes for gill lesions were used, where gills were ranked from 1 to 4 depending on the number and types of lesions. To investigate if gill lesions are dependent on collection site the nonparametric Kruskal Wallis test was used followed with the Wilcoxin paired T test. To test if there was a difference in the interrenal nuclear diameters with site of capture, two way ANOVAs with repeated measures were used. Post-hoc comparison of means using the Tukey HSD test was performed to clarify significant main effects.

### *Immune Status*

For general health and immune status, additional chinook salmon (*Oncorhynchus tshawytscha*) were sampled from the Kitimat hatchery (n=125), Kildala estuary (n=52), and the Kitimat estuary (n=53). The general health of each fish was examined from weight, fork length and condition factor measurements. Blood was collected from each fish to assess various hematological variables; hematocrit, erythrocyte cell counts and mean erythrocyte volume. The activity of the natural immune system was also examined in each fish. Head kidney material was isolated and the respiratory burst activity of glass adhered phagocytes determined using the nitro-blue tetrazolium (NBT) assay (Anderson 1992). In addition, the activity of plasma lysozyme was determined using the lysoplate method (Osserman & Lawlor 1966 with modifications by Yousif et al., 1994).

Differences between the sample sites for each of the above variables were determined using Kruskal-Wallis tests, and the comparisons between the sites were made using Dunn's pairwise comparison tests. Differences were noted where  $p < 0.05$ . The computer software program Sigmastat (Jandel Scientific, San Rafael, CA) was used for all the analyses.

### **PAH Metabolite Analysis**

Bile samples for PAH metabolite analysis were taken from a separate set of fish. 60 fish were combined in one composite sample and 4 composite samples were taken for each site. Gall bladders were removed from euthanized salmon, placed into precleaned amber vials, frozen over dry ice, stored at  $-20^{\circ}\text{C}$  and then at  $-80^{\circ}\text{C}$  until analysed. PAH metabolite conjugates in bile were measured by synchronous-scan fluorescence spectrometry (SFS) at Simon Fraser University following the method of Ariese et al. (1993). The samples of bile were diluted 1/500 with a 1:1 HPLC grade ethanol and HPLC water solution (Fisher Scientific Ltd.). The fluorescence response was measured on a Perkin Elmer Luminescence spectrophotometer LS-50 with FL data manager operating on an IBM-PC compatible computer. Both excitation and emission monochromators were scanned synchronously with a fixed wavelength difference of 37 nm. The area of the fluorescence emission response was measured from 335-356 nm on the emission monochromator. A six point calibration curve of 1-hydroxypyrene was used to calibrate the fluorescence spectrophotometer. The fluorescent

emission of the 1-hydroxypyrene standards was measured from 340-361 nm. A conversion factor of 2.2 was applied to account for the difference in fluorescence yield between 1-hydroxypyrene and its conjugate, 1-pyrenyl glucuronide, which is the major conjugated metabolite of pyrene in the bile. Protein analyses of fish bile were done by the method of Lowry et al. (1951) using a Bausch and Lomb Spectronic 20 spectrometer.

Principal components analysis (PCA) was performed using the multivariate statistical software package *Pirouette* v. 2.60. The data were autoscaled prior to performing the analysis.

## Results and Discussion

### *Histology*

Histologically the liver tissues examined from all the juvenile chinook salmon, *Oncorhynchus tshawytscha*, were normal. A two week exposure time to contaminated sediments is too short to reveal any idiopathic liver lesions. For instance, it takes approximately three years for English sole, *Pleuronectes vetulus*, inhabiting the contaminated sediments of Puget Sound to develop preneoplastic liver lesions (pers. comm. M.S. Myers, NOAA, Seattle, WA).

Analysis of the interrenal nuclear diameters (Figure 1 & Table 1) revealed a statistically significant increase ( $p > 0.001$ ) in fish collected from the hatchery and from Kitimat Harbor compared to those from the Kildala estuary. The mean diameters were as follows: Kildala estuary =  $5.2 \pm 0.27 \mu\text{m}$ , Kitimat Harbour =  $5.9 \pm 0.31 \mu\text{m}$  and Kitimat hatchery =  $5.9 \pm 0.32 \mu\text{m}$ . Interrenal cells in elasmobranchs and bony fishes represent the equivalent of the mammalian adrenal cortex. These cells produce corticosteroids, with cortisol being the most quantitatively important in teleosts. This hormone has potent effects on intermediary metabolism and is important for seawater adaptation. In addition, cortisol levels can be elevated after exposure of teleost fishes to some stressors, suggesting that it is a major factor in the piscine stress response (Ferguson 1989). Interrenal cell activity in salmonids has been shown to exhibit transient increases due to stress from: exposure of butoxyethanol ester of 2,4-dichlorophenoxyacetic acid; with density-related social interaction; and a transitory stress response when smolts are released from a hatchery directly into sea water (McBride et al., 1981; Donaldson et al., 1984; McDonald et al., 1988). Plasma cortisol changes and histological examination of interrenal tissues, has

been utilized as an indicator of primary effects of stress. For instance, Brown et al. (1986) reported rainbow trout exposed to high acid levels revealed plasma cortisol and interrenal cell nuclear diameters to be higher than for control levels.

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- Fig. 1 Chinook salmon head kidney showing interrenal cells (IR), kidney tubule (KT) and hemopoietic tissue (H), H & E. Bar = 50  $\mu$ m.
- Fig. 2 Gill. Primary and secondary lamella ( $1^{\circ}$   $2^{\circ}$ ), chondrocyte (c), pillar cells (p), mucus cell (m), and erythrocytes (e). H & E. Bar = 50  $\mu$ m.
- Fig. 3 Slight epithelial lifting (small arrow) of the secondary lamella and hyperplasia (large arrow) of the epithelial cells. H & E. Bar = 50  $\mu$ m.
- Fig. 4 Severe epithelial lifting and hyperplasia leading to fusion of the secondary lamella. H & E. Bar = 50  $\mu$ m.

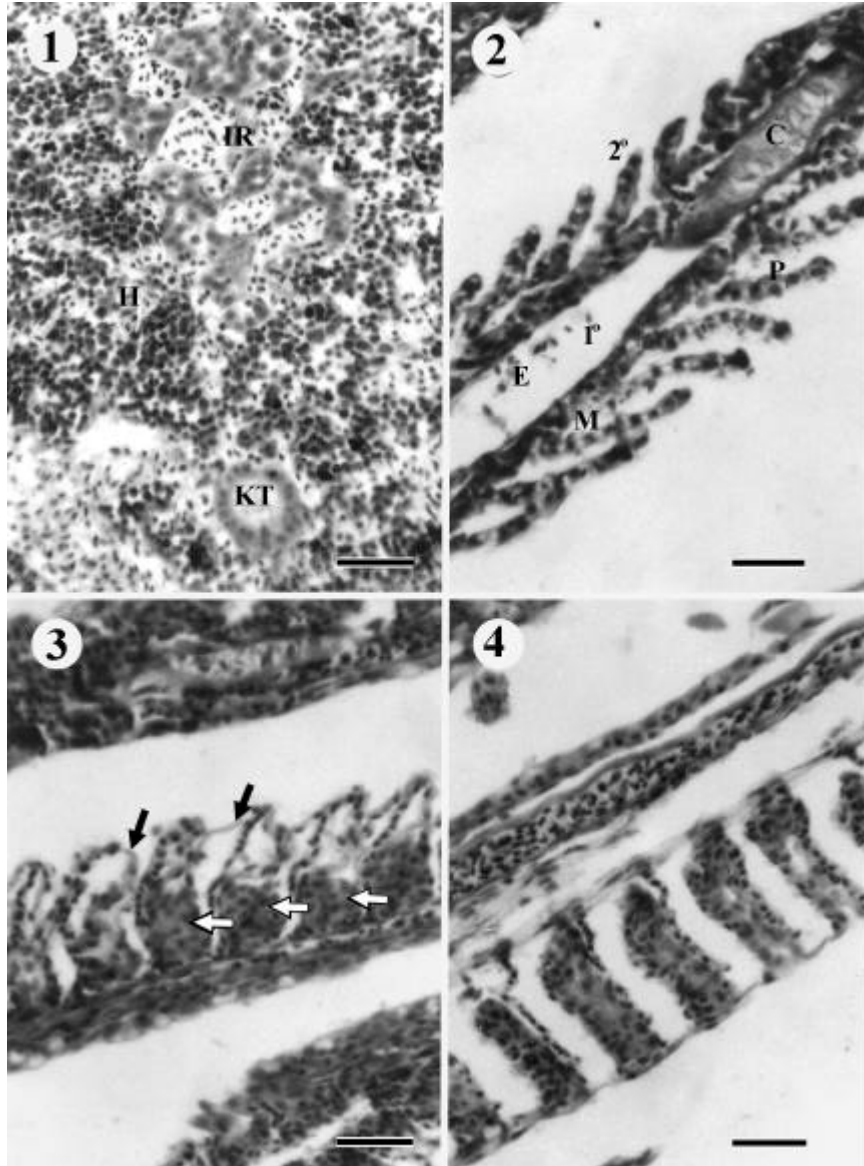


Table 1. Summary of general condition, hematological data and immune status for juvenile chinook salmon sampled from the hatchery and from the Kildala and Kitimat estuaries two weeks after release.

	Kitimat Hatchery	Kildala Estuary	Kitimat Estuary
Weight (g)	8.6 ± 0.11 (125) <sup>b</sup>	8.9 ± 0.22 (52) <sup>a</sup>	8.3 ± 0.12 (53) <sup>b</sup>
Fork Length (cm)	9.1 ± 0.04 (125) <sup>a</sup>	9.3 ± 0.05 (52) <sup>b</sup>	9.4 ± 0.04 (53) <sup>b</sup>
Condition Factor (x10 <sup>4</sup> g/cm <sup>3</sup> )	112 ± 0.86 (125) <sup>a</sup>	109 ± 2.33 (52) <sup>a</sup>	101 ± 0.66 (53) <sup>b</sup>
Hematocrit (%)	52.0 ± 0.5 (125) <sup>a</sup>	46.9 ± 0.8 (52) <sup>b</sup>	48.2 ± 0.6 (51) <sup>b</sup>
Erythrocytes (x10 <sup>6</sup> /mm <sup>3</sup> )	0.57 ± 0.00 (125) <sup>a</sup>	0.63 ± 0.015 (52) <sup>b</sup>	0.68 ± 0.017 (53) <sup>c</sup>
MEV (µm <sup>3</sup> )	941 ± 18.2 (125) <sup>a</sup>	760 ± 18.0 (52) <sup>b</sup>	736 ± 21.5 (51) <sup>b</sup>
Plasma Lysozyme Activity (U/mL)	144 ± 11.8 (109) <sup>a</sup>	69.4 ± 8.0 (46) <sup>b</sup>	75.8 ± 3.9 (49) <sup>b</sup>
Phag. Resp. Burst Activity (%NBT pos.)	2.45 ± 0.112 (125) <sup>a</sup>	5.65 ± 1.05 (51) <sup>b</sup>	1.86 ± 0.13 (53) <sup>a</sup>
Gill lesion index*	3.16 ± 0.18 (25) <sup>b</sup>	2.56 ± 0.23 (25) <sup>a</sup>	2.60 ± 0.18 (25) <sup>a</sup>
Interrenal nuclear diameter (µm)	5.9 ± 0.01 (750) <sup>a</sup>	5.2 ± 0.01 (750) <sup>b</sup>	5.9 ± 0.01 (750) <sup>a</sup>

<sup>a</sup> X±SE (n), different from b at the 95% confidence level, <sup>b</sup> X±SE (n), different from a at the 95% confidence level, <sup>c</sup> X±SE (n), different from a and b at the 95% confidence level. \*Gill lesion index is at the 93% confidence level

In this study, an increase in interrenal cell nuclear diameters was observed with respect to site of capture. In other words, fish collected from Kitimat hatchery and harbour have nuclear diameters that are 13.5% larger than in fish collected from the Kildala estuary. The differences observed in the interrenal cell nuclear diameters suggests that the hatchery salmon are exhibiting physiological stress due to crowded hatchery conditions. Recovery, after release appears to be occurring for the Kildala estuary fish, but is not evident in the Kitimat estuary stock.

The manner in which gill changes occur is often an accurate indicator of the causative agent, *e.g.*, bacteria, diet, or chemical. However, various agents may produce gill lesions simultaneously. Extensive damage from a specific agent may overshadow or mask gill injury produced by a second or third. The changes in gill lamellar structure however, can affect gas exchange, osmoregulation, as well as altering susceptibility to a variety of disease-causing organisms (Sinderman 1979). Normal gill tissue (figure 2) as well as gill lamellae with epithelial lifting (figure 3) or partial fusion and hyperplasia (figure 4) occurred at varying degrees of severity in all fish collected from all sites. Gill lesions were ranked from 1 to 4 depending on the number of and lesion types, present. Statistical analysis revealed that the degree of severity in gill lesions of the hatchery fish was significantly greater compared to the fish from Kitimat Harbour ( $p > 0.05$ ) and the Kildala estuary ( $p > 0.02$ ). This difference may be related to factors associated with overcrowding, such as changes in oxygen, urine, and pH levels.

#### *Immune Status*

The general condition of the fish was found to vary with the sample site (Table 1). The weight and fork length measurements were used to calculate the condition factor. The condition factor is an indicator of the nutritional state or “well being” of a fish (Busacker et al., 1990). Our sampling revealed that the condition factor in the fish from the Kitimat estuary was significantly lower than the other sites. Perhaps the growth rate of these fish was compromised due to lack of available food.

The hematological data (Table 1) reveals differences between the fish sampled from the 3 sites. Hematocrit values were significantly higher in the hatchery group, while the erythrocyte counts were significantly lower than the other sites. The mean erythrocyte volume (MEV), which is calculated from the hematocrit and erythrocyte count data, was significantly higher in the hatchery group.

These hematological results are likely due to the osmoregulatory status of the fish. At the Kildala and Kitimat estuary sites, the fish were in seawater and were perhaps dehydrated due to the hypertonic environment. The high erythrocyte counts observed in the Kildala and especially the Kitimat estuary fish, may also reflect reduced O<sub>2</sub> carrying capacity in the seawater or alternatively hemoconcentration due to gill damage

The status of the natural immune system of the fish from the 3 sites was found to be significantly different (Table 1). The plasma lysozyme activity was significantly higher in the hatchery fish. Lysozyme has been shown to decrease during smoltification, when cortisol (membrane stabilizer) levels increase and neutrophil (lysozyme producing leucocytes) numbers decrease (Muona and Soivio 1992). The respiratory burst activity of head kidney phagocytes was significantly higher in the fish sampled from the Kildala site. The reduced respiratory burst activity in the hatchery and Kitimat estuary fish may be associated with stress and/or contaminant exposure. Pentachlorophenol (PCP) has been shown to suppress the respiratory burst activity of phagocytes (Anderson & Brubacher 1993), and macrophages (Roszell & Anderson 1994). Benzo(a)pyrene has also been shown to inhibit respiratory burst activity (Lemaire-Gony et al., 1995). Various environmental contaminants have been shown to suppress the immune system of fish (Dunier & Siwicki 1993).

#### *PAH Metabolites*

The PAH metabolite conjugate concentrations in composited bile of 60 fish are reported as 1-pyrenyl glucuronide equivalents (Table 2). Although the methods show a high degree of specificity, it may overestimate the actual concentration of 1-pyrenyl glucuronide. Pyrene sulphates, other pyrene glucuronides, alkylpyrene glucuronides sulphates, and other conjugates of PAH having the pyrenoxy chromophore may be present within the bile and contribute to the signal (Ariese et al., 1993). These interferences would be minor, but nonetheless, the SFS method employed here is considered more for screening than for absolute quantitation.

The 1-pyrenyl glucuronide equivalent concentrations in bile composites from the hatchery and those caught after release at the head of Kildala estuary were all below the detection limit of 0.26 µg/ml with the exception of one composite of Kitimat River stock fish from the hatchery. The five composites from the Kitimat estuary ranged from 25.7 to 47.1 µg/ml.

Table 2: Synchronous-scan fluorescence spectrometric analysis of juvenile chinook salmon bile for PAH metabolite conjugate concentrations reported as 1-pyrenyl glucuronide equivalents (Pyr.gluc). Values expressed as mean  $\pm$  standard deviation of four composites with 60 fish each.

Sample ID*	Pyr.gluc. / Protein ( $\mu\text{g}/\text{mg}$ )	Detection limits ( $\mu\text{g}/\text{mg}$ )	Pyr.gluc. / Biliverdin ( $\mu\text{g}/\text{mg}$ )	Detection limits ( $\mu\text{g}/\text{mg}$ )
KID	0.067 $\pm$ 0.089	0.067 $\pm$ 0.089	0.783 $\pm$ 0.707	0.783 $\pm$ 0.707
KIT	0.029 $\pm$ 0.009	0.028 $\pm$ 0.010	1.010 $\pm$ 0.730	0.815 $\pm$ 0.799
KD	0.026 $\pm$ 0.003	0.026 $\pm$ 0.003	0.958 $\pm$ 0.104	0.958 $\pm$ 0.104
KT	3.174 $\pm$ 0.613	0.024 $\pm$ 0.002	180.6 $\pm$ 76.69	0.446 $\pm$ 0.153

\* KID, hatchery salmon-kildala stock; KIT, hatchery salmon-kitimat stock; KD, Kildala estuary salmon; KT, Kitimat estuary salmon

The PAH metabolite concentration depends on feeding status of the fish, where levels increase when the fish are not feeding and water in the bile is resorbed (Collier and Varanasi, 1991). Thus, for comparison purposes PAH concentrations in the bile are generally expressed relative to the concentrations of protein or biliverdin whose concentrations in bile also increase and decrease with feeding status.

The concentrations of proteins in the bile composites of juvenile chinook salmon from the Kitimat inner harbour varied slightly, indicating similar feeding status (not unexpectedly since composites of 60 fish were used). The PAH metabolite conjugate concentrations in the Kitimat harbour samples ranged from 2.40 - 3.88  $\mu\text{g}/\text{mg}$  bile protein which was 100 fold greater when compared with the samples from the Kildala estuary and hatchery.

#### *Data Summarised using Principal Components Analysis*

Principal Components Analysis modelling illuminates the differences in the fish from the two field sites and the hatchery (Figure 5). Thus, the first principal component distinguishes the two field sites from the hatchery, and the second,

the field sites from each other. The phagocytic respiratory burst activity, weight and condition factor make a strong positive contribution and the interrenal nuclear diameter makes a strong negative contribution to PC 2, resulting in a clear distinction between the Kildala estuary and the other two sites. The condition factor also loads positively to the PC 1, helping distinguish the Kildala estuary and hatchery from the Kitimat estuary. The mean erythrocyte volume, gill lesion index, plasma lysozyme activity, and % hematocrit load strongly positive on PC 1, while the fork length and erythrocyte concentration load strongly negative. These variables mainly contribute to the distinction between the hatchery and the other two sites along PC1. The plasma lysozyme activity and gill lesion index load almost the same on both PCs, indicating a particularly strong correlation between these two variables.

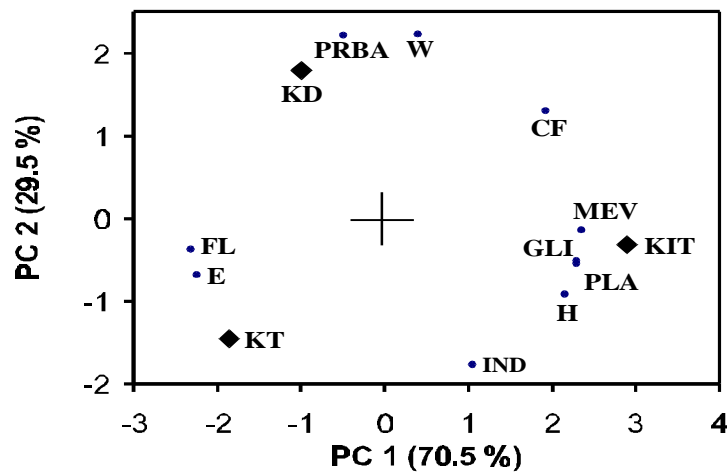


Figure 5. Biplot of sample site scores (♦) and scaled variable loadings (•) for PCA model for juvenile chinook salmon from three sites (KIT, KD and KT) in the Kitimat study area. Data for the model were autoscaled before processing. The scale factors for the variables in the biplot were 5.93 and 3.69 for PC1 and PC2, respectively. (W = weight, FL = fork length, CF = condition factor, H = hematocrit, E = erythrocyte, MEV = mean erythrocyte volume, PLA = plasma lysozyme activity, PRBA = phag. resp. burst activity, GLI = gill lesion index, IND = interrenal nuclear diameter)

## Conclusion

In general, the histopathological and biochemical differences could be explained by the differing rate of recovery from crowding stress in the hatchery and saltwater stress in the estuaries. Chemical exposure is known to cause impaired function in fish and our results suggest that the presence of chemicals related to aluminium smelting activity in the Kitimat estuary may hinder physiological processes involved in smoltification. Further work is required to determine whether the observed differences affect the juvenile chinook salmon's survival to adulthood.

## Acknowledgements

Part of this work was funded by the Canadian Toxic Chemicals Green Plan. A special thank you to the US Department of Agriculture and US Geological Survey for funding towards travel to the American Fisheries Society conference held in Aberdeen, Scotland.

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