Biochemical Responses Based on Cytochrome P450 Induction in Chinook Salmon (*Oncorhynchus tshawytscha*) Exposed to Bleached Kraft Mill Effluent on the Fraser River

by

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Abstract

Juvenile chinook salmon were collected from sites on the upper Fraser River downstream of bleached kraft pulp mills and municipal outfalls. Ethoxyresorufin-O-deethylase activity (EROD), CYP 1A1 density, and DNA adduct concentrations were measured in liver tissue. Liver histopathology was performed. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) were measured in carcasses. Polyaromatic hydrocarbon (PAH) metabolites were measured in bile. Biochemical, but not histopathological, responses were significantly different from those in controls at nearly all sites. Biochemical responses were not correlated with any of the contaminants analyzed. Fish sampled closest to effluent discharge showed the weakest responses. In fish experimentally exposed to effluent, significant increases in biological effects were seen at 2% (and higher) effluent concentrations. These results indicate that fish at this site may not be exposed to effluent concentrations previously thought, perhaps suggesting that chinook are mobile during winter months. Biological responses are not caused by the organic contaminants measured.

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Dedication

This thesis is dedicated to my mother, Yvonne Lamont and Mary Williams. It is dedicated in loving memory to Arnold Howe, who died before I finished, but always believed I could do anything.

1. Introduction

1.1 *Overview*

Salmonids are fish of great interest in the Pacific Northwest, in part because of their large fisheries and their particular life history which encompasses both fresh and marine habitats. In British Columbia, salmon runs are suffering from declines that are considered to be due to overfishing. The impact of contaminants on a potentially fragile population may be significant. The Fraser River is important because of its large contribution as a spawning ground for all of the Pacific salmonids. Chinook overwinter in the upper reaches of this system where pulp mill production is high. Before 1990, pulp mills were producing a large amount of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans. This was reflected in large contaminant concentrations and large EROD induction in chinook captured downstream of pulp mills. Chinook caught at a site near Quesnel had concentrations of 68 ng kg^{-1} 2,3,7,8- TCDD and EROD activities 55-fold over control fish (Rogers, *et al.*, 1989). Since the mill processes have changed and contaminant production has decreased, contaminant levels have greatly declined in the fish, paralleled by some declines in EROD activity and other biological responses (D. Martens and R. Gordon, personal communication). The biological responses, but not the contaminant concentrations, have continued to be significantly greater than those found in control fish (D. Martens and R. Gordon, personal communication). If the pulp mills are producing such low levels of PCDDs and PCDFs, why are the biological responses still present? The need to continue and broaden the investigation of contaminant toxicity in chinook salmon on the upper Fraser River was the impetus for this study.

1.2 *Chinook Salmon (Oncorhynchus tshawytscha)*

Chinook salmon are anadromous and spend a proportion of their life in both fresh and salt water. Chinook spawn in fresh water from August to November, although the peak time is early October (Fraser *et al.*, 1982). This change to fresh water results in the

death of the adult. The eggs incubate for 500 degree days (one day at 1°C = one degree day) and the alevin emerge from the gravel in April to May (Fraser *et al.*, 1982). The juveniles will then follow one of three life histories. The "immediate migrants" will migrate towards the estuary immediately after swim-up. These fish are usually found in coastal streams and are thought to originate from late run stocks (Fraser *et al.*, 1982). The extent of this life history pattern is unknown but it seems to be directly related to the size of the receiving estuary, with the larger estuaries producing a larger proportion of this type of chinook (Fraser *et al.*, 1982). The "ocean-type migrants" remain in fresh water for about 60 to 150 days before migrating towards the estuary in June to August. This seems to be the dominant life history pattern for eastern Pacific chinook (Fraser *et al.*, 1982). The third life history is the "stream-type migrants" which remain in fresh water for the winter and migrate the following spring. Although they tend to be less common than the ocean-type, they occupy spawning and rearing grounds that are large distances from the estuary. While in fesh water, chinook will feed mainly on dipterans (adult chironomids) but also larval or juvenile representatives of the orders Homoptera, Coleoptera, Hymenoptera and Arachnida (Levings and Lauzier, 1991; Emmett *et al.*, 1996). Smoltification occurs in the estuary as the fish prepare to enter salt water. Chinook return to fresh water in their 3, 4 or 5 year of age, although the 4 year old age class represents >50% of the returning adults (Fraser *et al.*, 1982). Two or three year old jacks (precocious males) are common in some races of chinook (Fraser *et al.*, 1982).

The stream-type chinook remain dormant during the winter period, hiding within the cobble during the day and foraging at night. Warm-water areas including industrial outfalls and domestic wastewater effluents may serve as important sources of food production in winter (Levings and Lauzier, 1991). Hatches of insects also occur during occasional periods of warm weather, leading to variability in growth and survival of chinook over the winter months (Levings and Lauzier, 1991). These salmon can only be caught by electrofishing, as they are not found in the water column. Range of movement during winter months is unknown as only limited mark-recapture studies have been done. Extended movement occurs in summer months but this seems to be caused by preferential movement of fish away from high suspended sediment levels in the mainstem Fraser river

(Scrivener *et al.*, 1993). Although preliminary data suggests that site fidelity in winter is not high (Emmett *et al.*, 1996), the range that these organisms may cover is still unclear. Movement is assumed to be limited, making this a useful monitoring species in which to study effects of local pollution.

1.3 *The Fraser River Watershed*

The Fraser River basin occupies an area of 230 000 km^2 in British Columbia, Canada. In total, it covers 9 (of 12) biogeoclimatic zones of the province, making it a very diverse watershed. The mainstem river is 1253 km long and has over 300 tributaries. The river has a high turbidity, with levels of suspended solids reaching 389 mg l^{-1} during freshet (Northcote, 1974). Low flow periods occur during winter months and high flow occurs in June (Fraser, *et al.*, 1982).

The Fraser River supports a valuable salmon run. All five species of Pacific salmon (sockeye, chinook, pink, chum and coho) and two species of anadromous trout (steelhead and cutthroat) spawn in these waters. There are at least 300 streams, rivers, and lakes in this watershed that support salmon spawning , with chinook and sockeye represented throughout (Birtwell, *et al.*, 1988). Pink, coho and chum spawn mainly in the lower areas of the watershed (Birtwell, *et al.*, 1988). Chinook salmon have been found spawning in 98 streams in the watershed including areas of the Stuart, Nechako, Quesnel, Chilcotin, Thompson (North, South and main), Lillooet and entire (upper, middle and lower) Fraser Rivers (Birtwell, *et al.*, 1988). Contributions of these areas to the fish stocks vary with the upper Fraser (east of Prince George), Nechako and Chicoltin Rivers contributing 1.1, 3.4 and 5.5 million juveniles and the Thompson, Lillooet and mainstem Fraser contributing 19.8, 14.2 and 12.7 million juveniles (Birtwell, *et al.*, 1988).

In the Fraser River, up to 90% of the adult chinook are classified as the ocean-type (Fraser *et al.*, 1982). Where distances to estuary are quite large, the stream-type chinook dominate. Chinook found north of the Thompson River are thought to be exclusively stream-type. Chinook in this area are potentially sensitive to pollution as they remain in the river during low flow periods and thus are exposed to the highest concentrations of

contaminants. Food chain contributions during this period may be significant if insect development is encouraged in areas of industrial discharge.

There are a number of sources of pollution in the upper Fraser River basin. Bleached kraft mills discharge effluent at Prince George, Quesnel and Kamloops and sewage outfalls discharge at Prince George and Quesnel. Non-point source inputs include agricultural pollutants from livestock ranching, wood preservatives from wood processing plants and herbicide use from the logging industry (Birtwell, *et al.*, 1988). The investigation of the effects of these contaminant sources, especially in the face of declining fish stocks is important (Birtwell *et al.*, 1988).

1.4 *Organic Contaminants*

Organic contaminants of main interest to this thesis are the polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). These four chemical classes consist of a large number of congeners that have similarities in shape, size, and chemistry. They are all planar, multi-ringed structures and all but PAHs have some chlorination. The degree to which the congener takes on a planar arrangement greatly increases its toxicity. This arrangement is necessary for the congener to bind to the Ah receptor (see section 1.4), which mediates its toxic effects.

PCDDs and PCDFs are three-ringed structures and those with 2,3,7,8-substitutions are the most toxic. PCBs are two ringed structures that may be coplanar or mono-*ortho* substituted. Coplanar PCBs have substitutions in the *para* and *meta* positions only, which increases the toxicity because they are planar. Substitution in *ortho* positions will reduce the ability of the molecule to take a planar arrangement and thus the toxicity of the compound will be reduced. Mono-*ortho* PCBs are nearly planar and thus have some potency. PAHs are hydrocarbons that have at least 2 fused benzene rings, with or without substitution. The diversity of congeners in this group is much larger than the other 3 classes. On the whole, these chemical classes are considered hydrophobic and are associated with sediment or suspended solids in the water column. The degree of

solubility varies across the groups. The four contaminant classes are shown in Figure 1. Naphthalene, the smallest PAH in the class, is shown as an example of a PAH.

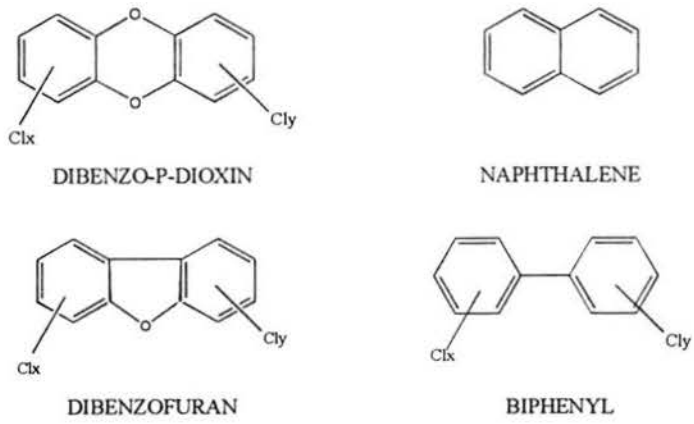

Figure 1 Organic Contaminant Classes

Adapted from Birnbaum, 1994

PCDDs, PCDFs and PAHs may be produced by natural sources. PAHs are produced by marine petroleum seeps and by some bacteria, fungi and plants. PCDDs and PCDFs may be produced by wood combustion (Nestrick and Lamparski, 1982). Like PCBs, PCDDs and PCDFs may be generated by natural precursors, but these must be converted during industrial processes. The vast majority of PCDDs, PCDFs, PAHs and all PCBs released into the environment are from anthropogenic activity. The introduction of these compounds into the aquatic food chain occurs when they are released in effluent and runoff into the surface waters. They are bound to sediment or suspended solids which are either in direct contact with or ingested by an organism. PCDDs, PCDFs and PCBs are lipophilic and accumulate in fatty organs as metabolism is limited with many congeners. PAHs are more easily metabolized and do not bioaccumulate in vertebrates. Invertebrates, which cannot metabolize PAHs, bioaccumulate PAHs.

1.5 *Biological Effects of Contaminants*

Organic contaminants have varied effects depending on the particular contaminant or contaminant class, the dose and the species exposed. The four main classes of contaminants outlined above have similar effects in most species. PCDDs, PCDFs, PCBs

and PAHs are inducers of the cytochrome P450 1A1 enzyme in nearly all species examined. In the specific case of PAHs and possibly PCBs, this induction can be related to another commonly measured biological effect, production of DNA adducts. The damage caused to DNA by reactive species has been shown to be a leading step to fixed DNA mutations and is thought to be critical in the multi-step pathway of chemical carcinogenesis. This is reviewed for mammalian systems in Beach and Gupta (1992). Over time, changes in the DNA may lead to altered tissue, pre-neoplastic conditions and ultimately a tumor. This depends on the ability of the organism to repair the adduct or the mutation before it is fixed. By measuring the induction of the P450 1A1 system and coupling this information with the presence of DNA adducts and histopathological changes, a linkage between biochemical and physiological changes may be established.

1.5.1 Cytochrome P450 1A1

The cytochrome P450 enzymes, denoted as CYP, are responsible for metabolism of a wide range of endogenous and exogenous compounds in an organism. These enzymes are found in every cell in the body (except red blood cells and skeletal muscle cells) and every kingdom including bacteria (Guengerich, 1993). P450 enzymes insert an oxygen molecule into the substrate according to the equation: $2\ NAD(P)H + O_2 + R \rightarrow 2\ NAD(P)^+ + H_2O + RO$, where R is the substrate for the reaction. They are located in the endoplasmic reticulum and thus microsomes made from tissue homogenates are capable of these reactions. Tissue and species specificity exists for many P450 enzymes, although certain enzymes seem to be fairly well represented across species and tissues. Enzymes are grouped according to families and subfamilies, depending on the percent homology between particular enzymes. The chemistry of the cytochrome P450enzymes have been reviewed by Guengerich (1993) and Gonzalez (1989) and by Stegeman (1993), referring specifically to fish.

The cytochrome P450 1A1 (CYP 1A1) enzyme is of particular interest to toxicologists because it is responsible for the metabolism of PCDDs, PCDFs, PCBs and PAHs, although this is limited to certain congeners. In addition, this enzyme is induced by these contaminants (i.e. *de novo* synthesis occurs after exposure). The induction of CYP

1A1 is thought to occur through a series of events within the cell that leads to transcription of the CYP 1A1 gene (and others). Initially, the contaminant binds to the Ah receptor, a cytosolic receptor bound to heat shock protein (HSP) 90. HSP90 is released upon binding and the receptor-ligand complex binds aryl hydrocarbon receptor nuclear transferase (ARNT). This complex moves into the nucleus and acts on xenobiotic response elements in the DNA to affect the transcription of several genes. For a review see Guengerich (1993). Increases in mRNA, protein and enzyme activity can be detected by a variety of techniques after exposure of the cell to contaminants.

CYP 1A1 induction after exposure to organic contaminants can be measured in several ways. Since induction causes *de novo* synthesis of the enzyme, measurement of mRNA, protein concentration or catalytic activity are possible. The measurement of catalytic activity is the most common in toxicological studies because it is a fast, cheap and reliable measurement. This assay makes use of specific substrates for CYP 1A1 which are converted into fluorescent metabolites. Ethoxyresorufin-O-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) activity are two catalytic measurements of CYP 1A1. They are differentiated by the substrate used, 7-ethoxyresorufin (EROD) or benzo[a]pyrene (AHH). EROD is more widely used, in part because benzo[a]pyrene is a carcinogen and more expensive than 7-ethoxyresorufin. The main problem with measuring catalytic activity is the need to preserve the functional enzyme and components of the oxidative process. The tissue must be quick frozen and microsomes must be stored at -80°C or colder. Repeated freezing can result in loss of activity and thus they cannot be used indefinitely. Unfortunately, there is no way to identify if there has been a loss of activity. Measurement of protein concentrations or CYP 1A1 densities overcomes the main difficulty of the EROD assay. This assay uses western blotting techniques to identify the concentration of protein in a given sample. Functional activity is unimportant to this assay. The identification of the protein is accomplished with the use of an antibody. A purified standard is also applied to each gel and thus quantification of protein concentrations is possible. The main difficulty is the lack of species specific standards available. If the standard and sample are not from the same species, differences in

antibody affinity for the protein will exist and conversion to concentration is not exact. This particular problem creates difficulty in the comparison of data to published literature.

1.5.2 DNA Adducts

DNA adducts are formed when chemicals covalently bond with a base in the DNA. Large ring PAHs (>4) can form reactive intermediates during metabolism by CYP 1A1 and epoxide hydrolase. PAHs are the most widely studied organic contaminants responsible for DNA adducts. This is due in part to their rapid metabolism by CYP 1A1, which allows a greater potential for DNA adducts to form. Initially, PAHs are metabolized through CYP 1A1 to form an arene oxide which is very unstable and can form phenols (by spontaneous rearrangement), diols (via epoxide hydrolase) or glutathione conjugates (via glutathione-S-transferase) (Varanasi *et al.*, 1989). For a detailed review of the metabolic activation of carcinogens see Guengerich (1992). Diol epoxides react preferentially with the N_2 position of guanine to form a large, bulky hydrophobic adduct (Beach and Gupta, 1992). The most common benzo[a]pyrene adduct, is shown in Figure 2.

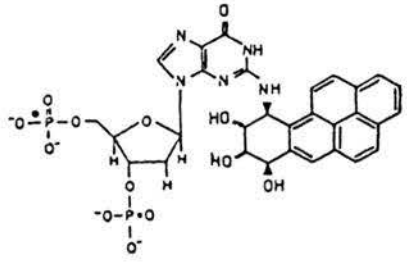

Figure 2 Benzopyrene-DNA Adduct

Taken from Gupta (1993)

Benzo[a]pyrene (BP), the classic PAH studied for DNA adducts, is metabolized to a diol epoxide through the following reactions: BP → BP 7,8-oxide → BP 7,8-diol → BP 7,8-diol 9,10-epoxide. Of course, this is only one of several pathways possible but it is the only pathway responsible for the formation of an epoxide. This epoxide can be conjugated to a final metabolite for excretion or it may react with macromolecules in the cell, including DNA. It is the balance between the conjugation reaction and epoxide formation that decides the extent of DNA adduct formation.

DNA adducts can be measured by postlabeling the adduct with ^{32}P and using thin layer chromatographic techniques and autoradiography to visualize them. This method is one of the most common DNA adduct quantification techniques. It is particularly useful when the exposure is a complex misture and unknown because it integrates the total number of adducts. Additionally, this technique has a higher sensitivity than other techniques and can identify 1 adduct in 10^6 - 10^8 normal nucleotides (Chang *et al.*, 1994). It has a low minimum DNA requirement (1-10 μg) which is useful when working with juvenile fish.

^{32}P-postlabeling follows six basic steps outlined in Figure 3. First, the DNA is digested into single bases by enzymatic hydrolysis with micrococcal endonuclease and spleen phosphodiesterase. Second, nuclease P1 converts normal nucleotides to a form that cannot be labeled. Third, the adducts are labeled with ^{32}P and the mixture is spotted on a thin layer chromatography (TLC) sheet. The adduct is labeled at the location marked with an asterisk in Figure 2. In step 4, the adducts are separated in 2 dimensions using chromatography solvents, optimized for pH and ionic strength according to the adducts of interest. The TLC sheets are exposed to film and a map is created of the location of the adducts. Last, the adduct zone can be removed from the sheet and quantified by scintillation counting or alternatively, the sheets can be exposed to phosphor screens and quantified by computer. This is a new technique that has been shown to be particularly effective in choosing an appropriate background and dealing with the problem of film quenching with high adduct concentrations (Reichert *et al.*, 1992).

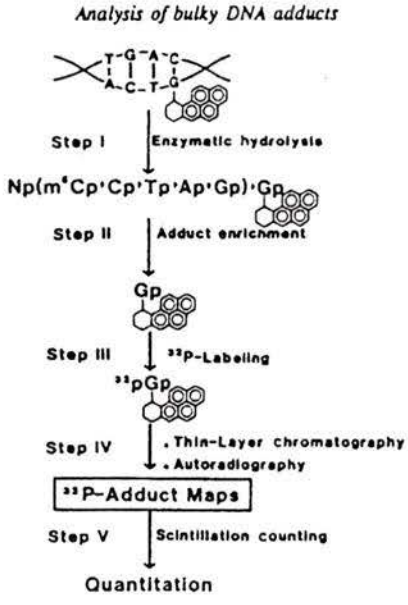

Figure 3 Steps to Postlabeling Adducts

Taken from Gupta (1993)

1.5.3 Histopathology and Tumors

Histopathological changes and tumors have been measured in a number of fish species, although they tend to be either bottom dwelling or bottom feeding. Although PCDDs, PCDFs, PCBs and PAHs are hydrophobic and are in greater concentration in the sediment or suspended solids than the water, only PAHs are quickly metabolized which makes biomagnification difficult. Therefore, those species that are in direct contact with the sediment are considered at the highest risk of tumors. Age seems to have a strong relationship to tumor prevalence and can be used to explain some variability between sites. Size is sometimes used as an indirect measurement of age and has been found to have a significant positive relationship to neoplasms (reviewed in Myers, 1991). With this in mind, juvenile fish (0 to 1+ age groups) can usually be examined for histopathological changes only, while older fish may be examined for tumors (Myers, 1991).

The main problem with measuring histopathological changes or tumor prevalence is that the data are reported in qualitatative terms simply as positive or negative which makes statistical analysis difficult. Histopathological analysis is more subjective than other measurements and efforts must be taken to "blind" the histopathologist from the exposure or study site. Ensuring that a proportion of samples are examined by more than one histopathologist will help to ensure the validity of the results. The advantage of these measurements is that they are more closely related to higher order effects (in this case death) than many biochemical changes. Unfortunately, laboratory work showing cause and effect relationships with organic contaminants has not been performed and viral or bacterial infections may cause similar changes.

1.6 *Biomonitoring Effects of Contamination*

Measuring the effects of contaminant toxicity can be performed in three basic ways: field studies, controlled laboratory studies, or a hybrid approach. Field studies examine *in situ* problems associated with pollution and effluent discharge. These studies have many uncontrolled factors which makes it difficult to show clear relationships between exposure and effects and interpret results. For example, the mobility of the organism is often unknown and therefore the exposure is questionable. Exposure is usually estimated by chemical residue analysis of tissues but some contaminants are easily metabolized which means that tissue concentrations reflect recent exposure only. Environmental samples may be used to estimate exposure but these do not address bioavailability and bioaccumulation or biomagnification through the food chain. In addition, there is no guarantee that the measured contaminants cause the effects measured. Field studies have the advantage of estimating toxicity and population effects in real situations. Chronic, low level contamination is most prevalent in the wild but this type of exposure is more difficult in a laboratory situation. There is a need to verify that under variable conditions toxicity is still measurable since a combination of conditions not tested in the laboratory may alter the response.

Laboratory studies are most useful for short term toxicity testing. They allow for methods development and illustrate cause and effect relationships. The exposure is

controlled and thus confidence can be expressed in differences between exposure concentrations. The main problem with this type of study is that chronic tests are very expensive and difficult to run and the results are difficult to extrapolate to field exposures. These studies are useful for the study of mechanisms of toxicity and generally allow any life stage to be tested. The flexibility in size and numbers of organisms required for adequate tissue samples and for statistical confidence makes this type of study beneficial.

Hybrid studies attempt to overcome the limitations of field and laboratory studies. In a controlled field study the exposure is known and some variables are controlled or measured to try to limit the caveats of field work. This type of study has increased variability over a traditional laboratory study and is designed to imitate the field situation. This type of study is a good way to test specific questions that may arise from field data.

The object of any of these approaches is to test responses of organisms to contaminant exposure and establish a measure of toxicity to that contaminant. Higher order effects can be difficult to measure except for acute toxicity measurements such as death or birth defects. Other effects such as tumor development or reduced reproduction may take a long time to appear or may occur only after chronic exposure. Effects that will result in population level changes are preceded by smaller biochemical and physiological changes. In field studies, it is important to examine these changes as population level effects may be so severe that species may be at risk of decline. These early changes are referred to as biomonitors or bioindicators of toxicity. These changes alone do not necessarily mean that the organism will die or the population will decline but they do indicate an increased risk if the biomonitor has a clear relationship to a higher order effect.

The use of biomonitors has been of special focus at recent scientific meetings (Ecological Relevance of Toxicity Tests and Biomarkers, Second SETAC World Congress, 16th Annual Meeting, November 5-9, 1995) and the need to establish the relationship of these measurements to a defined toxic endpoint has been highlighted (Munkittrick and McCarty, 1995). In particular, biomonitors should be sensitive, relatively inexpensive and reproducible tests. The measurement of biomonitors, in addition to contaminant levels in organisms, may improve the assessment of impact on exposed species (Stein *et al.*,1991). The following factors are important in the use of

biomonitors: (a) it must be possible to measure the responses in indigenous species, (b) concurrent use of several tests should provide a better assessment of toxicity, and (c) further knowledge of the relationship of these outcomes to higher order effects is required to estimate population or community effects (Stein *et al.*,1991).

1.7 *Objectives*

Field studies often measure early biological changes without establishing clear links to higher order effects. This problem can be avoided by either including a wide range of different responses to show a "whole organism response" or by including responses with clearly established relationships to higher order effects. In this study, the latter was chosen.

This study was designed to examine integrated biological responses to organic contaminants in chinook salmon in the upper Fraser River and to attempt to answer the question as to whether pulp mill effluent could be a major contributor to the biological responses that have been found at these sites. This project had of two major components:

1. measurement of biological responses and contaminants in chinook salmon overwintering in the upper Fraser River and its major tributaries;
2. measurement of biological responses and contaminants in chinook salmon exposed to bleached kraft mill effluent in a controlled field study.

2. Materials And Methods

2.1 *Sampling of Juvenile Chinook*

Juvenile chinook salmon (*Oncorhynchus tshawytscha*), 1+ years were killed by a blow to the head, weighed and measured. The liver and gall bladder were removed and frozen in liquid nitrogen for transport. The carcasses were placed on dry ice. Livers, gall bladders and carcasses were stored at -80°C until analyzed.

2.2 *Microsome preparation*

Microsomes were used for ethoxyresorufin-O-deethylase activity and CYP 1A1 density analysis. Microsomes were prepared as described by Hodson *et al.* (1991). Two livers (approximate weight 100 mg each) were pooled for microsome preparation because the livers were small. Liver samples were thawed on ice and homogenized in 4 ml g^{-1} of homogenizing buffer (50 mM Tris[hydroxymethyl]aminomethane hydrochloride(Tris-HCl), pH 7.4 at 4°C; 150 mM potassium chloride, 2 mM ethylenediaminetetraacetic acid (EDTA)). Homogenates were centrifuged at 10 000×g for 20 minutes at 4°C. The supernatant was removed and ultracentrifuged at 100 000×g for 60 minutes at 4°C. The 10 000×g pellet was used for DNA extraction. The 100 000×g supernatant was discarded and the pellet was washed with microsome wash buffer (10 mM EDTA, pH 7.4; 150 mM potassium chloride). The pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.5 at 4°C).

2.3 *Protein Determination*

Protein concentrations were determined by the Bradford assay against a standard curve of bovine serum albumin (BSA) (Bradford, 1976). For microsomal protein concentrations, 5 µl of microsomes were added to 95 µl of water and 5 ml of Bradford reagent. Samples were analyzed on a Perkin Elmer spectrophotometer (Perkin Elmer, Norwalk, CT) at 595 nm against a reagent blank. Samples and 1/3 dilutions were read in duplicate. For bile protein concentrations, 5 µl of diluted sample (1/20) was added to 245

µl of Bradford reagent in a 96 well plate. The plates were shaken and read at 590 nm on a Dynatech MRX plate reader (Dynatech Laboratories, Chantilly, VA) against a reference filter of 405 nm. Samples were analyzed in triplicate.

2.4 *Ethoxyresorufin-O-deethylase (EROD) Activity*

EROD activity is a measure of the catalytic activity of the CYP 1A1 enzyme. It is an indirect measure of CYP 1A1. EROD activity was measured as described by Hodson *et al.* (1991). Briefly, 100 µg of microsomes were added to an incubation mixture containing: 6 mM magnesium chloride, 1.4 g l^{-1} BSA, 88 mM sodium phosphate buffer (pH 7.5 at 4°C) and 24 mM nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was initiated with the addition of 6 µl 7-ethoxyresorufin (in dimethylsulphoxide) to achieve a final concentration of 1.7 µM. The reaction was allowed to proceed for two minutes at room temperature (25°C) and was stopped with the addition of 2.5 ml of cold acetone or methanol. The samples were centrifuged at 3000×g for 5 minutes and the supernatants were read on a Perkin Elmer fluorescence spectrophotometer (Perkin Elmer, Norwalk, CT) at excitation and emission wavelengths of 535 and 585 nm, respectively. Resorufin concentrations in unknowns were calculated based on a standard curve of reaction mixture spiked with known concentrations of resorufin. All samples were analyzed in triplicate with a sample blank. The sample blank was prepared by adding the methanol or acetone before the 7-ethoxyresorufin. EROD activity was expressed as pmol resorufin (mg protein)$^{-1}$ minute^{-1}. The detection limit was 21 pmol mg^{-1} min^{-1}.

2.5 *Western Blotting for CYP 1A1*

Until antibodies against CYP 1A1 were developed, catalytic activity was the only measure of CYP 1A1 induction. With the development of antibodies, a direct measure of CYP 1A1 by western blotting, could be performed. Western blotting for CYP 1A1 was based on the methods of Arlotto and Parkinson (1989).

2.5.1 Antibody Production and Purified 1A1 Standard

Primary antibody was prepared by Dr. S. Bandiera (Department of Pharmaceutical Sciences, UBC). A trout CYP 1A1 oligopeptide identical to a hydrophilic sequence found in the C-terminal half of the trout CYP 1A1 (Myers *et al.*, 1993) and linked to keyhole limpet hemocyanin (KLH) was used to produce a polyclonal antibody in rabbits. The polyclonal antibody was not back absorbed to remove affinity for KLH.

Purified CYP 1A1 standard was prepared by Dr. S. Bandiera (Department of Pharmaceutical Sciences, UBC) from rat liver according to the method of Ryan *et al.* (1982).

2.5.2 Sample Preparation

Samples were diluted with a solution containing a dye (Bromophenol blue) to track the electrophoresis of the proteins and with 2- mercaptoethanol, a reducing agent. Sample diluting buffer was prepared fresh from stock solution (0.062 M Tris, 10% glycerol, 0.001% Bromophenol blue, 1.0% sodium dodecyl sulphate (SDS) v/v/v) and 2-mercaptoethanol at a ratio of 19:1. Microsomes and CYP 1A1 standard were diluted with sufficient sample diluting buffer to yield a concentration of 2.5 $\mu g\ \mu l^{-1}$ and 25 μM, respectively. The preparations were denatured for 3 minutes at 95°C.

2.5.3 Acrylamide Gels

Separating gels (0.375 M Tris, pH 8.8 at room temperature; 7.5% acrylamide:N,N'methylenebisacrylamide (BIS), 0.042% ammonium persulphate, 0.01% SDS, 0.03% N,N,N',N'-tetramethylethylenediamine (TEMED)) were layered with hydrated butanol and allowed to polymerize for a minimum of one hour. TEMED and ammonium persulphate initiate the polymerization of the acrylamide. When polymerized, the butanol was poured off and the gel was washed with separating gel wash buffer (0.68 M Tris, pH 8.8 at room temperature; 0.182% SDS). The separating gel could be stored for up to 48 hours, with buffer on top, before use. The stacking gel (0.125 M Tris, pH 6.8 at room temperature; 3% acrylamide:BIS, 0.08% ammonium persulfate, 0. 1% SDS, 0.05% TEMED) was allowed to polymerize for a minimum of one hour before the combs

were removed and the wells were washed out with stacking gel wash buffer (0.23 M Tris, pH 6.8 at room temperature; 0.182% SDS).

2.5.4 Electrophoresis

Each well was loaded with 20 µl of Pyronin Y (0.165 mM Pyronin Y, 20% glycerol, 0.1% SDS; mixed 1:9 with sample diluting buffer), CYP 1A1 standard or sample. Pyronin Y was loaded in the outside lanes to monitor time required for electrophoresis and the transfer efficiency. Gels were electrophoresed for approximately 60 minutes at 15 mamp per gel (constant current) in electrode buffer (50 mM Tris[hydroxymethyl] aminomethane (Trizma), 6.9 mM SDS, 384 mM glycine). The apparatus was kept in ice during electrophoresis.

2.5.5 Western Blotting

Polyvinyldifluoride (PVDF) membranes were moistened in methanol and water followed by equilibration in transfer buffer (24.7 mM Trizma, 128 mM glycine, 0.35 mM SDS, 20% methanol) for one hour. When electrophoresis was complete, the stacking gel was discarded and the membrane was placed on the back of the separating gel in a sandwich assembly. Proteins were transferred at 100 volts (constant voltage) for one hour in cold transfer buffer.

2.5.6 Visualization of Bands

After transfer, the membranes were placed in membrane blocking buffer (5% Carnation non-fat milk powder, 50 mM sodium chloride, 10 mM Tris, pH 7.4; 1 mM EDTA) for two hours. The membranes were incubated in antibodies diluted in antibody diluting buffer (300 mM sodium chloride, 5 mM potassium chloride, 16 mM sodium phosphate, 3 mM potassium phosphate, 0.4 mM EDTA, pH 7.4; 20 g l^{-1} BSA, 0.05% polyoxyethylenesorbitan monolaurate (Tween 20), 5% goat serum). Between each antibody incubation, membranes were washed with wash buffer (0.05% Tween 20, 0.26 M sodium chloride, 4.75 mM potassium chloride, 15 mM sodium phosphate, 0.3 mM potassium phosphate, 0.04 mM EDTA, pH 7.4) and placed in blocking buffer. The antibodies and incubation time are as follows:

Antibody	Concentration	Incubation Time
1. Primary Ab	0.63 μg ml^{-1}	overnight
2. goat *anti*-rabbit IgG	2.00 μg ml^{-1}	4 hours
3. rabbit peroxidase *anti*-peroxidase	2.65 μg ml^{-1}	2 hours

The primary antibody recognizes CYP 1A1 and the secondary antibody, which is linked to a horseradish peroxidase enzyme , recognizes the primary antibody. The tertiary antibody recognizes the peroxidase enzyme and increases the signal to noise ratio. All incubations were performed at room temperature. After the third incubation, the membranes were washed with wash buffer and incubated with colour developer (4-chloro-1-napthol membrane peroxidase substrate kit) for 4.5 to 5.5 minutes. The membranes were air dried overnight and stored in foil until densitometry could be performed.

2.5.7 Densitometry

Densitometry was performed using a PDI 4oe densitometer (PDI, Huntington Station, New York). Bands were analyzed with Quantity One, version 2.6 (PDI, Huntington Station, New York) for area under the curve. The data is in average reflective density units (RD) and are converted to relative RD by expressing each band as a fraction of the purified CYP 1A1 standard (0.5 pmoles). All analyses were performed in triplicate and averaged for each sample. The average detection limit was 0.1 RD.

2.6 *Contaminant Analyses*

2.6.1 Carcass Analyses

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), mono-ortho polychlorinated biphenyls (mo-PCBs) and coplanar polychlorinated biphenyls (co-PCBs) were analyzed by the Regional Dioxin Laboratory (Department of Fisheries and Oceans, Sidney BC) according to MacDonald *et al.* (1996) and Rantalainen *et al.* (1996). Whole carcasses, minus the liver and gall bladder, were pooled to ≈30 g for high resolution gas chromatography mass spectrometry (HRGC/HRMS) analysis. Samples were analyzed with a procedural blank and a certified reference material standard.

One sample was analyzed in duplicate for quality control measures. Percent moisture and percent lipid were determined for each pool analyzed. Concentrations were reported in pg g^{-1} and had been corrected for the percent recovery of the reference standard. The non-detectable samples were set to the detection limit. In a few samples, the 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD) congener was found to be unusually high due to glass contamination (M. Ikonomou, personal communication) and these were set at the average OCDD value. The detection limits for PCDDs and PCDFs ranged from 0.06-0.14 and 0.05-0.12 pg g^{-1}, respectively. The detection limits for mo-PCBs and co-PCBs ranged from 0.01-0.14 and 0.04-0.20 pg g^{-1}, respectively.

Contaminants are expressed as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent (pg/g wet weight) based on toxic equivalent factors (TEFs) from Ahlborg *et al.* (1994) and NATO-CCMS (1988). Worst case contaminant values were calculated to include all PCBs measured, with TEFs of the most toxic PCB of each class (mono-ortho substituted or coplanar) used as surrogate TEFs. Toxic equivalent quotients (TEQs) were calculated for each sample by summing the concentration of each congener (in TCDD equivalents).

2.6.2 Bile Analyses

Bile analyses for PAH metabolites were performed by Curtis Eickhoff (Environmental Toxicology Program, Simon Fraser University). Bile was analyzed using synchronous scan fluorimetry (SFS) for 1-hydroxypyrenol according to Ariese *et al.* (1993), and converted to ng pyrene-1-glucuronide (ml bile)$^{-1}$. The detection limit was 760 ng ml^{-1}.

2.7 *^{32}P-Postlabeling for DNA Adducts*

^{32}P-postlabeling for DNA adducts is a measure of total adducts. These methods are preferential for large, hydrophobic adducts. DNA is enzymatically hydrolyzed and the normal nucleotides are modified to prevent labeling. The DNA is enzymatically labeled with ^{32}P-ATP and spotted onto a thin layer chromatography plate. The plate is developed in two directions and the amount of radioactivity in the adduct zone is quantified. The DNA adduct concentrations were determined from DNA extracted from liver tissue. The

methods outlined below were from Reichert and French (1994). Each segment of the assay is briefly explained below.

2.7.1 Preparation of Thin Layer Chromatography (TLC) Plates

A 0.75% polyethyleneimine (PEI) - cellulose slurry, pH 6.0, was spread to 4 mm thick with a Desaga Plate Spreader (Desaga, Heidelberg, Germany) on a matte plastic sheet and dried overnight at room temperature. The sheets were cut and developed overnight with distilled water. The sheets were cut to 16 × 20 cm dimensions and dried for 1 to 2 hours. The sheets were stored at -20°C.

2.7.2 [γ-^{32}P] Adenosine Triphosphate (ATP)

[γ-^{32}P]ATP (50 mCi ml^{-1}) was purchased with a specific activity of 3000 Ci (mmol ATP)$^{-1}$. It was diluted to 33 mCi ml^{-1} with kinase buffer (0.1 M bicine, 0.1 M magnesium chloride, 0.1 M dithiothreitol, 10 mM spermidine pH 9.0). The purity of the ATP was verified by spotting 0.1 - 0.3 µl of ATP on a TLC plate and developing in 1 M lithium chloride. The plate was dried and exposed to film for 10 seconds. A single spot was visible on the film, with little tailing.

2.7.3 Determination of [γ-^{32}P]ATP Specific Activity

The specific activity of the [γ-^{32}P]ATP was used for adduct determination (see 2.7.13). The specific activity was determined by labeling a known amount of 2'-deoxyadenosine-3'-monophosphate (2'-dA-3'MP) with [γ-^{32}P]ATP. A labeling mix (6.5 × 10^{-13} moles of 2'-dA-3'MP, 20.6 µCi of [γ-^{32}P]ATP, 6.25 U polynucleotide kinase (PNK), 14 mM bicine, 14 mM dithiothreitol, 14 mM magnesium chloride and 1.4 mM spermidine, pH 9.0) was incubated for 45 minutes at 37°C. This mixture was diluted 27-fold with water and 10 µl was spotted on a TLC plate. The plates were developed in bases buffer (0.24 M ammonium sulphate, 8 mM sodium phosphate, pH 7.4) and dried. The plates were exposed to film for 30 minutes to 1 hour at room temperature and to phosphor screens for 5 minutes. The 3',(^{32}P)5'-deoxyadenosine bisphosphate spot (3', (^{32}P)5'-dA-

BP) was located by autoradiography and quantified by storage phosphor imaging. The specific activity was determined according to equation 1.

$$\frac{(\text{dpm of } 3',(^{32}P)5'\text{-dA-BP}) \times (\text{spotting aliquot factor})}{\text{moles of } 2'\text{-dA-}3'\text{MP available for}}$$

Equation 1 Specific Activity of [^{32}P]ATP

2.7.4 DNA isolation

DNA was isolated from the 10 000×g pellet formed during microsome preparation (see Microsome preparation). This pellet contains the nuclei and cellular debris. The pellet was resuspended in 850 µl of 1% SDS/20 mM EDTA (pH 7.4) and incubated with 4 µl of a RNAse/amylase solution (5 µg µl^{-1} α-amylase, 5 µg µl^{-1} RNAse A, 5 U µl^{-1} RNAse T_1, 25 mM Tris-HCl, pH 7.4) for 30 minutes at 37°C. Four microlitres of a 0.04 mg µl^{-1} proteinase K/2 M Tris-HCl (pH 7.4) solution was added and the samples were incubated for 30 minutes at 37°C with occasional mixing. Proteins were removed by sequential organic solvent extractions with 1 volume of phenol, phenol:chloroform:isoamyl alcohol (25:24:1) (twice), and chloroform:isoamyl alcohol (24:1) (twice). The aqueous phase was mixed with 0.1 volumes of 5 M sodium chloride. One volume of cold (-20°C) 95% ethanol was added to precipitate the DNA. The sample was centrifuged at ≈10 000 ×g for 5 minutes to pellet the DNA and the supernatant was removed with a pipette. The DNA was washed with 70% ethanol and the residual ethanol was removed. The DNA was resuspended in 50 µl of 10 mM Tris, 1 mM EDTA (pH 7.4) (TE). Because salmon DNA is particularly hard to dissolve, the sample was left for several days at 5°C and then resuspended by pipetting.

2.7.5 Protein and RNA Contamination

A 1/10 dilution and 1/100 dilution was prepared for verification of RNA and protein contamination. The 1/10 dilution was mixed with loading dye (5µl DNA: 1µl loading dye) and run on a 1% agarose gel with ethidium bromide. The spots were made

visible under UV light. The DNA should be large strand DNA with little shearing and there should be very little RNA contamination (seen as a spot at the bottom of the gel). The 1/100 dilution was measured on a spectrophotometer against TE at 280, 260 and 230 nm. The ratios of absorbances at 260/230 and 260/280 should be greater than 2.3 and 1.8, respectively to demonstrate absence of protein contamination. The DNA concentration was determined from the absorbance at 260 nm.

2.7.6 Enzymatic Hydrolysis of DNA and Enrichment of Adducts

DNA samples were hydrolyzed to single nucleotides for labeling. An enzyme incubation mixture was prepared with 25 μg of DNA, 0.8 μg μl^{-1} spleen phosphodiesterase, 0.2 μg μl^{-1} micrococcal endonuclease, 4 mM sodium succinate and 2 mM calcium chloride (pH 6.0). This mixture was incubated at 37°C for 3 hours with mixing every hour. After the three hours, 5 μl was diluted to 500 μl with water for the analysis of bases (see Bases) and 10 μl removed for adduct determination. Salmon sperm DNA was analyzed with the samples as a negative control and one sample from each run was performed in duplicate for quality control.

Normal nucleotides were enzymatically modified to prevent them from being labeled. A nuclease P_1 incubation mixture containing 0.5 μg μl^{-1} of nuclease P_1, 0.05 M sodium acetate (pH 5.0), 0.24 M zinc chloride and 10 μl of enzyme hydrolysate was placed in a 37°C water bath for 45 minutes. Three microlitres of 0.5 M Tris (pH 9.6) was added to raise the pH of the mixture for labeling. Duplicates of 1.5 and 3 fmol of *7R*,8*S*, 9*S*-trihydroxy-10*R*-(N^2-deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene were prepared as standards. These standards are benzo[*a*]pyrene diolepoxide molecules adducted to guanosine and gives information on efficiency of labeling between runs. A labeling mixture with the enriched hydrolysate or standard, 100 μCi of [γ^{32}P]ATP, 8 U PNK, 22.5 mM bicine, 22.5 mM magnesium chloride, 22.5 mM dithiothreitol and 2.25 mM spermidine (pH 9.0) was incubated at 37°C for 45 minutes.

2.7.7 Chromatography of Adducts

The labeled DNA was spotted (20μl) onto a TLC plate (2 samples per plate) with a wick (Whatman no. 17 CHR paper) attached to the end nearest the origin. It was placed in 1.0 M sodium phosphate (pH 6.0) for development in D1 (Figure 4) overnight to remove normal nucleotides and nonspecific radioactivity from the plate. The plates were removed and the wick cut off. Between each chromatography step the plates were rinsed to remove all chromatography solvent and cut, if necessary. The plates were completely dried before the next step. The edge was wetted with distilled water and the plates were placed in 7.2 M urea, 3.8 M lithium formate (pH 3.5) for development in the D3 direction (Figure 4). After D3 had run to the top, the plates the plates were cut in half, rinsed and dried. The edge was dipped in D4 dip (0.05 M Tris, 0.8 M lithium chloride, pH 8.0) and the plates were placed in 7.2 M urea, 1.5 M lithium chloride, 0.5 M Tris (pH 8.0) for development in the D4 direction (Figure 4). The plates were cut 1 cm from the top and side, at the origin marks and at the corner of the origin. The plates were taped face down on paper for exposure to film and phosphor screen.

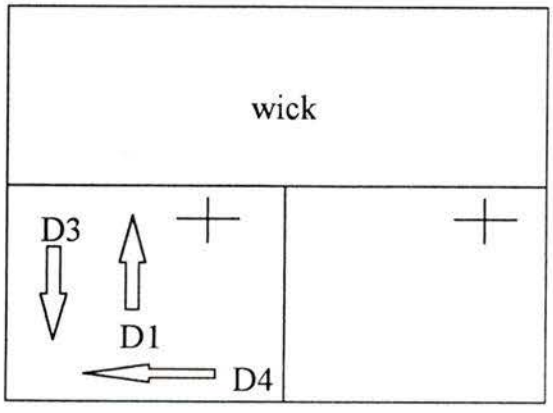

Figure 4 Thin Layer Chromatography Sheet

The cross indicates the origin, where samples were spotted. The arrows indicate the direction of each chromatography development. The wick was attached for D1 only and then removed.

2.7.8 Exposure of Plates to Film and Phosphor Screens

The plates were exposed to film for 24 and ≈72 hours at -80°C. Calibration strips were prepared by spotting diluted labeling mix onto small squares of TLC plates in volumes equivalent to 150, 300, 450 and 600 dpm. One set of calibration strips (150-600 dpm) was taped down to each sheet of plates. The plates were exposed to phosphor screens for 24 hours at room temperature.

2.7.9 Bases

Bases analysis determines the total number of bases in the enzyme hydrolysate. This is calculated in Equation 3. Base analyses were performed on the diluted enzyme hydrolysate (see Enzymatic Hydrolysis of DNA and Adduct Enrichment). An incubation mixture containing 10 µl of diluted enzyme hydrolysate, 8 U PNK, 1.25 µCi [γ^{32}P]ATP, 1.1×10^{-4} M ATP, 36.5 mM bicine, 36.5 mM magnesium chloride, 36.5 mM dithiothreitol and 3.65 mM spermidine (pH 9.0) was incubated at 37°C for 45 minutes. Ten microlitres of each sample was spotted on a TLC plate and developed in bases buffer. The sheets were dried and exposed to film for 30 minutes and to phosphor screens for 5 minutes at room temperature.

2.7.10 Bases Specific Activity

The bases specific activity is used for bases determination (2.7.12). An aliquot (≈0.2 µl) of the labeling mix (8 U PNK, 1.25 µCi [γ^{32}P]ATP, 2.2×10^{-4} M ATP, 73 mM bicine, 73 mM magnesium chloride, 73 mM dithiothreitol and 7.3 mM spermidine, pH 9.0) was spotted onto a TLC plate and developed in 1M lithium chloride. There should be one major spot on the plate due to [γ^{32}P]ATP. Any other spot or tailing is due to breakdown of [γ^{32}P]ATP. The two spots are analyzed by phosphor imaging and percentage of radioactivity due to [γ^{32}P] is calculated. Five microlitres of the labeling mix was placed in 5 ml of scintillation fluor and counted on a scintillation counter. The specific activity was determined according to Equation 2.

$$\frac{[(\%\ \text{radioactivity due to } [^{32}P]ATP) \times (\text{average dpm of 5 } \mu l \text{ aliquot}) \times (\text{spotting aliquot factor})]}{\text{concentration of ATP in the aliquot}}$$

Equation 2 Bases Specific Activity

2.7.11 Phosphor Imaging

The phosphor screens (Molecular Dynamics, Sunnyvale, CA) were prepared by exposing them to the Image Eraser (Molecular Dynamics, Sunnyvale, CA) for 6 minutes. The screens were then exposed to the sheets as outlined in the appropriate section above. The phosphor imager (Molecular Dynamics, Sunnyvale, CA) read the screens at 176 μ pixel^{-1}. The images were analyzed with Image Quant, version 3.3 (Molecular Dynamics, Sunnyvale, CA). The prepared calibration strips or 4 excised spots were placed in scintillation vials with ≈10 ml scintillation and read on a scintillation counter for 10 minutes per vial. For each sheet, the correction factor was determined: (phosphor imaging number/cpm in the vial); averaged for the four strips. This number was used to convert the phosphor imager number into cpm.

2.7.12 Bases Determination

The base analyses were used to determine the total amount of DNA analyzed for adducts. One clear base spot was used (the adenosine, cytosine combined spot or the thymine spot) for quantitation. The total amount of DNA analyzed is determined according to Equation 3.

$$\frac{[(\text{dpm of the base spot}) \times (100\% / \%\ \text{base in DNA}) \times (\text{spotting aliquot factor}) \times (\text{dilution factor for enzyme hydrolysate})]}{\text{specific activity of bases labeling mix}}$$

Equation 3 Total Amount of DNA

2.7.13 Adduct Determination

The adducts form a diagonal zone on the TLC plate. This whole zone was analyzed with the Image Quant software. The adducts (fmol) in each sample was determined according to Equation 4.

$$\frac{\text{(dpm for the DNA adduct zone)} \times \text{(spotting aliquot factor)}}{\text{specific activity of } [^{32}P]ATP}$$

Equation 4 DNA Adducts in Sample of DNA

The DNA damage is expressed as nmol adducts (mol DNA)$^{-1}$. The detection limit was 1 - 2.5 nmol (mol DNA)$^{-1}$.

2.8 *Histopathology for Lesions*

Histopathology slides were prepared by Dave Barnes, DFO, Cultus Lake Laboratories (Cultus Lake, B.C.). Livers were preserved in Bouin's fixative for histological preparation. They were embedded in paraffin, sectioned at 7 μm and stained with hematoxyin and eosin. Sections were examined by Dawna Brand (Shawnigan Lake, BC) using a light microscope.

2.9 *Chemicals*

BSA, NADPH TEMED, ammonium persulphate, acrylamide, RNase A, RNase T_1, amylase, PEI, micrococcal endonuclease, spleen phosphodiesterase, nuclease P_1, salmon testes DNA, 2'-dA-3'mp, and ATP were from Sigma (St. Louis, MO). Coomassie Blue was from Eastman Kodak (Rochester, NY). Resorufin and 7-ethoxyresorufin were from Molecular Probes (Eugene, OR). BIS was from BDH (Poole, England). PVDF membranes were from BioRad (Hercules, CA) or Millipore (Bedford, MA). Antibodies were from Cappell Research Products (Durham, NC) and 4-chloro-1-naphthol colour development kits were from Kirkegaard and Perry Laboratories (Gaithersburg, MA). Proteinase K was from Life Technologies (Gaithersburg, MA). Cellulose was from Machery Nagel (Düren, Germany). [γ-^{32}P]ATP was from New England Nuclear Products

(Wilmington, DE) and *7R*,8*S*, 9*S*-trihydroxy-10*R*-(N^2-deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene was from Midwest Research Institute (Kansas City, MI). PNK was from US Biochemical (Clevland, OH). All other chemicals were reagent grade or better.

3. Juvenile Chinook in the Upper Fraser River

3.1 *Abstract*

Juvenile chinook salmon (*Oncorhynchus tshawytscha*) were captured at 7 sites on the upper Fraser, Nechako and Thompson Rivers. Biological responses, including ethoxyresorufin-O-deethylase (EROD) activity, CYP 1A1 density, DNA adduct concentration and histopathology in liver tissue, were measured to assess the effects of contaminants on the fish before they began migration downstream. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) were measured in the carcasses. Polyaromatic hydrocarbon (PAH) metabolites were measured in the bile. There were strong correlations between the biological responses, except for those determined for liver histopathology. There was no clear correlation between these responses and contaminant concentrations. Significant increases in EROD activity, CYP 1A1 density and DNA adduct concentrations compared to those at control sites indicate that these fish may be at risk for contaminant toxicity. The contaminants responsible for these effects are not apparent although polyaromatic hydrocarbons are suspected.

3.2 *Introduction*

The upper Fraser River and its tributaries are spawning grounds and overwintering sites for chinook salmon. Recent declines in stocks of this species have been attributed to overfishing and habitat degeneration (Slaney *et al.*, 1996). Overwintering juveniles may be affected by contaminants from industrial and municipal discharge (Servizi *et al.*, 1993) which may result in decreased survival through smoltification. The chinook in the upper Fraser River watershed spend over one year in the river system before migrating downstream to smolt. This is therefore a good target species in which to investigate contaminant toxicity, since fish have been exposed to contaminants for a significant length of time and throughout early development.

Several bleached kraft pulp mills and municipal sewage outfalls discharge directly into the mainstem Fraser River. Other industrial discharges, including wood processing

plants, discharge into the Fraser River's main tributaries. Several biological responses were chosen to assess the effect of contaminants on juvenile chinook just before migration. The responses were chosen to assess both exposure to contaminants and impact of the exposure.

Ethoxyresorufin-O-deethylase (EROD) activity and CYP 1A1 induction have been used as biological monitoring tools in a number of studies. They are effective biomonitors of exposure to organic contaminants. Their combined use is particularly effective to overcome the particular difficulties in each measurement. EROD activity may be reduced due to improper handling of liver tissue (Hodson, *et al.*, 1991) or due to enzyme inhibition after exposure to complex mixtures (Tysklind *et al.*, 1995). CYP 1A1 measurement was developed to overcome the limitations of the EROD assay but methods are not yet standardized between laboratories and results are therefore difficult to compare to other studies. The two assays measure different levels of the cytochrome P450 1A1 response and thus their integrated use is preferable when exposure to complex mixtures occurs. Since this system is induced with exposure to certain chemical classes, the induction of EROD activity or CYP 1A1 density can be used as a bioassay of exposure to organic contaminants. The toxicological implications of this response are unclear and thus it is important to relate the induction of this enzyme to other biological responses with established relationships to higher order effects.

Measurement of DNA adducts by the ^{32}P-postlabeling assay is a useful tool to assess PAH exposure and toxicity. This method is particularly useful for complex chemical exposure as it integrates all adducts into one measurement. PAH-DNA adducts are formed by binding of reactive intermediates, created during metabolism by cytochrome P450 1A1, to DNA and other macromolecules in the cell. DNA adduct concentrations have been correlated to enzyme activities similar to EROD in field studies with English sole (*Parophrys vetulus*) (Collier, *et al.*, 1996), which indicates that the combined use of these measurements is useful. The presence of DNA adducts has been linked to neoplasia and tumour growth in English sole (Collier *et al.*, 1996).

3.3 *Materials and Methods*

Juvenile (1+years) chinook salmon (*O. tshawytscha*) were captured by electrofishing at several sites on the upper Fraser, Thompson and Nechako Rivers. They were killed by a blow to the head, weighed and measured. The liver and gall bladder were removed and frozen in liquid nitrogen for transport. The carcasses were placed on dry ice. Livers for histopathology were dissected and placed in Bouin's fixative. Livers, gall bladders and carcasses were stored at -80°C until analyzed.

Microsomes were prepared from pools of two frozen liver samples according to Hodson *et al.* (1991). Microsomes were analyzed for protein concentration, EROD activity and CYP 1A1 density. Protein concentrations were determined by the method of Bradford (1976). EROD activity was determined by the methods of Hodson *et al.* (1991). CYP 1A1 density was determined by western blotting as described by Arlotto and Parkinson (1989). CYP 1A1 density was expressed in reflective density units, as a ratio to the 1A1 standard. An average reflective density is calculated by densitometry for all bands on a particular membrane and then each unknown band is expressed in terms of the standard from that membrane. This helps to remove day to day variation in the intensity of the bands.

DNA adducts were analyzed according to Reichert and French (1994). Liver samples were homogenized and centrifuged for postmitochondrial supernatant (to prepare microsomes) and the pellet was removed for DNA extraction. The adducts were enriched by the nuclease P1 method. Adduct concentrations were determined using phosphor imaging techniques.

Livers for histopathology were embedded in paraffin, sectioned at 7 μm and stained with hematoxyin and eosin. Histopathology examinations were done using a light microscope.

Polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls were analyzed in the carcasses by high resolution gas chromatography/mass spectrometry according to MacDonald *et al.* (1996) and Rantalainen *et al.* (1996). Two pools per site were analyzed. Bile sacs were centrifuged to break them open and the bile was removed for analysis. Polycyclic aromatic

hydrocarbon metabolites were analyzed by synchronous scan fluorimetry for 1-hydroxypyrenol equivalents according to Ariese *et al.* (1993). Protein concentrations in the bile were analyzed using a modified Bradford assay (Bradford, 1976).

All statistical analyses were performed using Statistica 5.1 (Statsoft, Tulsa OK). Differences between site means were analyzed using ANOVA and Fisher's least significant difference test, when Levine's test for homogeneity of variance was non-significant and examination of mean versus standard deviation plots showed no significant relationship. Pearson's correlations were used to examine relationships between variables. Data are reported as mean ± standard deviation.

3.4 *Study Sites*

Juvenile chinook salmon were captured at several sites on the upper Fraser, Thompson and Nechako Rivers during March and April of 1995. The Thompson and Nechako Rivers are major tributaries of the upper Fraser River. The Nechako River joins the Fraser River below Prince George, while the Thompson River joins the Fraser at Lytton.

The Nechako River has no known source of contamination and these sites have been found to be low in contaminants in previous studies (R. Gordon and D. Martens, personal communication). There are three bleached kraft pulp mills at Prince George, all of which discharge to the Fraser above the confluence of the Nechako. There is a sewage outfall from the city which enters below the confluence. There is one bleached kraft mill at Quesnel, along with a number of wood processing plants and a sewage outfall. The sewage and pulp mill effluent are discharged directly into the Fraser River. There is a bleached kraft mill at Kamloops, upstream of the study site, but the effluent travels through Kamloops Lake before it enters the Thompson River.

In March, fish from the Thompson River were caught at Spences Bridge. In April, fish were caught at the remaining sites. Control fish from the Nechako River were caught at Swanson's Creek and near Fort Fraser. On the upper Fraser River, fish were caught just below the diffuser at Northwoods Pulp Mill, Prince George; at Stoner and Longbar above

Quesnel and at Soda Creek, below Quesnel. The fish at the Northwoods site are estimated to be in 12-14% (volume / volume) effluent based on conductivity measurements (G. Kruzynski, personal communication). Water samples were not analyzed in this field trip. Figure 5 shows the study sites.

The delay in sampling between the Thompson and the other sites was due to the delay in ice break-up on the river of the more northern sites. Ice must clear from the sides of the river as chinook habitat is limited to the shallow waters where the flow is slower. An effort was made to collect 20 fish per site, although this was not possible at all sites.

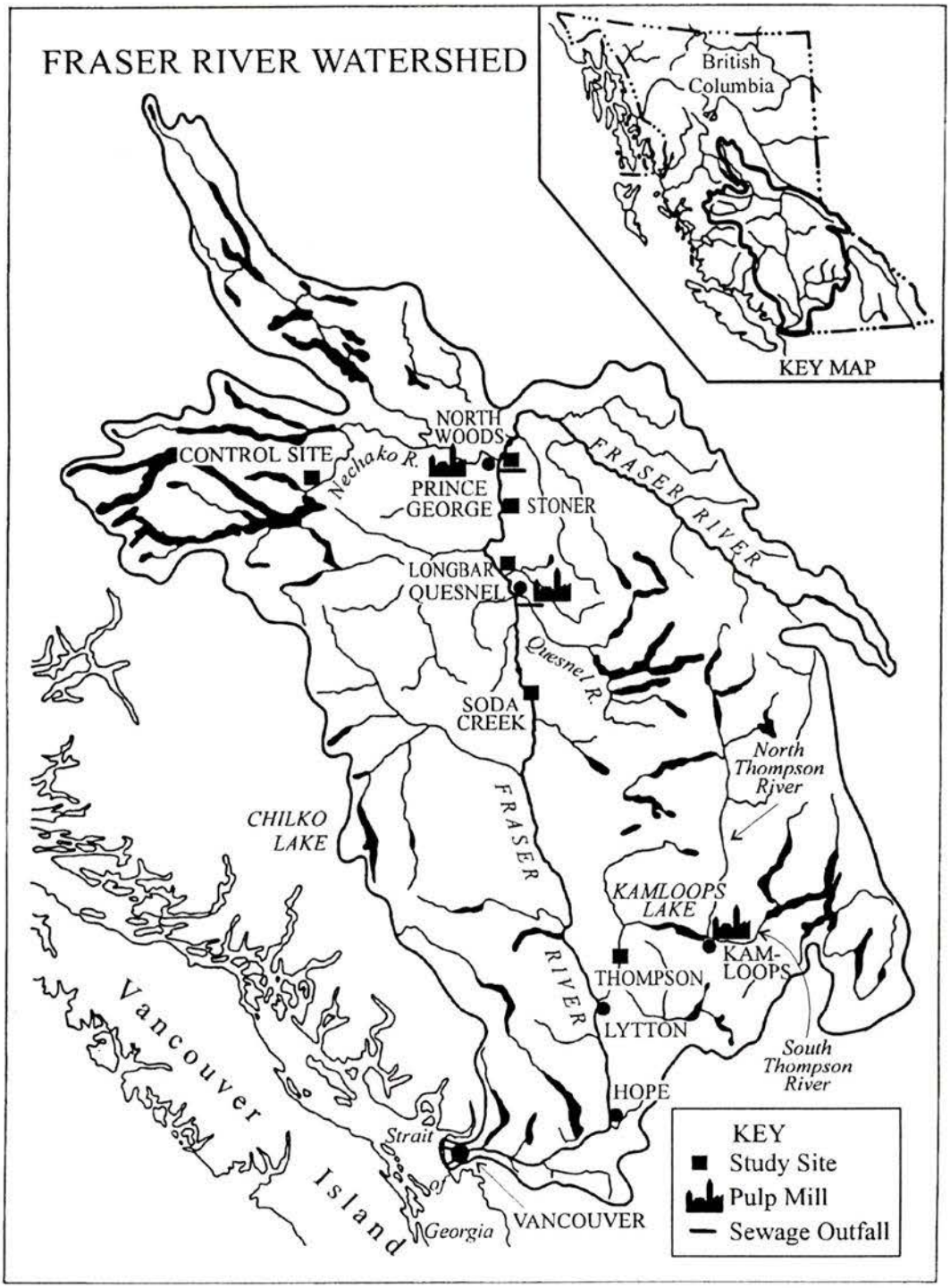

Figure 5 The Fraser Watershed

3.5 *Results*

3.5.1 General Characteristics

The juvenile chinook were approximately 18 months of age. Fish this age are easily differentiated from the younger age class (6 months) by length (≈8 cm vs 3-4 cm) and weight (≈7 grams versus 1-3 grams). The fish collected for this study had a mean fork length and weight of 8.19 cm and 6.94 grams, respectively. Table 1 shows the lengths and weights at all sites. The length data were normally distributed (Shapiro Wilk W test) but the weight data were not (P=0.02). There was a significant difference between the mean weights of the two Nechako collections (Swanson's Creek and Fort Fraser), which is a contributing factor to the non-normal distribution. However, even with the Fort Fraser collection (Nechako-2) is removed, the distribution remained non-normal (P=0.04). There were significant differences in the length and weight between sites. As seen in Table 2, which lists the P values based on a MANOVA test, most sites were statistically different in both length and weight. Correlations were performed on all biological response measurements with respect to these two variables to ensure that differences in response between sites were not due to differences in length or weight. There was no correlation between either mean length or weight and any other measurement. The differences in length and weight do not appear to be large enough to affect the biological responses or the contaminants load. The fish were not mature enough to be separated by sex via gonadal development. A few individuals had internal parasites, but none exhibited any difference in organ appearance, weight or length. The fish caught from the Nechako River near Fort Fraser exhibited the largest number of internal parasites. The parasites were not kept for identification. Several fish from Soda Creek had an increase in mesenteric fat. In previous years' sampling, increased mesenteric fat was associated with pale, mushy livers, and increased weight, and the occasional fin clip (R. Gordon, personal communication). These fish were thought to be from a hatchery due to their different PCB content which probably came from contaminated feed (R. Gordon, personal communication). The fish

caught in 1995 had no difference in liver appearance and there was no relationship to increased weight in these fish. No fin clips were found on any captured fish.

Table 1 Mean Lengths and Weights for Juvenile Chinook Salmon from the upper Fraser River

Site	Length (cm)	Weight (g)
Nechako River -1 (n=11)	8.68 (0.52)	7.67 (1.13)
-2 (n=17)	9.53 (0.62)	10.6 7 (1.64)
-combined (n=28	9.20 (0.71)	9.49 (2.07)
Northwoods (n=16)	6.79 (1.02)	3.68 (1.72)
Stoner (n=20)	7.16 (0.71)	4.66 (1.55)
Longbar (n=20)	7.71 (0.55)	5.99 (1.20)
Soda Creek (n=20)	8.66 (0.74)	8.78 (2.30)
Thompson River (n=20)	8.96 (0.80)	7.34 (1.86)
All Sites (n=124)	8.19 (1.17)	6.94 (2.77)

Data are reported as means with standard deviation in brackets. Nechako 1 and 2 include fish caught on Swanson's Creek and Fort Fraser, respectively.

Table 2 Significant Differences Between Sites for Length and Weight

p Values for Differences among sites for Length							
	Nechako-1	Nechako-2	Northwoods	Stoner	Longbar	Soda Creek	Thompson
Nechako-1	1	0.003	<0.001	<0.001	<0.001	NS	NS
Nechako-2		1	<0.001	<0.001	<0.001	<0.001	0.02
Northwoods			1	NS	<0.001	<0.001	<0.001
Stoner				1	0.02	<0.001	<0.001
Longbar					1	<0.001	<0.001
Soda Creek						1	NS
Thompson River							1
p Values for Differences among sites for Weight							
	Nechako-1	Nechako-2	Northwoods	Stoner	Longbar	Soda Creek	Thompson
Nechako-1	1	<0.001	<0.001	<0.001	0.01	NS	NS
Nechako-2		1	<0.001	<0.001	<0.001	<0.001	<0.001
Northwoods			1	NS	<0.001	<0.001	<0.001
Stoner				1	0.01	<0.001	<0.001
Longbar					1	<0.001	0.014
Soda Creek						1	0.008
Thompson River							1

P values were determined by MANOVA analysis. NS=not significant, $P>0.05$. Nechako 1 and 2 include fish caught on Swanson's Creek and Fort Fraser, respectively.

3.5.2 Ethoxyresorufin-O-deethylase (EROD) Activity

The non-transformed EROD data were not distributed normally, $P<0.001$ (Shapiro-Wilks W test), and thus for all analyses the log transformed data were used. The mean EROD activity (non-transformed data) is shown in Figure 6. There was no statistical difference between the two collections from the Nechako River and thus they were combined for further analyses. The Nechako River fish had a mean EROD activity of 24.1 ±11.0 pmol (mg protein)$^{-1}$ minute^{-1}. Fish from the Northwoods and Thompson River sites had EROD activities of 33.3±13.8 and 26.5±10.7 respectively, not significantly greater than those from the Nechako River site. Fish from Northwoods were significantly different from Soda Creek and Longbar. Fish from the Thompson River were significantly different from Stoner, Longbar and Soda Creek. Fish from Stoner (56.6±32.6), Longbar (59.2±36.0) and Soda Creek (73.1±29.0) had EROD activities significantly greater than those from the Nechako site. There was no difference between these three sites.

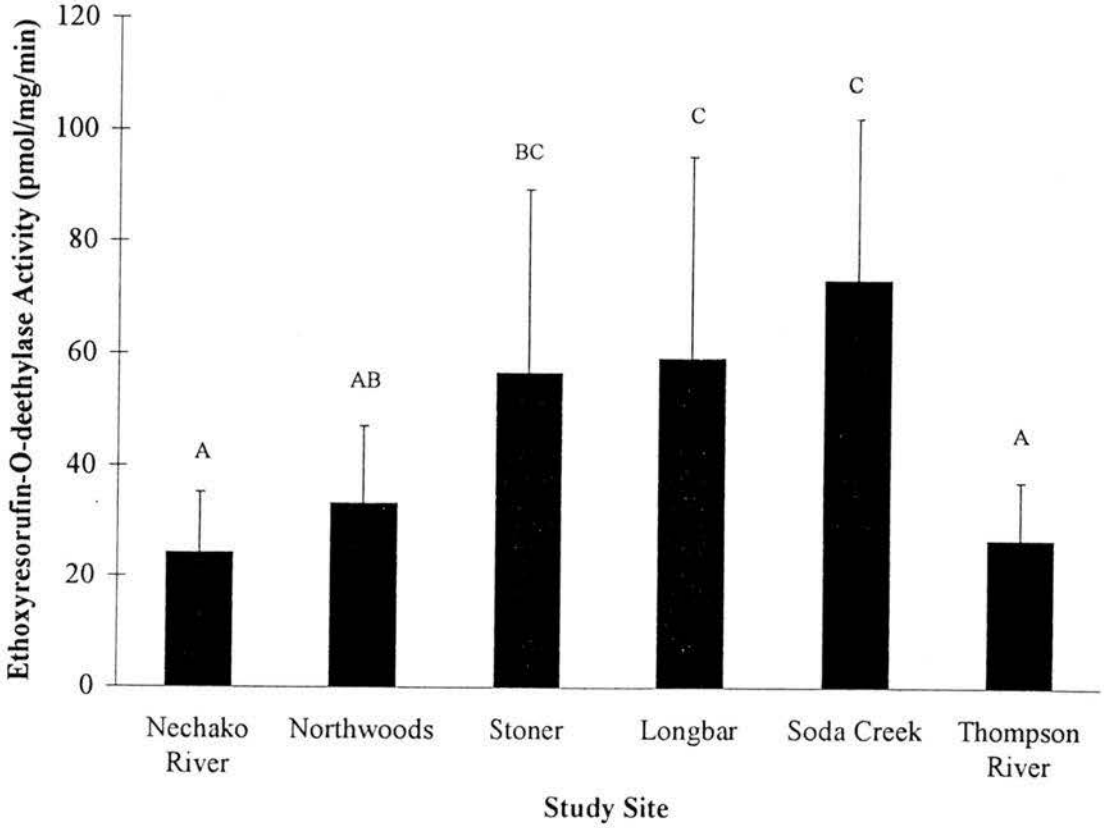

Figure 6 Ethoxyresorufin-O-deethylase Activity in Livers of Juvenile Chinook from the upper Fraser River

Data shown are untransformed; log transformed data were used for analyses. Error bars represent the standard deviation. Means with different letters differ significantly, $P<0.05$.

3.5.3 CYP 1A1 Density

The non-transformed CYP 1A1 data were also not normally distributed, $P<0.01$ (Shapiro-Wilks W test), and thus for all analyses the log transformed data were used. The mean CYP 1A1 data (non-transformed) are shown in Figure 7. These data are expressed as a ratio of the unknown sample to 0.5 pmol CYP 1A1 standard, in reflective density units (the data are not expressed in pmoles because there may be a difference in the affinity of the primary antibody for the standard and the unknowns, as the protein is derived from different species). Fish from the Nechako River, with a mean reflective density of 1.03±0.38, were found to be significantly different from all other sites, $P<0.001$. Fish from Northwoods (2.24 ±1.26) and Thompson River (2.02 ±0.83), were found to be significantly different from those from Stoner and Longbar with P values of <0.02 and <0.01, respectively. There was no difference between Stoner (3.50 ±1.26), Longbar (3.12 ±0.84), and Soda Creek (2.79 ±1.24).

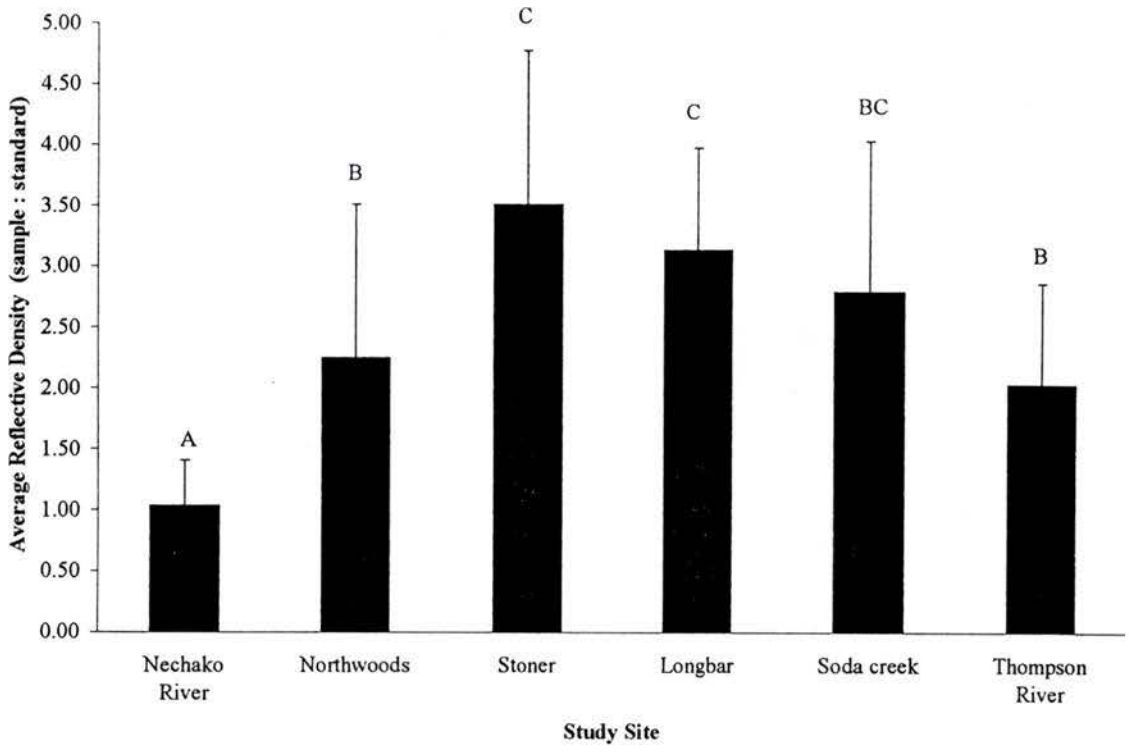

Figure 7 CYP 1A1 Density in Livers of Juvenile Chinook from the upper Fraser River
Data shown are untransformed; log transformed data were used for analyses. Error bars represent the standard deviation. Means with different letters differ significantly, $P<0.05$.

3.5.4 DNA Adducts

The non-transformed DNA adduct concentrations were not normally distributed, $P<0.01$ (Shapiro-Wilks W test), and thus for all analyses the log transformed data were used. The mean DNA adduct concentrations (non-transformed) are shown in Figure 8. The mean concentration at the Nechako River was 4.57±2.85 nmol (mol nucleotides)$^{-1}$. This site was significantly different from all other sites at a P value <0.05. There were significant differences between fish from Thompson (7.41 ±4.25), Stoner (16.49 ±13.24) and Longbar (12.94 ±6.60). Fish from Northwoods and Soda Creek had 9.49±5.45 and 9.52±3.88 nmol adducts (mol nucleotides)$^{-1}$, respectively. There were no differences between Northwoods, Stoner, Soda Creek and Longbar.

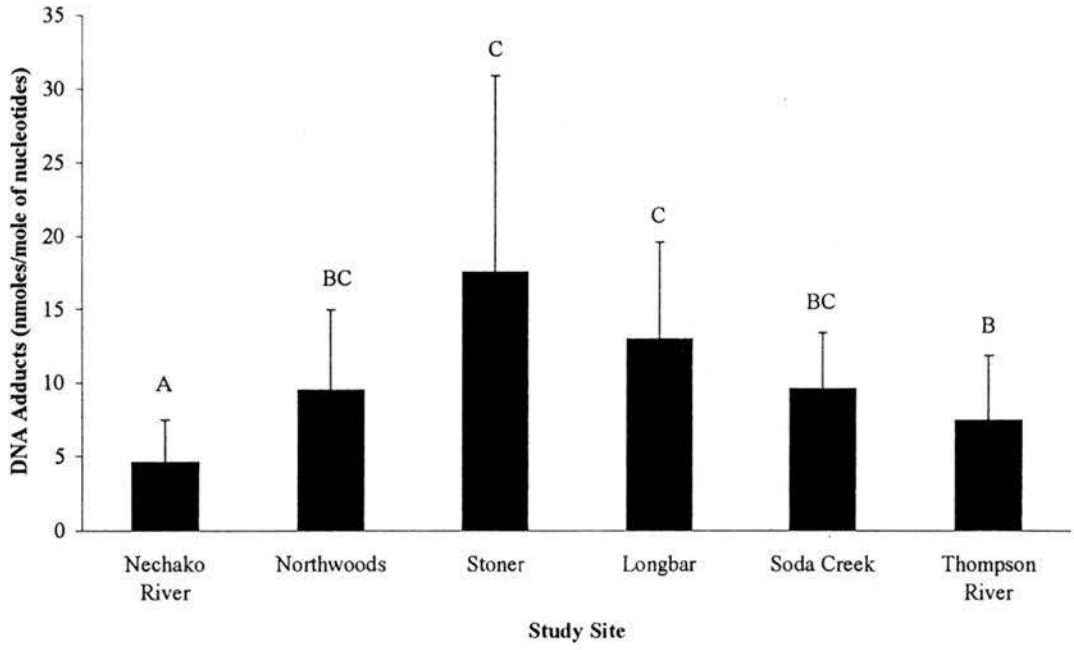

Figure 8 DNA Adducts in the Livers of Juvenile Chinook from the upper Fraser River
Data shown are untransformed; log transformed data were used for analyses. Error bars represent the standard deviation. Means with different letters differ significantly, $P<0.05$.

3.5.5 Histopathology

Livers from all sites except Northwoods, where there were not enough fish caught, were kept for histopathology. Slides were prepared and coded for the analysis. The histopathologist did not know the codes before the analysis was done. Three conditions were found: vacuolation, hepatocellular steatosis and hydropic vacuolation. The most frequent condition found was vacuolation which is characterized by extensive cytoplasmic vacuolation in combination with pycnotic (condensed) nuclei. This condition was found at all sites studied. Hydropic vacuolation, which is characterized by a clear cytoplasm, small compact nuclei and marked vacuolation (Hinton *et al.*, 1992), was found in fish from Nechako and Stoner. Hepatocellular steatosis is an intracytoplasmic storage disorder. Lesions of this type are non-nodular, diffusely distributed in a non-zonal pattern and have

normal muralial architecture but are characterized by fatty changes within the hepatocytes that are visible as round smooth-edged vacuoles peripherally displacing the nuclei. The histopathology findings are summarized in Table 3, which lists the frequency (number of fish with condition/total number examined) of each condition at each site. The Nechako site is split into the two distinct collection areas because they exhibit different frequencies. The frequency of lesions had no significant correlation with either length or weight of the fish.

Table 3 Histopathology in Livers of Juvenile Chinook from the upper Fraser River

Liver Histopathology			
Site	Vacuolation	Hepatocellular Steatosis	Hydropic Vacuolation
Nechako River- Site 1 (n=7)	0.29	0.00	0.14
- Site 2 (n=12)	0.83	0.00	0.17
-combined	0.63	0.00	0.16
Northwoods	NA	NA	NA
Stoner (n=16)	0.63	0.19	0.13
Longbar (n=20)	0.30	0.20	0.00
Soda Creek (n=20)	0.15	0.20	0.00
Thompson River (n=19)	0.47	0.00	0.00

Data are expressed as frequency of lesions (number of fish with condition/total examined) from that site, NA is not analyzed. Nechako 1 and 2 include fish caught on Swanson's Creek and Fort Fraser, respectively.

3.5.6 Bile Contaminants

There was very little bile in each gall bladder and thus up to 9 gall bladders had to be used for one pool and in some cases, significant dilution was required to achieve the volumes needed for analysis. Nechako samples from one site required no dilution but significant dilution was required in samples from the other. Fish from Northwoods,

Longbar, Soda Creek and Thompson had at least one sample which did not require any dilution. Fish from Stoner required dilution for all samples submitted. In all samples, protein concentrations were above zero and ranged from 6.69-17.21 mg ml^{-1}. All samples except the Thompson River and one Soda Creek sample had non-detectable concentrations of 1-hydroxypyrenol equivalents. The Thompson River samples had a mean of 3883 ng ml^{-1} or 139.7 ng (mg protein)$^{-1}$. The undiluted Soda Creek sample had trace amounts only and the other 2 samples were above detection.

3.5.7 Carcass Contaminants

Carcasses were pooled to attain ≈30 g for high resolution gas chromatography mass spectrometry analysis for polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), mono-ortho substituted polychlorinated biphenyls (mo-PCBs) and coplanar polychlorinated biphenyls (co-PCBs). Data are reported in pg g^{-1} and have been corrected for the percent recovery of the reference standard. Two pools from each site were analyzed. The contaminant data included many congeners whose concentrations were near the detection limit which caused replicates to appear different if non-detectable samples were given the value of zero. Thus all non-detectable concentrations were given a value equivalent to the detection limit for statistical calculations.

Toxic equivalent factors (TEFs) relate the toxicity of organic contaminants to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This allows the contaminant burden to be expressed as one amount, the toxic equivalent quotient (TEQ). The TEQ is the sum of each congener, expressed in TCDD equivalents (pg/g). TEFs were used from NATO-CCMS (1988) for PCDDs and PCDFs (see Table 4) and from Ahlborg *et al.*(1994) for mo-PCBs and co-PCBs (see Table 4) to calculate TEQ values for each sample. TEQs were calculated for PCDDs, PCDFs, mo-PCBs and co-PCBs. In addition, a total TEQ value, referred to as the total burden was calculated. This value is the sum of each group of contaminants. A worst case total burden was also calculated to include additional information on certain PCB congeners not previously included. The average TEQ value

for each site was used for all analyses. The differences in TEQ values between sites are difficult to test because there is inadequate replication in the data to get a good measure of variance. With only 2 pools per site, the standard deviations are quite large in some cases

Table 4 Toxic Equivalent Factors (TEFs) for PCDDs, PCDFs and PCBs

PCDDs	TEF	co-PCBs	TEF
2,3,7,8-TCDD	1	PCB 77	0.0005
1,2,3,7,8-PeCDD	0.5	PCB126	0.1
1,2,3,4,7,8-HxCDD	0.1	PCB169	0.01
1,2,3,6,7,8-HxCDD	0.1		
1,2,3,7,8,9-HxCDD	0.1		
1,2,3,4,6,7,8-HpCDD	0.01		
OCDD	0.001		
PCDFs	TEF	mo-PCBs	TEF
2,3,7,8-TCDF	0.1	PCB123	0.0001
1,2,3,7,8-PeCDF	0.05	PCB118	0.0001
2,3,4,7,8-PeCDF	0.5	PCB114	0.0005
1,2,3,4,7,8-HxCDF	0.1	PCB105	0.0001
1,2,3,6,7,8-HxCDF	0.1	PCB167	0.00001
1,2,3,7,8,9-HxCDF	0.1	PCB156	0.0005
2,3,4,6,7,8-HxCDF	0.1	PCB157	0.0005
1,2,3,4,6,7,8-HpCDF	0.01	PCB189	0.0001
1,2,3,4,7,8,9-HpCDF	0.01		
OCDF	0.001		

Summarized from NATO-CCMS (1988) and Ahlborg *et al.*(1994).

and artificially small in others which leads to heterogeneity in the data and invalidates the use of ANOVA. As well, there are only 15 data points, which tends to give non-normal distributions that are not alleviated by log transformation.

PCDD concentrations in the fish had TEQ values in ranging from 0.14 to 0.41 pg g^{-1} TCDD equivalents. Fish from Stoner and the Thompson River had the highest TEQs at 0.40 and 0.41 pg g^{-1}, respectively. Fish from the Nechako had the lowest value at 0.14 pg g^{-1}. Fish from the two Nechako collections had similar contaminant concentrations and thus were pooled for the analysis. Fish from Longbar, Soda Creek and Northwoods had very similar TEQ values (0.27, 0.28, 0.28 pg g^{-1}). PCDFs ranged from 0.09 and 0.26 pg g^{-1} (TEQ value), with Nechako fish being the lowest and fish from the Thompson River being the largest. Fish from Northwoods, Stoner, Longbar and Soda Creek all had approximately the same TEQ value (0.13, 0.17, 0.18, 0.16). The PCDD and PCDF TEQ values are summarized on Figure 9.

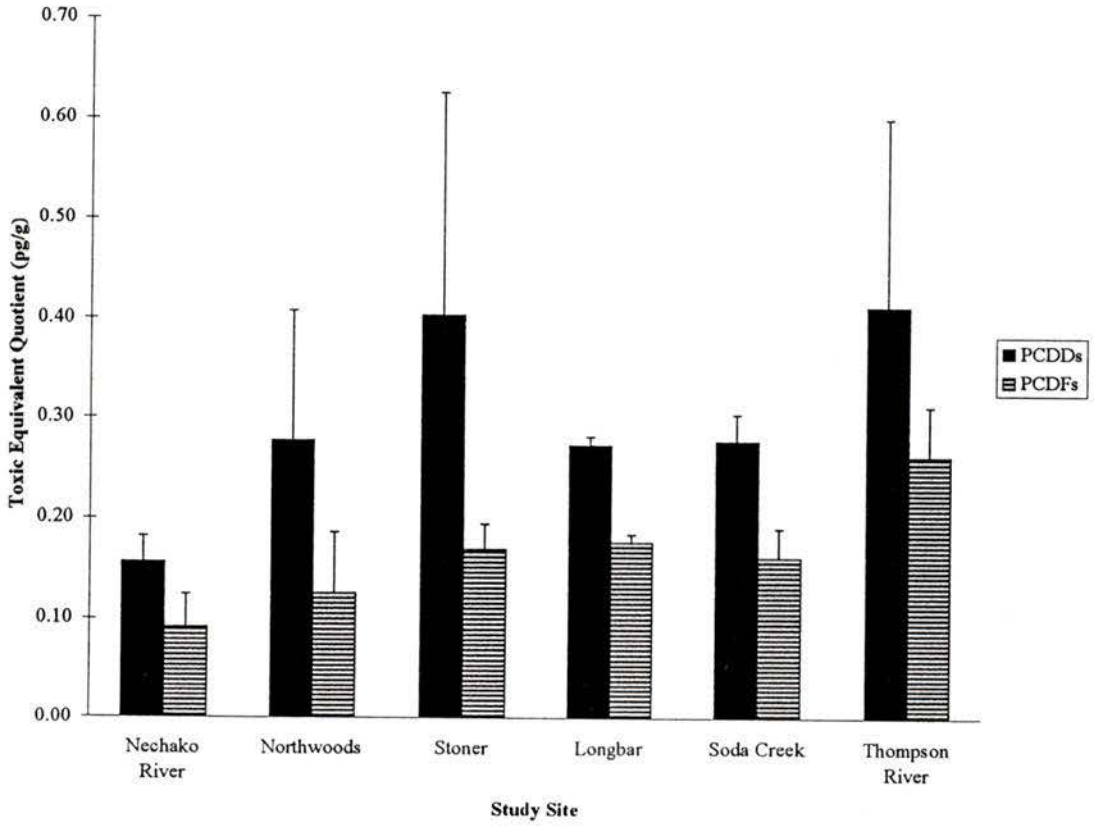

Figure 9 PCDDs and PCDFs in Carcasses of Juvenile Chinook salmon from the upper Fraser River

Data are expressed in TCDD equivalents; see text for explanation of calculation. Error bars represent standard deviation, based on two pools per site.

Both mono-*ortho* substituted and coplanar PCBs were detected with the HR/GC analysis of carcasses. For the mono-*ortho* PCBs, only 8 congeners have established TEFs, although 22 congeners were measured. For the coplanar PCBs, only 3 congers have established TEFs, while 18 congeners were measured. For the PCB-TEQ calculations (Figure 10), only those congeners with a TEF are included; however, a second calculation was performed by substituting the largest TEF for all of the congeners lacking TEFs. This is the so-called "worst case scenario", where an unknown congener is assumed to be as toxic as the most toxic in its class. Although the worst case PCB data are not shown, they replaced the PCB-TEQs for a second calculation of total burden, the worst case total burden. The PCB TEQ data are shown in Figure 10. The mo-PCBs range from 0.02 to 0.06 pg g^{-1} TEQ, and show very little variation control to exposed sites. The co-PCBs range from 0.08 to 0.16 pg g^{-1} TEQ. Fish from Northwoods had the largest TEQ value, but all other sites had comparable TEQ concentrations (0.08-0.12 pg/g). The Northwoods site has a very large standard deviation, due to large differences in the contaminant concentrations in the duplicate pools of carcasses for this site. As there were only 2 samples, it is impossible to decide which of these duplicates represent the site best.

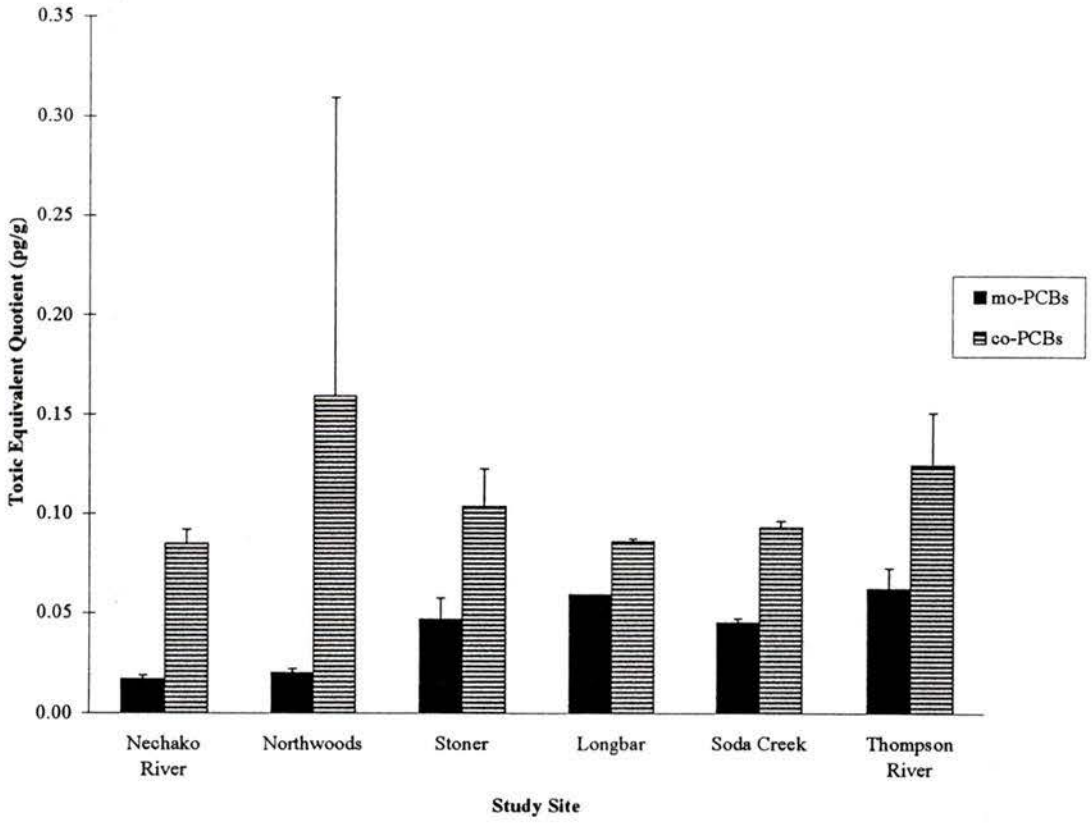

Figure 10 Mono-ortho and Coplanar PCBs in Carcasses of Juvenile Chinook Salmon from the upper Fraser River

Data are expressed in TCDD equivalents; see text for explanation of calculation. Error bars represent standard deviation, based on two pools per site.

The total organic contaminant burden was calculated by summing the TEQ values of each class of congener (PCDDs, PCDFs, mo-PCBs, and co-PCBs). This number is a simple way to express contaminant load. The total burden of each site has been expressed in two ways, the total contaminant burden and the worst case total. The worst case total is a sum of the PCDDs, PCDFs and the worst case PCB values for each PCB class, as explained above. The overall trend of the two total burden calculations is essentially the same, with the worst case toxic burden 2.2-3.2 fold greater than the total burden (see Figure 11). In both calculations, the Nechako River had the lowest burden. There was little difference between the other sites. This trend seems to be the same regardless of how the total burden is calculated; e.g., if the non-detectable congeners are assigned a

value of 0 or if a conservative TEF is substituted for those PCBs lacking published TEFs, the trend remains the same and the values are approximately equal to the total burden data shown in Figure 11.

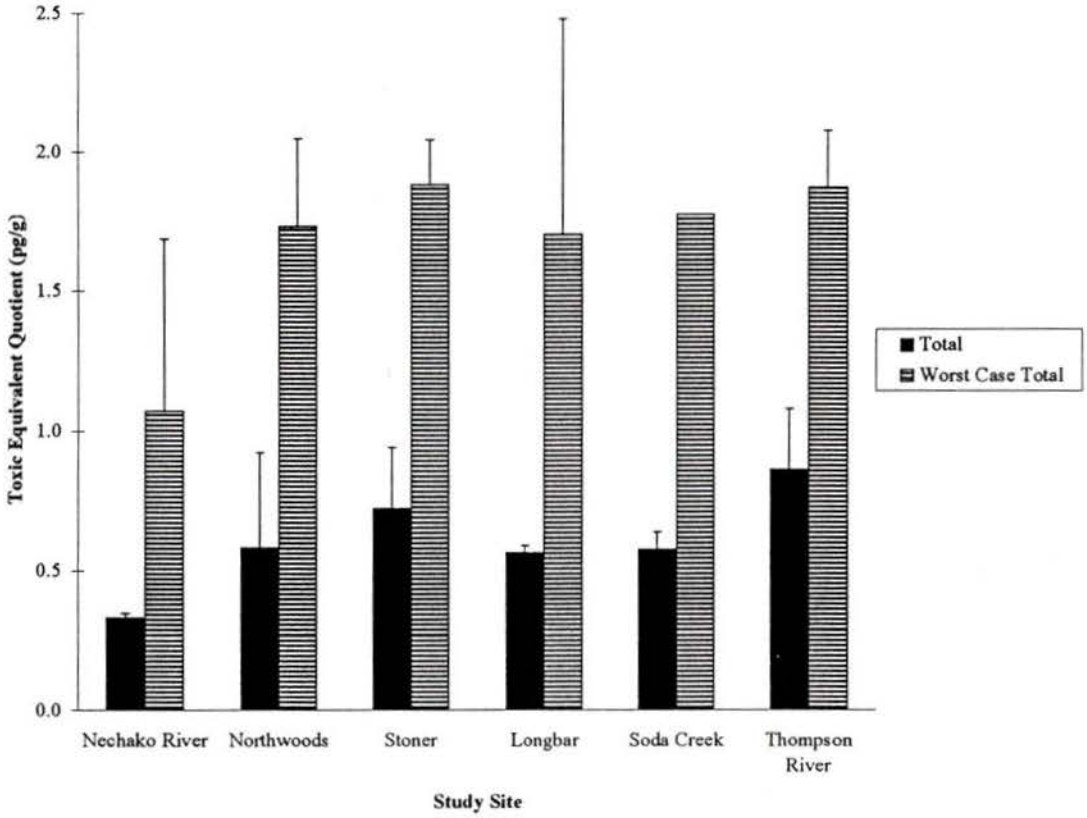

Figure 11 Total Contaminant Burden in Carcasses of Juvenile Chinook Salmon on the upper Fraser River

Data are expressed in TCDD equivalents; see text for explanation of calculation. Error bars represent standard deviation, based on two pools per site

3.5.8 Relationships Between Biological Responses

EROD, CYP 1A1 and DNA adducts were all analyzed on the same pool of livers for each sample. There was a significant correlation between EROD activity and both CYP 1A1 and DNA adducts (Table 5) and between CYP 1A1 density and DNA adducts (Table 5).

Table 5 Correlation Matrix for Biological Variables

Correlations for Biological Variables								
	EROD	CYP 1A1	Adduct	PCDDs	PCDFs	mo-PCBs	co-PBCs	Total Burden
EROD	1.00	**0.35**	**0.35**	0.38	0.25	0.52	-0.08	0.35
CYP 1A1		1.00	**0.40**	0.71	0.53	0.72	0.19	0.69
Adduct			1.00	0.71	0.48	0.64	0.22	0.66

Based on N=60 for correlations between biological variables
Based on N=7 for correlations between biological variables and contaminants
bold coefficients are significant at $p<0.05$

EROD, CYP 1A1, and DNA adducts can only be compared to the liver histopathological data on a site mean basis because the measurements were made in different fish samples. For this analysis, the Nechako site was split into the two collections, because there were different histopathology frequencies in the two collections. Hepatocellular steatosis was found to be correlated with EROD, CYP 1A1 and DNA adducts ($P<0.05$, N=6). There was no correlation between either vacuolation nor hydropic vaculoation with any of the other biological measurements.

3.5.9 Relationships between Contaminants

Multiple correlations between the different classes of organic contaminants were found. PCDDs, PCDFs and mo-PCBs were found to be correlated, but co-PCBs were found to be unrelated to the other contaminants. The total burden TEQ correlated with PCDDs, PCDFs and mo-PCBs but not with co-PCBs. The worst case total TEQ was correlated with PCDDs and PCDFs. The correlation coefficients are listed in Table 6, based on an analysis with the Nechako collections separated. Those coefficients which are statistically significant ($P<0.05$) are in bold.

Table 6 Correlation Coefficients for Organic Contaminants in Juvenile Chinook from the upper Fraser River

Correlation Coefficients for Contaminants						
	PCDDs	PCDFs	mo-PCBs	co-PCBs	Total Burden	Worst Case Burden
PCDDs	1	**0.861**	**0.758**	0.442	**0.973**	**0.826**
PCDFs		1	**0.889**	0.255	**0.916**	**0.762**
mo-PCBs			1	-0.016	**0.817**	0.681
co-PCBs				1	0.493	0.384
Total Burden					1	**0.804**
Worst Case Burden						1

coefficient in bold are significant at $p<0.05$

Based on mean TEQ values (pg/g TCDD equivalents), N=7

3.5.10 Relationships between Contaminants and Biological Responses

Contaminants and biological responses could only be compared using site means, as the pools were not equivalent (Table 5). The Nechako collections were not pooled for this analysis. EROD activity was not correlated with any contaminant group. CYP 1A1 was found to be significantly correlated with the worst case total (P=0.04). Its correlation with PCDDs, mo-PCBs, and the total burden was also borderline significant (P=0.07, 0.07, and 0.08, respectively). DNA adducts were significantly correlated to the worst case total burden (P=0.03) and were borderline significant with PCDDs (P=0.07). The lesions found did not correlate with any contaminant measurement except for hydropic vacuolation, which was negatively correlated with mo-PCBs (P=0.03). There was no significant correlation between 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD or 2,3,7,8-TCDF, the major PCDDs and PCDFs discharged by pulp mills (Yunker and Cretney, 1995, 1996), and any biological response (data not shown).

3.6 *Discussion*

The lengths and weights of the fish differed significantly between sites, even though they cover a very narrow range of values. There was no apparent relationship between either length or weight and any other measured variable. The fish were pooled for all analyses, therefore there could be no direct comparison of fish size and any of the

measured variables. This difference in size along the Fraser River has been noted in another study, where it was suggested that differences in fish growth were related to differences in invertebrate food supply (Levings and Lauzier, 1991).

EROD activity and CYP 1A1 density were correlated extremely well. They followed the same basic pattern in site trends. A general pattern emerged from this data which is also seen in other biological responses such as inter-renal nuclear diameter and blood chemistry (R.Gordon and D. Martens, unpublished data). Fish from the Nechako River had low concentrations or activities that were distinguishable from the other sites. Those from Northwoods and Thompson River had equivalent responses. The Thompson River fish were expected to be low, because of the dilution of the effluent by a lake and the distance from the pulp mill. Fish from Stoner, Longbar and Soda Creek all had similar responses, even though they represent different distances from the point source inputs for this system. There was no downstream gradient of response, since otherwise a decreased response in Longbar compared to Stoner would be seen. This suggests that either non-point source inputs play a significant role in the contaminant load of these rivers, or else that the fish are more mobile in winter months than previously thought. One mark-and-recapture study indicated that site fidelity in chinook on the Fraser River was not high but recovery of marked animals both in and outside the original site was low (Emmett *et al.*, 1996). Additionally, only a small area around one site was examined and any results indicating movement of chinook outside the capture site cannot be directly related to such large distances between the sites in this study. It is not known if freeze branding individuals for mark and recapture is stressful enough to cause behavioural changes in habitat selection. Therefore, it is hard to judge whether juvenile chinook exhibit wide mobility in winter months.

The Northwoods site was surprisingly low in EROD and CYP 1A1, considering its proximity to the pulp mill. This lack of response could be due to several possibilities. First, the fish at Northwoods may be unexposed (or exposed to low concentrations) due to inadequate mixing of effluent. Chinook prefer shallow, low flow areas near the edge of the river while the diffuser discharging effluent is in the middle of the river, where flow is higher. Second, the effluent from the pulp mill could be insignificant in comparison to the

municipal waste effluent. The pulp mill effluent would then represent a small proportion of the contaminant load on the river and downstream from the Northwoods site other inputs would result in higher response. However, for this scenario, the dilution must be large enough that the fish were not being exposed to 12-14% effluent as suggested, since this concentration would be predicted to cause significant induction (Servizi *et al.*, 1993). Third, migration started before or during sampling and fish downstream would be exposed for longer periods than at Northwoods, where the fish would be newly exposed to the effluent zone. This scenario is unlikely, since EROD induction is a fast response with only 24 hours required to see a difference over control fish (J. Wilson, unpublished data), although maximal induction is seen from 2-5 days, depending on the species and contaminant (Addison *et al.*, 1981, Collier and Varansi, 1991; Parrott *et al.*, 1995a).

The EROD activities seen in this experiment are comparable to other data in chinook. The highest EROD activity in this study was approximately 3-fold over control. Juvenile migrating chinook from two urban estuaries in Washington were found to have EROD activities 1.5 - 2.5 fold higher than those from a non-urban estuary (Stein *et al.*, 1995). Laboratory exposures to bleached kraft mill effluent (BKME) have shown similar levels of EROD activity to the highest sites in this study. After 60 and 144 days exposure to 0.3 to 4% BKME, juvenile chinook exhibited 2-fold induction over control exposures (Servizi, *et al.*, 1993). These effluent exposures were chosen to represent the exposures found at Stoner and Longbar in winter conditions (D. Martens, personal communication). In previous years' sampling, EROD activity has been much higher in the Fraser River chinook (Rogers, *et al.*, 1989). This response was associated with much higher PCDD and PCDF concentrations in the carcass. Since changes in the mills were completed to reduce the PCDD and PCDF concentrations in the effluent, the EROD activity has been at approximately the same level as seen in this study (R. Gordon and D. Martens, unpublished data).

CYP 1A1 density is not as commonly used in field studies as EROD activity. The two measurements are rarely used in concert and little has been published on their relationship. Although the expectation is that these two markers would produce the same results, it is not proven that they are interchangeable in all cases. In various field studies

EROD and CYP 1A1 density have been found to have similar responses to contaminants in a variety of species, such as European flounder (*Platichthys flesus)* (Addison and Edwards, 1988 and Stegeman *et al.*,1988) and longnose sucker (*Catostomus catostomus*) and mountain whitefish (*Prosopium williamsoni*) (Kloepper-Sams *et al.*, 1994). EROD activity has also been found to correlate with levels of CYP 1A1 mRNA in dab (*Limanda limanda*) (Renton and Addison, 1992). These findings agree with the results of this study and indicate that measurement of various components of the CYP 1A1 system can be useful for biomonitoring. Goksøyr *et al.* (1991) found some discrepancies between these measurements in dab, flounder and plaice (*Pleuronectes platessa*) at one of 4 sites studied in Norway and recommended that together, these two measurements can give information not apparent with one method alone.

The presence of DNA adducts suggests that there are sources of polycylic aromatic hydrocarbons in this watershed. The methods used for detection of adducts select for large, bulky hydrophobic adducts. Although recently some work has investigated the formation *in vitro* of PCB-DNA adducts in rat microsomes (McLean *et al.*, 1996), the chromatography solvents were different from those used in this study. As the pH and ionic strength of the solvent determines the mobility of the adducts on the TLC plate, it is not clear whether PCB adducts could be detected with the current methods. Fish from the control site had extremely low concentrations which were distinguishable from all other sites. Differentiation between sites was more difficult, in part because of high background on the chromatograms. The concentrations found in these fish were very similar to concentrations of adducts in juvenile chinook from Washington State estuaries (Stein *et al.*, 1995) and red drum (*Sciaenops ocellatus*) from Tampa Bay (McCain *et al.*, 1996), but lower than those found in English sole, rock sole and starry flounder (*Platichthys stellatus*) from Washington State (Stein *et al.*, 1991), winter flounder (*Pseudopleuronectes americanus*) from Long Island, New York (Grondlund *et al.*, 1991), and longnose killifish (*Fundulus majalis*), gulf killifish (*F. grandis*) and hardhead catfish (*Arius felis*) from Tampa Bay, Florida (McCain *et al.*, 1996). The clear relationship to EROD activity and CYP 1A1 induction is similar to that reported by Collier *et al.* (1996). In most studies, correlations between biological measurements are not performed, but,

EROD (or aryl hydrocarbon hydrolase, AHH) activity and DNA adducts seem to give similar results in chinook (Stein *et al.*, 1995), English sole, starry flounder (Stein *et al.*, 1991), and longnose killifish (McCain *et al.*, 1996).

The histopathology results were difficult to interpret because of the low frequency and because of the large number of some lesions in fish from the control site. With frequency data, there is no estimation of error associated with the site data because there is no range of response. However, it is interesting to note that hepatocellular steatosis occurs only at the sites with the highest EROD and CYP 1A1 response. This condition is a degenerative disorder suggestive of metabolic disorders (Myers *et al.*, 1987) commonly associated with dietary deficiencies or toxic chemical administration. Its role in the progression of lesions toward neoplasm formation in fish is presently unknown (McCain *et al.*, 1982).

Hydropic vacuolation is particularly difficult to interpret since it occurred only at the control site and Stoner, and at low frequency. Although this lesion has been highly correlated with cholangiocytic neoplasms and less well with hepatocellular neoplasms (Harshbarger and Clark, 1990), it may be found in livers free of neoplasia. It has been detected in rock sole and starry founder from contaminated sites in Puget Sound, Washington (Stehr *et al.*, 1990). These findings have led to this lesion being suggested as a specific biomarker for contamination (Hinton *et al.*, 1992). However, as in this study, it has been found in fish from reference sites (M. Myers, personal communication) and no laboratory studies have shown induction of the lesion after contaminant exposure.

The vacuolation found in these fish is ambiguous, since the highest frequency was at one of the control sites. The pattern of vacuolation among sites did not match any other variable measured, including length or weight. This is particularly important since the degree of vacuolation has been associated with increased size in laboratory studies (D. Brand, personal communication). However, the fish from that study were growing rapidly, in contrast to the Fraser River fish that were most likely growing relatively slowly. Fish caught from an area polluted with arsenic (Sorenson *et al.*, 1980) or kraft pulp mill effluents (Lehtinen *et al.*, 1984) contained this lesion. The condition has also been induced in fish with reduced food intake due to stress (Khan and Kiceniuk, 1988).

It is possible that the fish in this study were not old enough to show significant lesions resulting from low level chronic exposure of contaminants. It may take longer for these conditions to develop. On the Fraser River, juveniles have been found with granulomas that have been attributed to resolved bacterial kidney disease (D. Martens, personal communication). This disease may result from contaminant exposure through effects on immunocompetence. Hepatic megalocytosis was found in both wild and farmed adult chinook, but seemed to be related to exposure to an algal toxin and rather than contaminant exposure (Stephen *et al.*, 1993). There are no other published reports of tumors in chinook salmon. Rainbow trout (*O. mykiss*) had no increase in lesions after exposure to 1.3 to 5.1% BKME in experimental streams (Hall *et al.*, 1992). Mountain whitefish (*Prosopium williamsoni*) exposed to BKME showed no differences in histopathology compared to fish from control sites in the Wapiti and Smoky Rivers (Kloepper-Sams *et al.*, 1994). There was no correlation between histopathology and contaminants in longnose sucker during the same study (Kloepper-Sams *et al.*, 1994). A strong relationship between exposure and lesions seems to be found only in benthic or bottom-feeding fish caught in highly contaminanted areas (Harshbarger and Clark, 1990).

PAH metabolite concentrations in the bile were detectable in fish from only one site. The amount of bile collected from such small fish was minimal and dilution was required in some cases to generate enough solution for both protein and synchronous scan analysis. Although the protein analysis indicates that the dilution was not excessive, the PAH metabolite concentrations may have dropped below detection following dilution. Since the 1-hydroxypyrenol metabolite spectrum was analyzed, instead of the pyrene-1-glucuronide conjugate which shows a fluorescence intensity 2.2 fold higher, dilution may have been critical (Ariese *et* al., 1993). Standards for the pyrene-1-glucuronide conjugate are not commercially available. For other studies, larger amounts of bile should be collected or the size of pools increased to ensure that dilution is not required. The fact that PAH metabolites were not detected may indicate that exposure to PAHs with 4 or more rings is minimal on the Fraser River, although the presence of DNA adducts suggests otherwise. The method used to analyze PAH metabolites is optimized for 1-hydroxypyrenol metabolites, the metabolites that account for a large proportion of the

total PAH metabolites in flatfish exposed to combustion related PAHs (Krahn *et al.*, 1987). It is not known if the same PAH spectrum would be found in the suspended solids of the Fraser River. Metabolites of hydrocarbons which form DNA adducts may have different spectral properties from 1-hydroxypyrenol. Metabolites of PAHs were also absent from English sole exposed to BKME, as detected by synchronous scan fluorimetry, but HPLC analysis for benzo[a]pyrene metabolites indicate significant exposure (D. Brand, personal communication). Additional testing of bile by HPLC analysis in the benzo[a]pyrene metabolite spectrum and SFS optimized for smaller ring PAH metabolites would be necessary to eliminate significant PAH exposure in these fish.

PCDDs, PCDFs and PCBs in the carcass were low in fish from all sites, with some congeners near the detection limit. In all cases, fish from the Nechako had the lowest concentrations of contaminants. There were no statistical differences between sites because of the large standard deviations in some sites. Fish from the other sites had very similar concentrations of all chemical groups and the trends seen in the biological responses were absent. There was no correlation between the biological responses and any chemical group, nor with the total contaminant burden. In addition, there was no correlation with either 1,2,3,6,7,8-HxCDD or TCDF, which are considered to be marker chemicals for pulp mill effluent exposure (Yunker and Cretney, 1995; 1996). The lack of correlation to chemical class or toxic burden may be due to the difficulty of resolving small differences in low contaminant concentrations in the fish. This is in part due to the lack of adequate replication in the contaminant data, but it is also due to errors introduced by the use of TEFs.

TEFs are important for reducing chemical data into limited number of data for comparison to biological variables. There are problems in their use as they are not well defined in all species. Depending on species chosen and endpoint test, there are significant differences in TEFs. Dose response curves of various PCDDs and PCDFs were not parallel in rainbow trout (Parrott *et al.*, 1995b). This raises the question of how much error may be introduced into contaminant burden calculations if the level of contamination is different from the level where the TEF was calculated. Complex mixtures would

require fairly high contamination to overcome the errors associated with contaminant burden calculation.

The contaminants are probably not high enough to cause the biological effects seen. For a significant EROD induction, TEQ values in chinook must be at least 3 pg/g (Servizi *et al.*,1993) a level not achieved at any site, even under the worst case scenario. In laboratory tests of rainbow trout, threshold concentrations of 16 pg/g TCDD in the liver were required for EROD induction (Parrott *et al.*, 1995b). Even though the contaminant concentrations are low, the biological responses differ significantly from the control site. This information may indicate that exposure to other inducing chemicals may have occurred. The problems with the synchronous scan data leaves open the question of high molecular weight PAH exposure. Considering the presence of DNA adducts in the fish, PAH exposure is probable and warrants further investigation.

3.7 *Conclusions*

Juvenile chinook on the upper Fraser River had significant increases in EROD activity, CYP 1A1 density and DNA adduct concentrations over control sites on the Nechako River. The EROD activity and CYP 1A1 data show a pattern of induction that increases at sites downstream of the Northwoods mill. The reason for this pattern is not known, although it is possible that the fish at the Northwoods site are outside the effluent plume. Non-point source inputs may be significant along the Fraser, accounting for the similar responses at sites of varying distances from the point sources. DNA adducts were similar at all of the exposed sites, although they were significantly greater than the control site. Efforts to improve on technique would probably reduce background and allow differentiation between DNA adducts at the exposed sites. There was a strong correlation between EROD activity, CYP 1A1 density and DNA adducts in these fish. Liver histopathology results are ambiguous and were not correlated with the other measured variables.

Contaminant concentrations in the fish were low and were not correlated with any of the biological effects. PAH exposure cannot be ruled out and HPLC analysis for

benzo[a]pyrene metabolites is suggested for further research. The contaminant concentrations measured in this study do not account for the biological effects seen in the fish. The presence of DNA adducts suggests PAH exposure had occurred. It is probable that PAH exposure alone would not be high enough to account for the biological effects measured. It is more likely that the total contaminant burden, including the PAH exposure, may have been high enough to suggest a relationship to the biological responses. There was a consistent pattern of response in the biological responses over time and this pattern is seen with other biomonitors such as interrenal diameter and blood chemistry measurements (D. Martens, personal communication). This indicates that there was a whole organism response which may result in decreased viability of the chinook in the Fraser River.

4. Juvenile Chinook Exposed to Bleached Kraft Mill Effluent

4.1 *Abstract*

Juvenile chinook salmon (*Oncorhynchus tshawytscha*) were exposed to 0, 2, 4, 8 or 16% effluent from a bleached kraft pulp and paper mill. Fresh effluent was combined with river water from upstream of the effluent line diffuser. Fish were exposed for 1 month and then biological responses and contaminant concentrations were measured. Ethoxyresorufin-O-deethylase (EROD) activity, CYP 1A1 density, and DNA adduct concentrations were measured in liver tissue. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) were measured in carcasses and polyaromatic hydrocarbon (PAH) metabolites were measured in bile. Fish were tested for biological responses to identify if the effluent, at concentrations similar to that found in the river, would cause significant effects. In previous field studies EROD activity, CYP 1A1 density and DNA adduct concentrations were significantly elevated in chinook located downstream of this mill. Fish sampled adjacent to the diffuser had lower responses than those downstream, where dilution was higher. In the experimental exposures, EROD activity was significantly increased over control values at all effluent exposures and CYP 1A1 levels were found to be increased in all but 2% effluent. DNA adduct concentrations were significantly increased at 8 and 16% effluent. These data indicate that the effluent can cause significant increases in all three responses at concentrations similar to those found in the river near the diffuser. Carcasses contained low concentrations of PCDDs and PCDFs. PCBs were at higher levels than field exposed fish but contaminant concentrations do not appear to have bioaccumulated in this study and were present before the exposure. PCBs account for 77% of the total contaminant burden. It is probable that the PCB concentrations were due to contaminated fish oil in the commercial feed. Bile analysis did not indicate any exposure to PAHs.

4.2 *Introduction*

Bleached kraft mill effluent (BKME) has been a focus of study for effects in the aquatic environment. In recent years, the response of the P450 system has been the subject of several

studies. Impacts on fish from one of the early field studies include reduced gonad growth, enlarged liver, induction of EROD activity, elevated ascorbic acid, abnormal carbohydrate metabolism and changes in white and red blood cell patterns and ion balance in perch (Andersson *et al.*, 1988). Since then, numerous studies have been conducted in a number of receiving waters in Canada and elsewhere. The effects seen in various fish species in Canada include: increased EROD activity (or a similar assay of CYP 1A1) (Hodson *et al.*, 1992, Kloepper-Sams *et al.*, 1994; Munkittrick *et al.*, 1991, 1992 a,b; Servizi *et al.*, 1993; Servos *et al.*, 1992), changes in blood variables (Hodson *et al.*, 1992), increased liver size or liver somatic index (Hodson *et al.*, 1992, Kloepper-Sams *et al.*, 1994; Munkittrick *et al.*, 1992a; Servos *et al.*, 1992), altered steroid concentrations (Kloepper-Sams *et al.*, 1994; Munkittrick *et al.*, 1991, 1992a,b), various changes in reproductive systems (Munkittrick *et al.*, 1991, 1992a), and lesions (Munkittrick *et al.*, 1992a). The one response common to these studies in an increase in EROD activity. Although the original focus in the early research was the toxic effects of PCDDs and PCDFs, process changes have reduced the production of these contaminants. In 84% of sites studied, fish downstream of bleached pulp mills in the USA have declining TCDD concentrations (Abbott and Hinton, 1996). In coastal BC, pulp mill TCDD concentrations have been declining since 1989 (see Figure 12). Contaminant concentrations in chinook salmon from the Fraser River follow this trend in both PCDDs and PCDFs (D. Martens and R. Gordon, personal communication).

Chinook salmon (*Oncorhynchus tshawytscha)* use the upper Fraser River for spawning and overwintering grounds. Chinook were collected downstream from pulp mill and municipal waste discharges in 1995. These fish showed increases in EROD activity, CYP 1A1 density, and DNA adduct concentrations (Chapter 3). Fish captured adjacent to the effluent diffuser of one mill were found to have increased CYP 1A1 density and DNA adduct concentrations, but no increase in EROD activity. The effluent concentrations at this site were thought to be 12-14%, based on conductivity measurements (G. Kruzynski, personal communication), but this concentration of effluent should result in significant EROD induction based on a previous study of this effluent in which chinook exposed to 0.3-4% effluent for 60 or 144 days exhibited 2-fold EROD induction over control fish (Servizi *et al.*, 1993). In 1995, this level of induction was not seen at the site adjacent to the mill, but was found at sites much farther downstream. Three hypotheses were raised as possible explanations for this finding. First, the fish at the mill site

could have missed the effluent plume. Second, the effluent from the mill could be insignificant to the total contribution of contaminants to the river. This scenario is problematic if the fish are being exposed to the 12-14% effluent as suggested. Much larger dilution or a significant change in the effluent characteristics would need to have occurred in order to account for the absent EROD response. Third, migration may have started before sampling began and the fish at the site adjacent the diffuser were newly exposed to effluent.

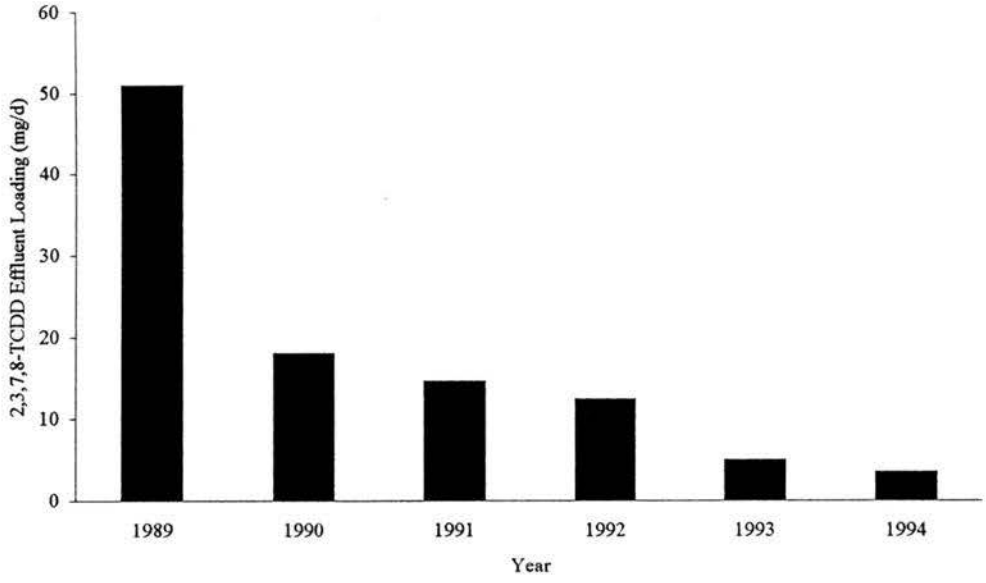

Figure 12 2,3,7,8-TCDD Effluent Trends in BC Coastal Pulp Mils

Data were taken from W. Knapp (Department of Fisheries and Oceans)

To investigate the potency of BKME on juvenile chinook fish were exposed to fresh effluent diluted with river water. This study simulates field exposure near the diffuser and would identify what concentrations of effluent were required to cause significant increases in EROD activity, CYP 1A1 density, and DNA adduct concentrations. It is recognised that in this study, the contamination due to diet is removed, which may remove an important component of the exposure. Commercial fish food was used to feed the animals throughout the study and although water-borne contaminants may adsorb to the food, the food chain component was absent.

4.3 *Materials and Methods*

The experiment took place on site at Northwood Pulp and Timber, Prince George, British Columbia. This pulp mill is a bleached kraft mill producing on average 14 000 air dried tonnes (ADT) per day of bleached kraft pulp. Chlorine dioxide is used for bleaching. Effluent is treated with both primary and secondary treatments, with secondary treatment being an aerated lagoon. 140 000 m^3 of effluent is produced by the mill each day with average TCDD, TCDF and AOX concentrations in the effluent of 0 ppq, 6.5 ppq and 0.4 kg ADT^{-1} (0.04 g L^{-1}), respectively.

Juvenile (1+years) chinook salmon were transported from Penny Hatchery (near Prince George, BC) to tanks held on site at a bleached kraft mill. They were not fed for 24 hours prior to transport and then acclimated in river water for 24 hours. Fish were fed a commercial diet of Bio-Diet (Bioproducts, OR) at 0.5 % body weight per day. Fish were placed with a loading density of 3 grams l^{-1} in tanks at 0.5 - 3 °C and exposed for 1 month to 0, 2, 4, 8, or 16% bleached kraft mill effluent (BKME). The temperatures in the tanks increased with concentration of effluent because the effluent was warmer than the river water. Water flow was 10 l min^{-1} so that 95% replacement occurred in less than one hour. The dissolved oxygen and conductivity ranged from 61 - 85 % saturation and 200 - 510 μsiemens cm^{-1} , respectively in the exposure tanks. Effluent was diluted with river water taken above the diffuser to simulate conditions directly below the diffuser. After the exposure, fish were killed with a blow to the head, weighed and measured. The liver and gall bladder were removed and frozen in liquid nitrogen. Carcasses were placed on dry ice. Livers, gall bladders and carcasses were stored at -80°C until analyzed.

Before any of the exposures were performed, a subsample of fish were taken for analysis. These represent the baseline or zero time fish. These fish were analyzed for contaminant concentrations, EROD activity and CYP 1A1 density. Bile analysis and DNA adduct concentrations were not performed on these fish. Although these fish were not collected at the same time as the fish used in this study, they were reared at the same time in the same hatchery and represent pre-exposure conditions for all the experiments at the mill.

Microsomes were prepared from pools of two livers as described by Hodson *et al.* (1991). Protein concentration, ethoxyresorufin-O-deethylase (EROD) activity and CYP 1A1 density were determined in the microsomes. Protein concentrations were determined by the method of Bradford (1976). EROD activity was determined by the method of Hodson *et al.* (1991). CYP

1A1 density was determined by western blotting as described by Arlotto and Parkinson (1989). CYP 1A1 is expressed in reflective density units, as a ratio to the CYP 1A1 standard. An average reflective density is calculated by densitometry for all bands on a particular membrane and then each unknown band is expressed in terms of the standard from that membrane. This helps to remove day to day variation in the intensity of the bands.

DNA adducts were analyzed according to Reichert and French (1994). Pooled liver samples were homogenized and centrifuged for postmitochondrial supernatant (to prepare microsomes) and the pellet was removed for DNA extraction. The adducts were enriched by the nuclease P1 method. Adduct concentrations were determined using phosphor imaging techniques.

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) were analyzed in the carcasses by high resolution gas chromatography/mass spectrometry according to MacDonald *et al.* (1996) and Rantalainen *et al.* (1996). Bile sacs were centrifuged to break them open and the bile was removed for analysis. Polycyclic aromatic hydrocarbon metabolites in the bile were analyzed by synchronous scan fluorimetry for 1-hydroxypyrenol equivalents according to Ariese *et al.* (1993). Protein concentrations in the bile were analyzed using a modified Bradford assay (Bradford, 1976).

All statistical analyses were performed using Statistica 5.1 (Statsoft, Tulsa OK). Differences between site means were analyzed using ANOVA and Fisher's least significant difference test, when Levine's test for homogeneity of variance was non-significant and examination of mean versus standard deviation plots showed no significant relationship. Pearson's correlations were used to examine relationships between biomarkers. Data are reported as mean ±standard deviation.

4.*4* ***Results***

4.4.1 General Characteristics

Chinook salmon were approximately 18 months old. The fish had a mean weight and length of 8.78 g and 9.17 cm, respectively. Table 7 shows the lengths and weights in all exposures.

The weight and length data were normally distributed (Shapiro Wilks W test). The lengths of the fish in 16% effluent were significantly different from those in 0, 2 and 4% effluent ($p<0.05$). The weights of fish in 0% effluent were significantly different from those in 8 and 16% effluent. The fish from 2% effluent were significantly different from those in 16% effluent ($p<0.05$). The mean lengths and weights of fish increased with increasing concentration of effluent. There were significant correlations between the lengths and weights of the fish and all biological responses. There is no correlation between the lengths and weights of the fish and any contaminant concentration. The fish were not mature enough to be distinguished between sex by gonadal development.

Table 7 Mean Lengths and Weights of Juvenile Chinook Salmon Exposed to BKME

Effluent Exposure	Length (cm)	Weight (g)
Control (0%) (n=20)	8.81 (0.86) a	8.09 (1.58) a
2% (n=20)	9.16 (0.56) a	8.44 (1.63) ab
4% (n=20)	9.11 (0.68) a	8.72 (1.88) abc
8% (n=20)	9.28 (0.47) ab	9.11 (1.39) bc
16% (n=20)	9.52 (0.50) b	9.56 (1.47) c
All Groups (n=100)	9.18 (0.66)	8.78 (1.65)

Standard deviations are shown in brackets. Data with different letters differ significantly, $P<0.05$.

4.4.2 Ethoxyresorufin-O-deethylase (EROD) Activity

The EROD data were log transformed for all analyses. The mean EROD activity (non-transformed data) is shown in Figure 13. The fish exposed to 0 and 2% BKME had a mean activity of 80.7± 34 and 109± 39 pmol mg^{-1} min^{-1}, respectively. These activities were significantly different from those from all other treatments ($p<0.05$). Fish exposed to 4, 8 and 16% BKME had mean EROD activities of 159± 53, 186± 44, and 207± 66 pmol mg^{-1} min^{-1}, respectively. There were no significant differences between these exposures. The regression of EROD versus concentration of effluent was significant, (log (EROD)= 0.02 × % concentration + 1.98, $p<0.001$, $R^2 = 0.39$).

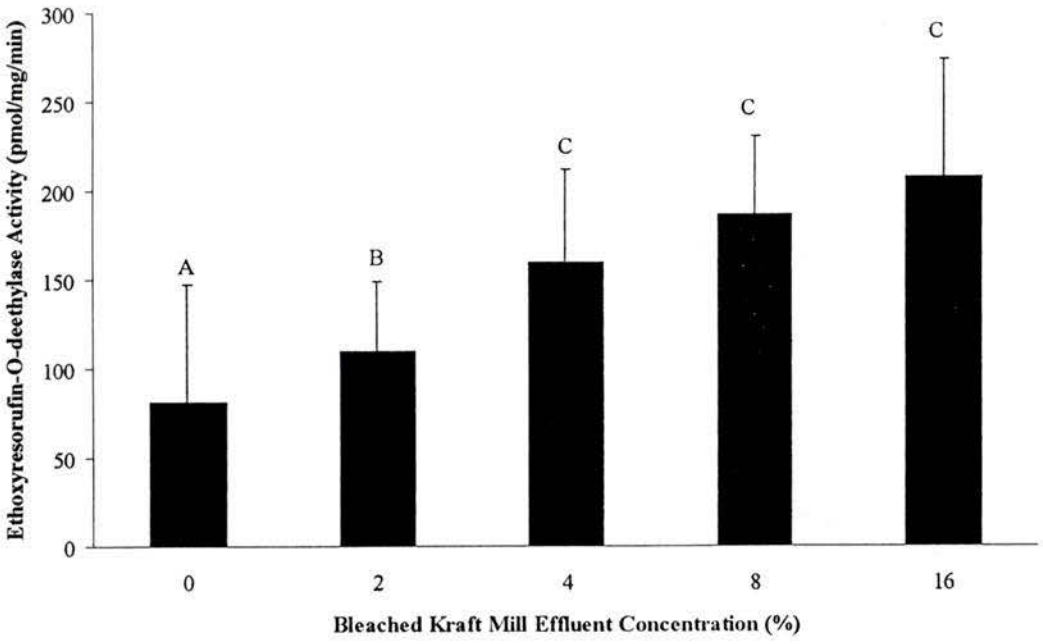

Figure 13 Ethoxyresorufin-O-deethylase Activity in Livers of Juvenile Chinook Exposed to BKME

Data shown are untransformed; log transformed data were used for analyses. Error bars represent the standard deviation. Means with different letters differ significantly, $P<0.05$.

4.4.3 CYP 1A1 Density

CYP 1A1 data were log transformed for all analyses. The mean CYP 1A1 density (non-transformed data) is shown in Figure 14. These data are expressed as a ratio of the unknown sample to 0.5 pmol CYP 1A1 standard, in reflective density units (RD). The CYP 1A1 data are not expressed in pmol because there may be a difference in the affinity of the primary antibody for the standard and the unknowns, as the protein is derived from different species. Control fish (0% BKME) had a mean CYP 1A1 density of 1.26±0.36 RD and were found to be significantly different from those exposed to 4, 8, and 16% BKME ($p<0.003$). Fish exposed to 2 and 4% BKME had mean CYP 1A1 densities of 1.94 ±0.87 and 2.50±1.02 RD, respectively. They were significantly different from fish exposed to 8 and 16% BKME ($p<0.01$). Fish exposed to 8% BKME had a mean CYP 1A1 density of 3.75±0.99 RD and were significantly different from those

from 16% BKME (5.94 ±3.12). The regression of CYP 1A1 levels versus concentration of effluent was significant, (log (CYP 1A1)= 0.03 × % concentration + 0.39, $p<0.001$, $R^2 = 0.66$).

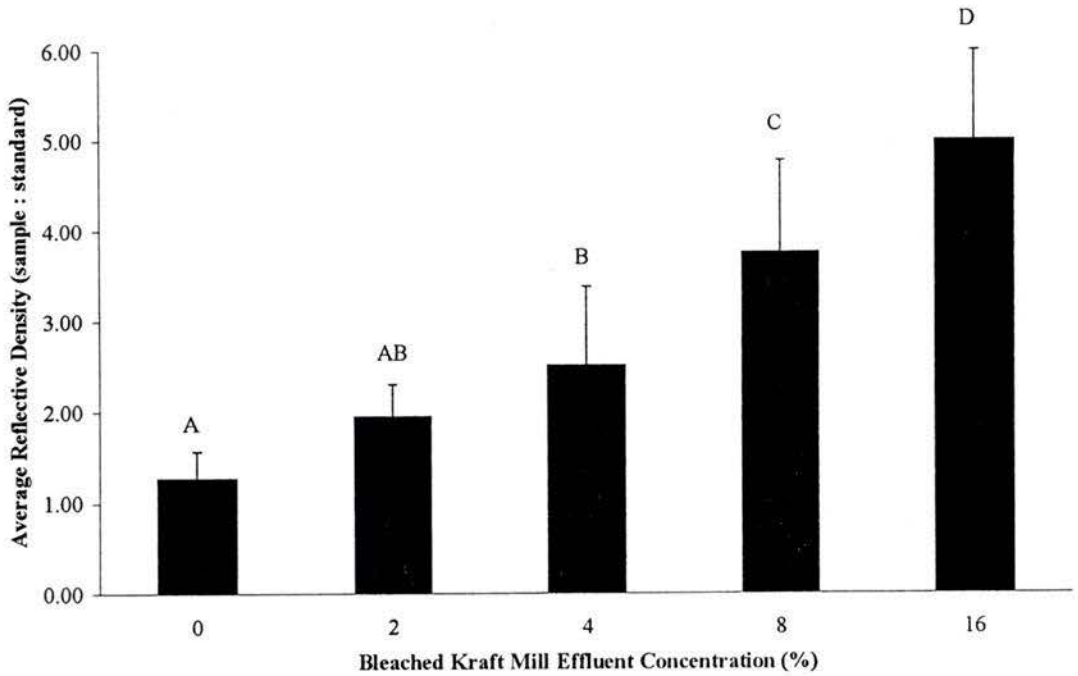

Figure 14 CYP 1A1 Density in Livers of Juvenile Chinook Salmon Exposed to BKME

Data shown are untransformed; log transformed data were used for analyses. Error bars represent standard deviation. Means with different letters differ significantly, $P<0.05$.

4.4.4 DNA Adducts

The DNA adduct data were log transformed for all analyses. The mean DNA adduct concentrations (non-transformed data) are shown in Figure 15. Fish from 0% and 2% BKME had mean DNA adduct concentrations of 18.58±4.54 and 20.83±10.10 nmol (mol nucleotides)$^{-1}$. Fish from 0 and 2% BKME were significantly different from those from 8 and 16% BKME ($p<0.005$). Fish from 4 and 8% BKME had mean DNA adduct concentrations of 27.12±13.92 and 38.18±18.02 nmol (mol nucleotides)$^{-1}$, respectively. These exposure groups were significantly different from 16% BKME fish (74.15±34.46) ($p<0.002$). The regression of DNA adduct

concentrations versus concentration of effluent was significant, (log (DNA adducts)= 0.04 × % concentration + 1.28, $p<0.001$, $R^2 = 0.57$).

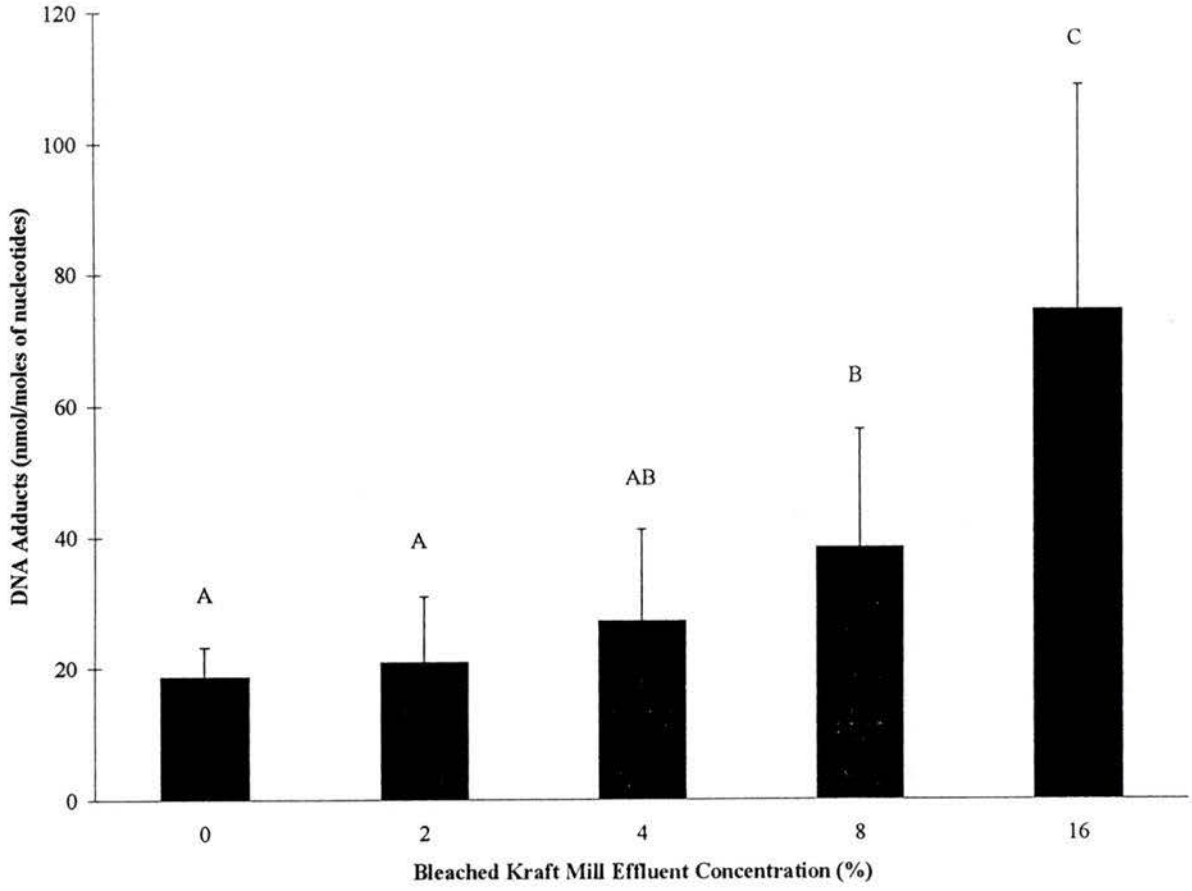

Figure 15 DNA Adducts in Livers of Juvenile Chinook Salmon Exposed to BKME

Data shown are untransformed; log transformed data were used for analyses. Error bars represent standard deviation.. Means with different letters differ significantly, $P<0.05$.

4.4.5 Bile Contaminants

Bile was collected and pooled for each treatment group. Two pools were analyzed for each concentration of effluent. None of the samples contained detectable PAH metabolites. Protein levels in the bile samples ranged from 5.9 - 9.1 mg ml^{-1}.

4.4.6 Carcass Contaminants

Carcasses were pooled to ≈30 g for high resolution gas chromatography mass spectrometry analysis for polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), mono-*ortho* substituted polychlorinated biphenyls (mo-PCBs) and coplanar substituted polychlorinated biphenyls (co-PCBs). One pool per site was analyzed. The non-detectable samples were set to the detection limit. Data are reported in pg g^{-1} and have been corrected for the percent recovery of the reference standard.

Toxic equivalent factors (TEFs) are conversion factors which relate the toxicity of organic contaminants to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This allows the contaminant burden to be expressed as one amount, the toxic equivalent quotient (TEQ). The TEQ is the sum of each congener, expressed in TCDD equivalents (pg g^{-1}). TEFs used (Table 8) were from NATO-CCMS (1988) and Ahlborg *et al.* (1994). Differences between treatments could not be tested as too few pools were available.

Table 8 Toxic Equivalent Factors (TEFs) for PCDDs, PCDFs and PCBs

PCDDs	TEF	co-PCBs	TEF
2,3,7,8-TCDD	1	PCB 77	0.0005
1,2,3,7,8-PeCDD	0.5	PCB126	0.1
1,2,3,4,7,8-HxCDD	0.1	PCB169	0.01
1,2,3,6,7,8-HxCDD	0.1		
1,2,3,7,8,9-HxCDD	0.1		
1,2,3,4,6,7,8-HpCDD	0.01		
OCDD	0.001		
PCDFs	TEF	mo-PCBs	TEF
2,3,7,8-TCDF	0.1	PCB123	0.0001
1,2,3,7,8-PeCDF	0.05	PCB118	0.0001
2,3,4,7,8-PeCDF	0.5	PCB114	0.0005
1,2,3,4,7,8-HxCDF	0.1	PCB105	0.0001
1,2,3,6,7,8-HxCDF	0.1	PCB167	0.00001
1,2,3,7,8,9-HxCDF	0.1	PCB156	0.0005
2,3,4,6,7,8-HxCDF	0.1	PCB157	0.0005
1,2,3,4,6,7,8-HpCDF	0.01	PCB189	0.0001
1,2,3,4,7,8,9-HpCDF	0.01		
OCDF	0.001		

Summarized from NATO-CCMS (1988) and Ahlborg *et al.*(1994).

In all contaminant classes, there was little difference between contaminant levels across the different effluent exposures. The PCDD concentrations were approximately 0.14 pg g^{-1} for all treatments (Figure 16). PCDFs ranged from 0.13 - 0.17 pg g^{-1} for all treatments (Figure 16). PCBs contributed much more to TEQ levels than either PCDDs and PCDFs. Mo-PCBs and co-PCBs ranged from 0.44- 0.53 and 0.47- 0.55 pg g^{-1} (Figure 17). Total TEQ and worst case total TEQ values ranged from 1.20- 1.31 and 5.49- 9.28 pg g^{-1}, respectively (Figure 18). PCBs (mo-PCBs and co-PCBs combined) accounted for 77% of the TEQ, while PCDDs and PCDFs

accounted for 11 and 12%, respectively. These contaminant concentrations are very similar to concentrations present in the fish before they were exposed to BKME (data not shown).

The total organic contaminant burden was calculated by summing the TEQ values of each class of congener. This number is a simple way to express contaminant load. The total burden of each site has been expressed in two ways, the total contaminant burden and the worst case total. The total contaminant burden is a sum of each TEQ for the PCDDs, PCDFs and both PCBs. The worst case total is a sum of the PCDDs, PCDFs and the worst case PCB values for each PCB class. For the mono-ortho PCBs, only 8 congeners have established TEFs, while 22 congeners were measured. For the coplanar PCBs, only 3 congeners have established TEFs, while 18 congeners were measured. For the PCB-TEQ calculations (Figure 17), only those congeners with a TEF are included. A second calculation was performed by substituting the largest TEF for all of the congeners lacking TEFs. This is the "worst case TEQ," where an unknown congener is assumed to be as toxic as the most toxic in its class. Although the worst case PCB data are not shown, they replaced the PCB-TEQs to calculate the worst case total.

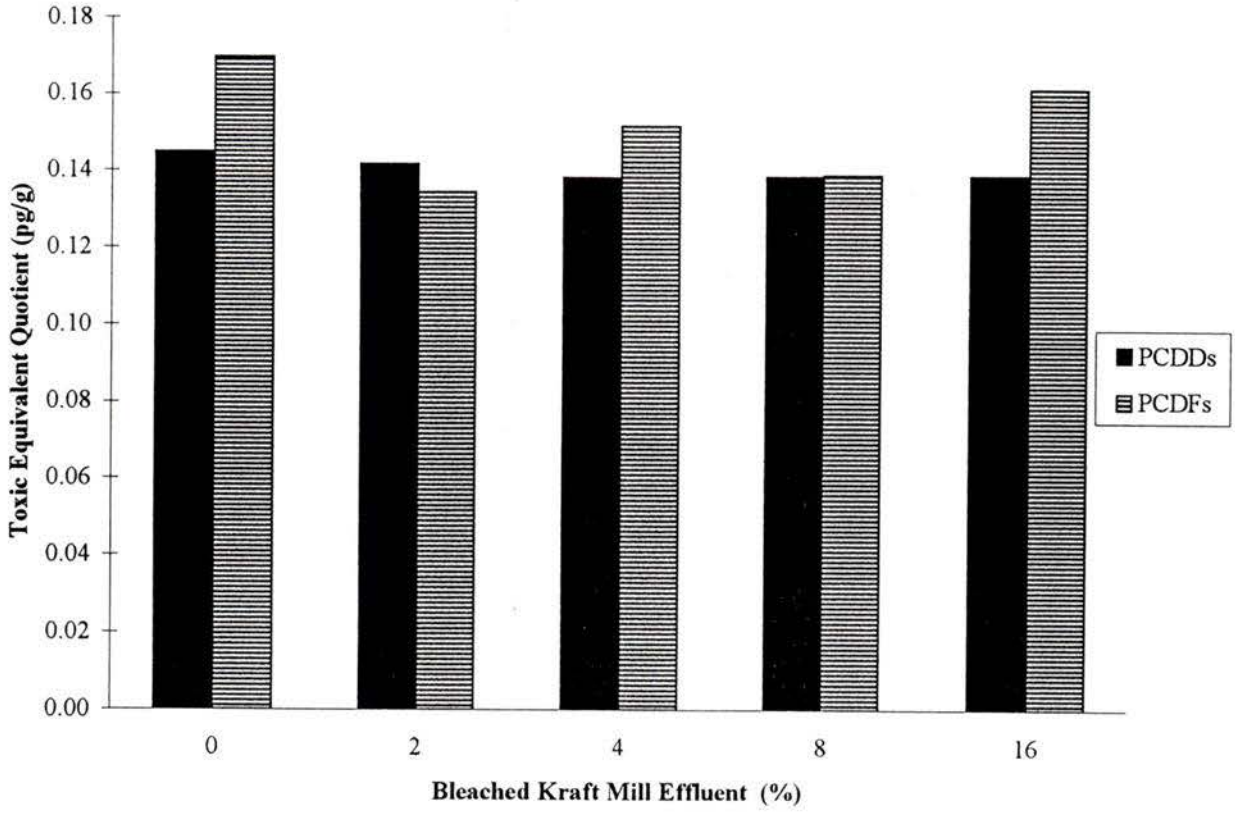

Figure 16 PCDDs and PCDFs in Carcasses of Juvenile Chinook Salmon Exposed to BKME

Data are expressed in TCDD equivalents; see text for explanation of calculation.

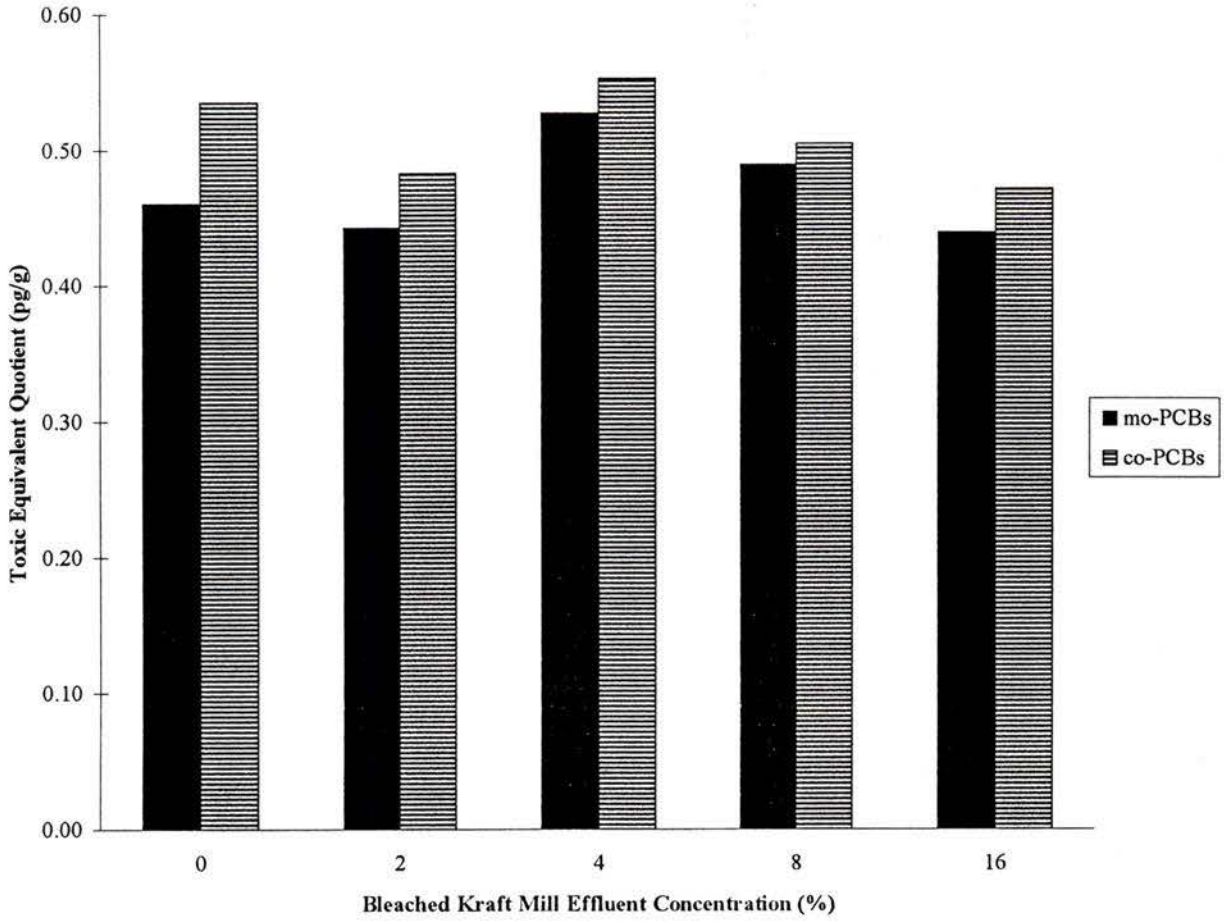

Figure 17 Mono-ortho and Coplanar PCBs in Carcasses of Juvenile Chinook Salmon Exposed to BKME

Data are expressed in TCDD equivalents; see text for explanation of calculations.

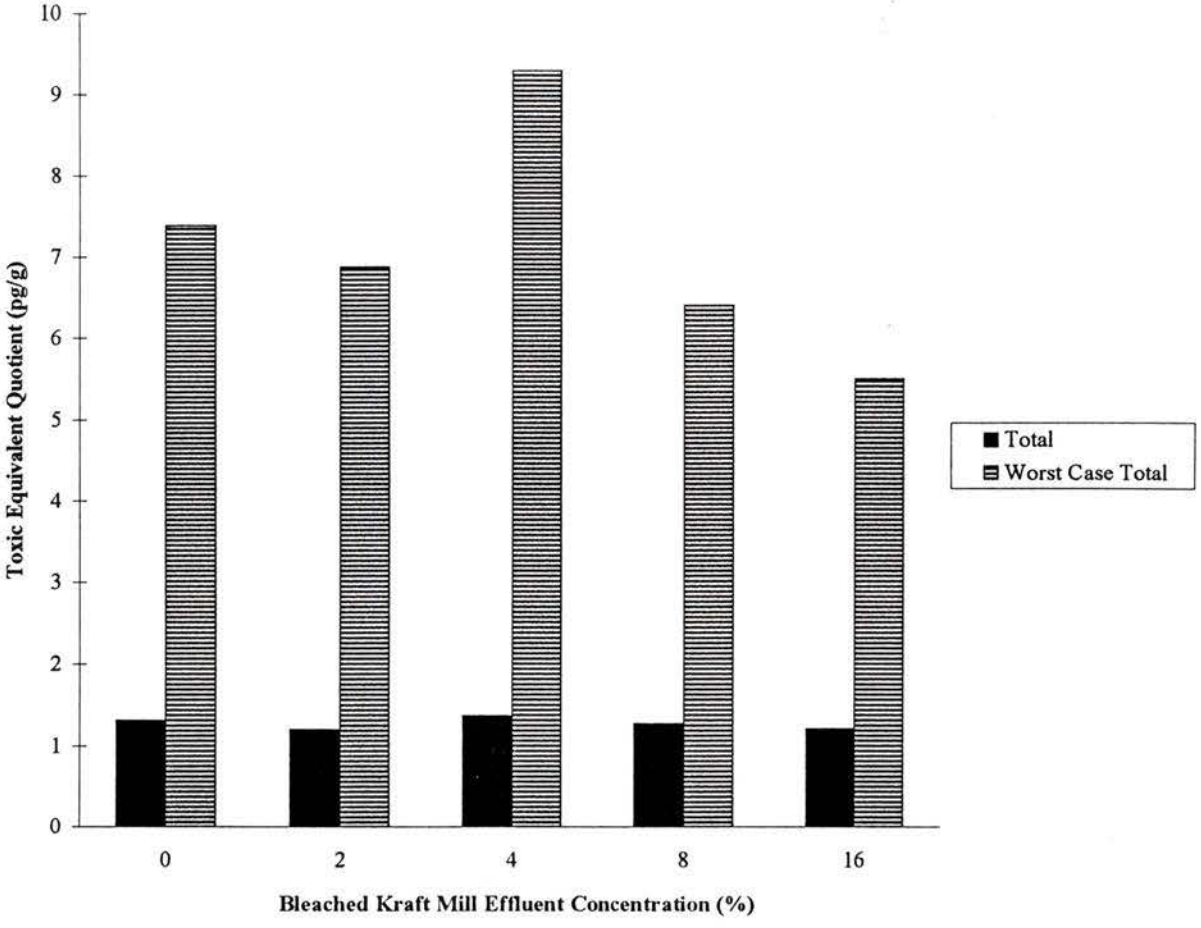

Figure 18 Total Contaminant Burden in Carcasses of Juvenile Chinook Salmon Exposed to BKME

Data are expressed in TCDD equivalents; see text for explanation of calculation.

4.4.7 Relationship Among Measurements

There was a significant correlation between EROD activity and both CYP 1A1 density and DNA adduct concentration (Table 9). A significant correlation between CYP 1A1 density and DNA adduct concentration was also found (Table 9). There were no significant correlations between the contaminant classes. Total TEQ was correlated with coplanar PCBs only. EROD activity was negatively correlated to PCDDs. No other correlation between a biological variable and contaminants was found.

Table 9: Correlation Matrix for Biological Variables

Correlations for Biological Variables								
	EROD	CYP 1A1	Adduct	PCDDs	PCDFs	mo-PCBs	co-PCBs	Total Burden
EROD	1.00	**0.65**	**0.45**	**-0.96**	-0.28	0.23	-0.34	-0.15
CYP 1A1		1.00	**0.64**	-0.82	-0.14	-0.06	-0.55	-0.36
Adduct			1.00	-0.65	0.13	-0.21	-0.56	-0.38

Based on N=46 for correlations between biological variables
Based on N=5 for correlations between biological variables and contaminants
bold coefficients are significant at $p<0.05$

4.5 *Discussion*

The literature on the topic of contaminant effects in fish exposed to BKME is quite diverse and includes such species as perch (*Perca fluviatilis*) (Andersson, *et al.*, 1988), suckers (*Catostomus* sp.) (Kloepper-Sams *et al.*, 1994; Munkittrick, *et al.*, 1991, 1992a, 1992b; Servos, *et al.*, 1992; Hodson *et al.*, 1992), whitefish (*Coregonus clupeaformis*) (Munkittrick *et al.*, 1992a, 1992b), and salmon (*Oncorhynchus* sp.) (Servizi *et al.*, 1993; Stein *et al.*, 1995; Burnison *et al.*, 1996; Hodson *et al.*, 1996). This diversity exists because each river system has its own particular community with dominant species that can be used as sentinel organisms for that watershed. In the Pacific Northwest, salmonids are of concern as they are an economically important species. Thus the literature discussed has been limited to focus on salmonids where comparisons are most meaningful. In addition there have been major changes in the pulping and effluent treatment process that have changed the composition of effluent and to a certain extent the effects in the fish downstream of mills. Therefore, the literature reviewed has also been limited to include only those studies on mills with similar basic processes and those completed after 1990. It should be noted that the changes in effluent characteristics have not necessarily resulted in changes in all effects downstream of pulp mills (Munkittrick *et al.*, 1992a). Chinook in the Fraser River have had large reductions in PCDD and PCDF concentrations, and EROD activity from the levels reported in Rogers *et al.* (1989). These reductions have been seen in fish collected from 1990 to present, after the new mill processes were installed (D. Martens and R. Gordon, personal communication).

The lengths and weights of the fish in these exposures were normally distributed. This is contrary to the field collections were food availability was thought to contribute to the non-normal distribution in weight (Levings and Lauzier, 1991) but in this study, food availability was controlled between tanks. Interestingly, the lengths and weights of the fish increase with increasing concentrations. There is no apparent reason why the fish would differ in size with concentration but this does lead to significant correlations between both length and weight and the biological measurements. It is unlikely that such small differences in size between treatment groups would significantly alter the biological responses measured. Immature rainbow trout (*O. mykiss*) between 1 and 30 grams showed no difference in EROD activity after exposure to 100% BKME (Hodson *et al.*, 1996).

EROD activity, CYP 1A1 density and DNA adduct concentrations were significantly correlated, but they did not necessarily produce the same trends. For EROD activity, significant differences between treatment groups were present in the lower concentration treatment groups only. For CYP 1A1 density, differences were seen in the fish exposed to higher concentrations of effluent. This suggests that EROD activity and CYP 1A1 density may produce different responses under certain circumstances. Field exposed chinook exhibited responses similar in both assays. Potentially, it is the range of concentrations that caused a varied response. Thus, when exposures cover a large range, the use of these responses in parallel would be preferable. DNA adduct concentrations seem to be similar in response to CYP 1A1 density and differed significantly at higher concentrations of effluent exposure only. This could be due to low concentrations of PAHs in the effluent, while CYP 1A1 density is caused by a combination of contaminants.

The relative EROD induction (1.4 - 2.6-fold over control) is similar to that seen in Servizi *et al.* (1993) where 0.3 - 4% effluent caused EROD activities 2-fold higher than controls. Other species have been found to have varying EROD levels after exposure to BKME, but this may be due to differences in age, sexual maturity, species response and effluent in the particular study. Laboratory exposure of rainbow trout to BKME resulted in EROD activities ranging from 1.3 (1% BKME) to 20 fold over control (100% BKME) (Hodson *et al.*, 1996). Rainbow trout exposed to 5% effluent gave an EROD response comparable to the highest response in this study (3-fold over control) (Hodson *et al.*, 1996).

The results indicate that at very low concentrations (2% effluent), significant EROD induction is detectable. The absolute EROD activities were much higher than those seen in the field and control fish (0% BKME) had higher activities than the highest EROD activity in field collected samples (chapter 3). The EROD activities in control fish were equivalent to those in baseline fish (i.e., before the exposure began). This high EROD activity is probably due to the high PCB contamination present in the fish before the effluent exposure began. Contaminated feed is suspected to be the source of PCBs to these fish. High PCB contamination has been seen in other samples of fish food (M. Ikonomou, personal communication) and food samples showed equivalent concentrations of contaminants as the those in the fish. However, the same lot and batch number were not available for testing and variation in the contaminant concentrations between batches is unknown. In future studies, where direct comparisons of contaminant levels and biological responses are being made to field data, samples of food should be analyzed for contaminants.

Considering the high EROD activities in fish exposed to 4, 8 and 16% BKME and the lack of difference between fish exposed to high concentrations, it is possible that maximum induction had occurred in fish exposed to ≥4% effluent. The combined field and laboratory data shown in Servizi *et al.* (1993) suggests that EROD activities may reach 50-fold over control without reaching maximum induction. If the entire contaminant burden in these fish was present before the study, the EROD induction caused by this contamination would be roughly 2-fold according to the dose-response curve in Servizi *et al.* (1993). Combined with the EROD activity from the BKME exposure, the total would be only 5-fold induction (induction due to pre-exposure contaminants + induction due to BKME). This is considerably less than the 50-fold induction suggested by Servizi *et al.* (1993). Of course this assumes that the EROD activities would be additive. It would be valuable to perform a complete dose-response curve for EROD activity in chinook.

CYP 1A1 densities for 0, 2 and 4% BKME were similar to densities found in the field (chapter 3). The control samples had very similar densities in both studies. Fish from Northwoods had a mean CYP 1A1 density of 2.24 RD which is similar to those fish from 2 and 4% BKME. Why a large increase in EROD induction is not paralleled by large changes in CYP 1A1 density is unknown. At 16% BKME, the CYP 1A1 density is considerably larger than the

field collections. CYP 1A1 densities in fish before the exposures began were identical to those exposed to 0% effluent. There are no significant differences in CYP 1A1 density between 0 and 2% BKME and 2 and 4% BKME exposed fish. This is perhaps because larger differences in protein must be present for adequate differentiation between samples. If a single sample is blotted in different amounts, only those lanes with approximately double the protein will appear different (J. Wilson, unpublished data). This suggests that samples that are less than 2-fold different will appear to be the same.

DNA adduct concentrations indicate that exposure to high molecular weight PAHs (4 or more rings) occurred during the experiment. The assay was not performed on samples collected before the exposure started. The DNA adducts were not significantly different at low concentrations, but were different among fish exposed to higher concentrations. This may indicate that the concentration of PAHs in the effluent is fairly low and therefore higher exposures are needed to produce a significant effect. These adduct concentrations are higher than those found in fish collected from the field. The control fish were much higher than all but those fish collected at one site (Stoner, chapter 3) in the field. Most laboratory experiments involve extracted sediment exposure in flatfish and thus comparisons are difficult.

The contaminants present in the fish exposed to BKME seem to be equivalent to concentrations found in pre-exposure fish. The PCBs were particularly high and collectively accounted for 77% of the total TEQ. In contrast, in the field collected chinook PCBs accounted for only 27% of the total TEQ (chapter 3). The PCB concentrations were approximately 10 (mo-PCBs) and 5 (co-PCBs) fold higher than chinook collected in the field (chapter 3). It seems that there is no identifiable contribution of the BKME exposure to the contaminant concentrations measured in the chinook. This indicates that the effects seen in the chinook in this experiment are most likely caused by something other than a PCDD, PCDF or PCB although they may account for the higher EROD activity seen in the 0% exposed fish. This finding is similar to Servizi *et al.* (1993) where after 60 days of exposure to 1.5 and 4 % BKME, there was no difference in concentrations of contaminants compared to control fish. TCDD and TCDF concentrations in the effluent have been measured at 0 and 0.65 ppq, respectively. With these concentrations, the expected accumulation of these congeners can be estimated based on a 60% efficiency of extraction of lipophilic contaminants from water by ventilation (McKim *et al.*, 1985) and a

ventilation rate of 150 ml kg^{-1} min^{-1} (rainbow trout at 11-12°C) (McKim and Heath, 1983). After 30 days, each fish would have been exposed to roughly 60 L of water, based on an average weight of 8.78 g. At 2% and 16% BKME exposure, this translates into 0.5 and 4 femtograms of TCDF potentially bioaccumulated. TCDD bioaccumulation cannot be calculated but it would be less than that of TCDF. In each of these cases, contaminant accumulation was not high enough to result in significant induction.

Bile concentrations of PAH metabolites were non-detectable. As there was no dilution in the bile before the analysis, either there was no significant PAH exposure or this technique is unsuitable for PAHs associated with pulp mill effluent. Since there are significant DNA adducts in fish exposed to the bleached kraft mill effluent, it is expected that PAH metabolites should be measurable in bile from 8 and 16% BKME exposed fish. PAHs have not been identified in another study of juvenile chinook exposed to BKME. Servizi *et al.* (1993) found no PAHs in the carcasses of juvenile chinook after 60 and 144 days of BKME exposure but they are probably not be identifiable in the carcass because of rapid metabolism. Additional testing of bile for PAH metabolites is needed to confirm that PAH exposure has occurred.

4.6 *Conclusions*

EROD activity was found to be induced in fish exposed to greater than 2% BKME. Fish exposed to higher concentrations of effluents (4, 8 and 16% BKME) were not significantly different. The absolute EROD activities were high even in the control and pre-exposure fish, which coupled with the lack of difference at the high concentrations, indicates that maximum EROD may have been reached in this exposure. A complete dose-response curve in juvenile chinook would be needed to confirm this hypothesis. CYP 1A1 density was not significantly different in response between the control and 2% and 2% and 4% BKME exposed fish. This is most likely due to the inability of this assay to detect minimal changes in protein concentrations. Samples that are not 2 fold different would rarely be found statistically different. Contrary to EROD activity, the absolute CYP 1A1 density is not elevated over other samples processed in this lab. Only the 16% effluent exposed fish have values that are high compared to other chinook samples. This is an interesting finding and reinforces the need to use these measurements in

parallel. Apparently our understanding of the mechanisms of EROD activity and the CYP 1A1 system is not absolute and that an increase in EROD activity will not always be paralleled by an increase in CYP 1A1. Considering the complexity of the system responsible for enzyme activity, including co-factors and the components of the oxidative process, it is not surprising that these measurements do not produce the same results in all circumstances. DNA adducts were significantly greater than control fish in those exposed to 8 and 16% BKME only. This suggests that PAH concentrations are low in the effluent and thus higher exposures are required to significantly increase them. This finding contrasts to field work where a significant increase in DNA adducts was seen at sites were the concentrations of effluent would be much less than those examined in this study. Of course in the field situation, this effluent is not the only contributor to contaminants in the river and it may be that PAH contributions from other sources may be sufficient to increase adduct concentrations. Since bile analysis did not detect any PAH metabolites at any concentration of effluent, conclusions regarding the usefulness of this assay from chapter 3 are confirmed. HPLC analysis of bile seems to be required to detect PAH exposure from a pulp mill source.

Contaminant concentrations are fairly low, with the exception of PCBs which are present at concentrations considerably higher than field exposed chinook. However, these contaminants were present in fish before the exposure occurred. There has been no bioaccumulation of PCDDs, PCDFs and PCBs in this study. Thus the contaminants that are causing the biological effects seen in these fish were not amongst those measured in this study.

5. General Discussion and Conclusions

There are several assumptions for the comparison of the field collected fish and the BKME exposed fish. First, the feeding component of experimentally exposed fish is very different from the field situation. Considering that the primary route of uptake for PCDDs and PCDFs appears to be dietary and may account for as much as 99% of the total burden (Batterman *et al.*, 1989 and Muir 1988) this is an important distinction. Fish in the field consume organisms which may have bioaccumulated contaminants from the effluent. The effluent plume, which is warmer than the Fraser River during winter months, may serve as an important source of food in winter (Levings and Lauzier, 1991). If this is true, then fish at Northwoods should be at higher risk for exposure to organic contaminants through the food chain. It is possible that a proportion of this food component could be included in the experimental fish when organic contaminants adsorb to the food pellets, but this does not account for the total food chain component.

Second, the biological responses in field collected fish are assumed to be pulp mill derived, particularly at the Northwoods site. At other sites this may not be true as municipal outfalls and non-point sources may account for a large proportion of the contaminant burden. Third, in comparing these responses, we assume that the absolute values are comparable because the assays were performed in the same manner. Fourth, the effluent characteristics and river conditions are assumed to be similar over different years of study. For example, the aerial photographs of the effluent plume, the conductivity measurements for estimating effluent concentrations, the field collections and the experiment were all performed in different years. Although there will be some year-to-year variability in river conditions such as temperature and flow, they are assumed to be minimal. The effluent dispersion should be identical between years. Fifth, it is assumed that the length of exposure is enough to simulate the field exposure. This is probably not true for the bioaccumulated contaminants which require a greater length in time. In a study by Servizi *et al.* (1993), bioaccumulation was only evident after 144 days of exposure to BKME. Sixth, hatchery reared chinook, which are potentially a different race of chinook, are assumed to respond to contaminants in the same way as Fraser River chinook.

The EROD activities and DNA adduct concentrations seen in field collected fish and experimentally exposed fish were very different. The EROD activities and DNA adduct concentrations of fish exposed to 0% BKME were higher than fish collected from any site on the Fraser River. This indicates that some other exposure has occurred before the experiment began. Interestingly, the CYP 1A1 densities do not exhibit this increase in response. In fact, the CYP 1A1 densities from BKME exposed fish compare very well with the field collected fish. This indicates that the relationship between EROD activity and CYP 1A1 density are not directly linear. More information is needed to understand the complex relationship between these two measurements. DNA adduct concentrations would be expected to relate with EROD activity since the formation of reactive metabolites is directly dependent on the functional activity of the enzyme. In order to compare the biological responses, relative increases (i.e. fold over control) will need to be used.

The controlled field experiment was performed to identify if BKME could induce significant responses in juvenile chinook at concentrations found at the Northwoods site on the Fraser River. The concentrations of effluent at the site where fish were collected were thought to be 12 - 14% BKME based on conductivity measurements of the river water (G. Kruzynski, personal communication). Based on aerial photographs of the river, the effluent plume has been shown to disperse directly through the site where the fish were collected. This indicates that if mobility was limited, the fish collected from the Fraser River should exhibit biological responses equivalent to those fish exposed to 8 or 16% BKME. However, the fish exposed to 8 and 16% BKME had much larger biological responses compared to the fish collected from Northwoods. By comparing relative biological responses (i.e. fold increase over control fish) from each study, the fish from the field had EROD activities and CYP 1A1 densities equivalent to fish exposed to 2 and 4% BKME, respectively. Only DNA adduct concentrations seemed to be similar to the Northwoods fish at 8% effluent. In all biological responses, fish exposed to 16% effluent exhibited very different responses than the field samples. This suggests that the fish captured at Northwoods were not exposed to 12 - 14% BKME but were exposed to much lower concentrations of effluent.

Contaminant concentrations in both field and experimentally exposed fish were low and there was little variation between exposed groups in each study. The concentrations were larger

in the experimentally exposed fish when total contaminants are compared. This increase is due to higher concentrations of mo-PCBs. Contaminant distribution in the two studies are outlined in Table 10. This indicates that the exposures to these contaminant classes were very different in the two studies, with higher proportion of PCBs in the experimentally exposed fish. However, the contaminant concentrations seen in the experimentally exposed fish were nearly identical to the concentrations found in the fish before the exposure began and in a sample of the brand of fish food used at the hatchery. Therefore, the contaminants in the experimentally exposed fish are not due to BKME exposure, but likely due to food contamination. Although fish in the Fraser River do bioaccumulate PCDDs, PCDFs and PCBs, the concentrations are too low to account for the biological effects measured. Servizi *et al.* (1993) found that concentrations of 3 pg g^{-1} were required to induce EROD activity. Studies by Parrott *et al.* (1995b) in the rainbow trout suggest that 16 pg g^{-1} may be required to induce EROD activity. TEQ concentrations were lower than these thresholds in the Fraser River fish. It is likely that there is some component of BKME, unmeasured in these studies, which is the cause of the biological effects. This component must be water soluble, otherwise no biological effects would be seen in the experimentally exposed fish. This finding supports other studies of bleach kraft mill effluent that suggest that inducer components of effluent would be water soluble and highly metabolized (Munkittrick *et al.*, 1992a). It is suggested that the inducers are likely planar, aromatic PAHs with a low degree of chlorine substitution based on the K_{ow} of fractions of effluent that still induce EROD activity in rainbow trout (Burnison *et al.*, 1996).

Table 10 Contribution of Each Contaminant Class to Total Burden

	Percent Total Burden	
Contaminant Class	Field Study	BKME Study
PCDDs	49	11
PCDFs	27	12
mo-PCBs	6	37
coPCBs	21	40

The BKME from Northwoods was capable of causing induction in chinook at concentrations as low as 2%. Fish from the Northwoods field site had EROD activities that would suggest the exposure to be lower than 2%. The fish caught at this site in 1995 must either have been more mobile than previously thought or had just recently moved into the site and thus been newly exposed to effluent. In the future, conductivity measurements of the site should be taken at the same time as the fish are caught. Caged fish experiments or exposure of fish to river water from the capture site would help to confirm this conclusion. In either case, mobility would be controlled and exposure of the fish to the concentrations of effluent the field fish are captured in would be assured.

5.*1* ***General Conclusions:***

Chinook exposed to BKME in the field and laboratory exhibit biological responses that suggest non-lethal toxicity. The long term effects of the biological changes measured are unclear.

There was some contaminant in both the field and experimentally exposed fish that is inducing the cytochrome P450 1A1 system and forming DNA adducts. There was a lack of correlation between contaminant concentrations and biological effects in the field exposures. In addition, there was no bioaccumulation of contaminants in the experimentally exposed fish. These results indicate that the contaminant responsible for the effects seen was not a PCDD, PCDF or PCB. The presence of DNA adducts in both the field and experimentally exposed chinook suggest a high molecular weight PAH molecule.

Future studies should focus experimentally on identifying the compound(s) responsible for these effects. Studies examining the distribution of these compound(s) in the Fraser River would be important to understand the downstream trends of biological effects seen in chinook.

Methods must be chosen carefully to address this problem. BKME derived PAHs cannot be measured by synchronous scan fluorimetry. HPLC analysis of bile is suggested. DNA adduct

concentrations are useful for biomonitoring BKME exposure in the field. Strong correlations were found between DNA adducts and measurements of the CYP 1A1 system. Under certain circumstances, the trends in EROD activity and CYP 1A1 densities do not agree. There is a general agreement between increasing EROD activity and increasing CYP 1A1 density as these measurements were positively correlated in both studies. It is suggested that these measurements be used together.

References

Abbott, J.D., and Hinton, S.W. 1996. Trends in 2,3,7,8-TCDD concentrations in fish tissues downstream of pulp mills bleaching with chlorine. Environmental Toxicology and Chemistry 15:1163-1165.

Addison, R.F., and Edwards, A.J. 1988. Hepatic microsomal mono-oxygenase activity in flounder *Platichthys flesus* from polluted sites in Langesundfjord and from mesocosms experimentally dosed with diesel oil and copper. Marine Ecology Progress Series 46: 51-54.

Addison, R.F., Zinck, M.E., and Willis, D.E. 1981. Time and dose-dependence of hepatic mixed function oxidase activity in brook trout *Salvelinus fontinalis* on pholychlorinated biphenyl residues: implications for 'biological effects monitoring'. Environmental Pollution (Series A) 25:211-218.

Ahlborg, U.G., Becking, G.C., Birnbaum, L.S., Brouwer, A., Derks, H.J.G.M., Feeley, M., Golor, G., Hanberg, A., Larsen, J.C., Liem, A.K.D., Safe, S.H., Sclatter, C., Wærn, F., Younes, M., and Yrjänheikki, E. 1994. Toxic equivalency factors for dioxin-like PCBs. Chemosphere 28:1049-1067.

Andersson, T., Förlin, L., Härdig, J., and Larsson,Å. 1988. Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. Canadian Journal of Fisheries and Aquatic Sciences 45:1524-1536.

Ariese, F., Kok, S.J., Verkaik, M., Gooijer, C., Velthorst, N.H., Hofstraat, J.W. 1993. Synchronous fluorescence spectrometry of fish bile: A rapid screening method for the biomonitoring of PAH exposure. Aquatic Toxicology 26: 273-286.

Arlotto, M., and Parkinson, A. 1989. Identification of cytochrome P450a (P450IIAI) as the principal testosterone 7α-hydroxylase in rat liver microsomes and its regulation by thyroid hormones. Archives of Biochemistry and Biophysics 270: 458-471.

Batterman, A.R., Cook, P.M., Lodge, K.B., Lothenbach, D.B., and Butterworth, B.C. 1989. Methodology used for a laboratory determination of relative contributions of water, sediment, and food chain routes of uptake for 2,3,7,8-TCDD bioaccumulation by lake trout in Lake Ontario. Chemosphere 19:451-458.

Beach, A.C., and Gupta, R.C. 1992. Human biomonitoring and the ^{32}P-postlabeling assay. Carcinogenesis 13:1053-1074.

Birnbaum, L.S. 1994. Mechanism of dioxin toxicity: relationship to risk assessment. Environmental Health Perspectives Supplement 9:157-167.

Birtwell, I.K., Levings, C.D., Macdonald, J.S., and Rogers, I.H. 1988. A review of fish habitat issues in the Fraser River system. Water Pollution Research Journal of Canada 23:1-30.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Burnison, B.K., Hodson, P.V., Nuttley, D.J. and Efler, S. 1996. A bleached kraft mill effluent fraction causing induction of a fish mixed-function oxygenase enzyme. Environmental Toxicology and Chemistry 15:1524-1531.

Chang, L.W., Hsia, S.M., Chan, P., and Hsieh, L. 1994. Macromolecular adducts: biomarkers for toxicity and carcinogenesis. Annual Review of Pharmacology and Toxicology 34:41-67.

Collier, T.K, Anulacion, B.F., and Myers, M.S. 1996. Relationships between biomarkers of contaminant exposure and toxicopathic lesions in marine fish. Paper presented at Second Society

of Environmental Toxicology and Chemistry World Congress (16th Annual Meeting). November 5-9, Vancouver, British Columbia.

Collier, T.K., and Varanasi, U., 1991. Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in English sole (*Parophrys vetulus*) exposed to environmental contaminants. Archives of Environmental Contamination and Toxicology 20:462-473.

Emmett, B. Kruzynski, G.M., and Birtwell, I.K. 1996. The use of a pulp mill effluent mixing zone during winter by juvenile Chinook salmon in the Fraser River at Prince George, British Columbia. Canadian Manuscript Report of Fisheries and Aquatic Sciences 2341: 38p.

Fraser, F.J., Starr, P.J., and Fedorenko, A.Y. 1982. A review of the chinook and coho salmon of the Fraser River. Canadian Technical Report of Fisheries and Aquatic Sciences 1126: 130p.

Goksøyr, A., Husøy, A. Larsen, H.E., Klungsøyr, J., Wilhelmsen, S., Maage, A., Brevik, E.M., Andersson, T., Celander, M., Pesonen, M., and Förlin, L. 1991. Environmental contaminants and biochemical responses in flatfish from the Hvaler Archipelago in Norway. Archives of Environmental Contamination and Toxicology 21:486-496.

Gonzalez, F.J. 1989. The molecular biology of cytochrome P450s. Pharmacology Reviews, 40:243-288.

Grondlund, W.D., Chan, S., McCain, B.B., Clark, R.C. Jr., Myers, M.S., Stein, J.E., Brown, D.W., Landahl, J.T., Krahn, M.M., and Varanasi, V. 1991. Multidisciplinary assessment of pollution at three sites in Long Island Sound. Estuaries 14:299-305.

Guengerich, F.P. 1992. Metabolic activation of carcinogens. Pharmacology and Therapeutics 54:17-61.

Guengerich, F.P. 1993. Cytochrome P450 enzymes. American Scientist 81:440-447.

Gupta, R.C. 1993. ^{32}P-Postlabeling analysis of bulky aromatic adducts. IN: Postlabeling methods for detection of DNA adducts. D.H. Phillips, M.Castegnaro and H. Bartsch (eds.) IARC Scientific Publication No. 124. Oxford University Press, Oxford.

Hall, T.J., Haley, R., Borton, D.L., Walsh, A.H. and Wolke, R.E. 1992. Histopathology of rainbow trout (*Oncorhynchus mykiss*) after long-term exposure to biologically treated bleached kraft mill effluent in experimental stream channels. Canadian Journal of Fisheries and Aquatic Sciences 49:939-944.

Harshbarger, J.C., and Clark, J.B. 1990. Epizootiology of neoplasms in bony fish of North America. The Science of the Total Environment 94:1-32.

Hinton, D.E., Baumann, P.C., Gardner, G.R., Hawkins, W.E., Hendricks, J.D., Murchelano, R.A., and Okihiro, M.S.. 1992. Histopathologic biomarkers. IN: Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress. R.J. Huggett, R.A. Kimerle, P.M. Mehrle and H.L.Bergman (Eds). Proceedings of the Eighth Pellston Workshop. SETAC Special Publications Series. Lewis Publishers. pp. 155-209.

Hodson, P.V., Efler, S., Wilson, J.Y., El-Shaarawi, A., Maj. M., and Williams, T.G. 1996. Measuring the potency of pulp mill effluent for induction of hepatic mixed function oxygenase activity in fish. Journal of Toxicology and Environmental Health 49(1):83-110.

Hodson, P.V., Kloepper-Sams, K.R., Munkittrick, K.R., Lockhart, W.L., Metner, D.A., Luxon, P.L., Smith, I.R., Gagnon, M.M., Servos, M., and Payne, J.F. 1991. Protocols for measuring mixed function oxygenases of fish liver. Canadian Technical Report of Fisheries and Aquatic Sciences 1829: 49p.

Hodson, P.V., McWhirter, M., Ralph, K., Gray, B., Thivierge, D., Carey, J.H., Van Der Kraak, G., Whittle, D.M., and Levesque, M. 1992. Effects of bleached kraft mill effluent on fish in the St. Maurice River, Quebec. Environmental Toxicology and Chemistry 11:1635-1651.

Khan, R.A., and Kiceniuk, J.W. 1988. Effect of petroleum aromatic hydrocarbons on monogeneids parasitizing atlantic cod, *Gadus morhua.* L. Bulletin of Environmental Contamination and Toxicology 41:94-100.

Klopper-Sams, P.J., Swanson, S.M., Marchant, T., Schryer, R., and Owens, J.W. 1994. Exposure of fish to biologically treated bleached-kraft effluent. 1. Biochemical, physiological and pathological assessment of rocky mountain whitefish (*Prosopium williamsoni*) and longnose sucker (*Catostomus castostomus*). Environmental Toxicology and Chemistry 13:1469-1482.

Krahn, M.M., Burrows, D.G., MacLeod, W.D. Jr., and Malins, D.C. 1987. Determination of individual metabolites of aromatic compounds in hydrolyzed bile of English Sole (*Parophrys vetulus*) from polluted sites in Puget Sound, Washington. Archives of Environmental Contamination and Toxicology 16:511-522.

Lehtinen, K.J., Notini, M., and Landner, L. 1984. Tissue damage and parasite frequency in flounders, *Platichtys flesus* L. chronically exposed to bleach kraft pulp mill effluents. Annales Zoologici Fennici. 21: 23-28.

Levings, C.D., and Lauzier, R.B. 1991. Extensive use of the Fraser River basin as winter habitat by juvenile chinook salmon (*Oncorhynchus tshawytscha*). Canadian Journal of Zoology 69:1759-1767.

MacDonald, D.D.; Ikonomou, M.G., Rantalainen, A-L., Rogers, I.H., Sutherland, D., and van-Oostdam, J. 1996. Contaminants in white sturgeon (*Acipenser transmontanus*) from the upper Fraser River, British Columbia. Environmental Toxicology and Chemistry, In Press.

McCain, B.B., Brown, D.W., Hom, T., Myers, M.S., Pierce, S.M., Collier, T.K., Stein, J.E., Chan, S., and Varanasi, U. 1996. Chemical contaminant exposure and effects in four fish species from Tampa Bay, Florida. Estuaries, 19(1):86-104.

McCain, B.B., Myers, M.S., Varanasi, U., Brown, D.W., Rhodes, L.D., Gronlund, E.D., Elliot, D.G., Palsson, W.A., Hodgins, H.D., and Malins, D.C. 1982. Pathology of two species of flatfish from urban estuaries in Puget Sound.. Interagency Energy -Environment Research and Development Program Report, EPA-600/7-82-001. Washington D.C.: Environmental Protection Agency.

McKim, J.M., and Heath, E.M. 1993. Dose determinations for waterborne 2,5,2',5'-[^{14}C]Tetrachlorobiphenyl and related pharmacokinetics in two species of trout (*Salmo gairdneri* and *Salvelinus fontinalis*): A mass balance approach. Toxicology and Applied Pharmacology 68:177-187.

McKim, J., Schmieder, P., and Veith, G. 1985. Absorption dynamics of organic chemical transport across trout gills as related to octanol-water partition coefficient. Toxicology and Applied Pharmacology 77:1-10.

McLean, M.R., Robertson, L.W., and Gupta, R.C. 1996. Detection of PCB Adducts by the ^{32}P-postlabeling technique. Chemical Research in Toxicology 9:164-171.

Muir, D.G. 1988. Bioaccumulation and effects of chlorinated dibenzodioxins and furans in fish, shellfish and crustacea: a brief review. Internal report prepared at the request of the Director, Physical and Chemical Sciences (Burlington, ON) for the Director, Oceanography, and Contaminants Branch, Department of Fisheries and Oceans, Ottawa, ON.

Munkittrick, K. and McCarty, L.S. 1995. A framework for detecting and identifying fish population responses: top-down, bottom-up or middle-out? Paper presented at Second Society of

Environmental Toxicology and Chemistry World Congress (16th Annual Meeting). November 5-9, Vancouver, British Columbia.

Munkittrick, K.R., Portt, C.B., McMaster, M.E., Van Der Kraak, G.J., Smith, I.R., and Dixon, D.G. 1992a. Changes in maturity, plasma sex steroid levels, hepatic mixed function oxygenase activity, and the presence of external lesions in Lake Whitefish (*Coregonus clupeaformis*) exposed to bleached kraft mill effluent. Canadian Journal of Fisheries and Aquatic Sciences 49:1560-1569.

Munkittrick, K.R., Portt, C.B., Van Der Kraak, G.J., Smith, I.R., and Rokosh, D.A. 1991. Impact of bleached kraft mill effluent on population characteristics, liver MFO activity, and serum steroid levels of a Lake Superior white sucker (*Catostomus commersoni*) population. Canadian Journal of Fisheries and Aquatic Sciences 48:1371-1380.

Munkittrick, K.R., Van Der Kraak, G.J., McMaster, M.E., and Portt, C.B. 1992b. Response of hepatic MFO activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. Environmental Toxicology and Chemistry 11:1427-1439.

Myers, C., Sutherland, L., Haasch, M., and Lech, J. 1993. Antibodies to a synthetic peptide that reacts specifically with rainbow trout hepatic cytochrome P450 1A1. Environmental Toxicology and Chemistry 12:1619-1626.

Myers, M.S. 1991. Relationships between hepatic neoplasms and related lesions and exposure to toxic chemicals in marine fish from the U.S. west coast. Environmental Health Perspectives 90:7-15.

Myers, M.S., Rhodes, L.D., and McCain B.B. 1987. Pathologic anatomy and pattern of occurrence of hepatic neoplasms, putative preneoplastic lesions and other idiopathic hepatic conditions in English sole (*Parophrys vetulus*) from Puget Sound, Washington. Journal of the National Cancer Institute 78: 333-363.

NATO-CCMS NATO Committee on the Challenges of Modern Society. 1988. International toxicity equivalency factor (I-TEF) method of risk assessment for complex mixture of dioxins and related compounds. NATO-CCMS Report 176.

Nestrick, T.J. and Lamparski, L.L. 1982. Isomer-specific determination of chlorinated dioxins for assessment of formation and potential environmental emission from wood combustion. Analytical Chemistry, 54:2292-2299.

Northcote, T.G. 1974. Biology of the lower Fraser River: a review. Westwater Research Centre, University of British Columbia, Vancouver Technical Report 3: 94p.

Parrott, J.L., Hodson, P.V., and Dixon, D.G. 1995a. Rainbow trout hepatic mixed-function oxygenase induction by polychlorinated dibenzo-*p*-dioxins (PCDDs) as a function of time and tissue concentration. Journal of Toxicology and Environmental Health 46:301-316.

Parrott, J.L., Hodson, P.V., Servos, M.R., Huestis, S.L., and Dixon, D.G. 1995b. Relative potency of polychlorinated dibenzo-*p*-dioxins and dibenzofurans for inducing mixed-function oxygenase activity in rainbow trout. Environmental Toxicology and Chemistry 14:1041-1050.

Rantalainen, A-L., Ikonomou, M.G., and Rogers, I.H. 1996. Lipid containing semipermeable membrane devices (SPMDs) as concentrators of toxic chemical in the lower Fraser River, Vancouver, British Columbia. Chemosphere, Submitted.

Reichert, W.L., and French, B. 1994. The ^{32}P-postlabeling protocols for assaying levels of hydrophobic DNA adducts in fish. U.S. Department of Commerce, National Oceanic and Atmospheric Administration Technical Memoradum NMFS-NWFSC-14, 89p.

Reichert, W.L., Stein, J.E., French, B., Goodwin, P. and Varanasi, U. 1992. Storage phosphor imaging technique for the detection and quantitation of DNA adducts measured by the ^{32}P-postlabeling assay. Carcinogenesis 13:1475-1479.

Renton, K.W., and Addison, R.F. 1992. Hepatic microsomal mono-oxygenase activity and P450IA mRNA in North Sea dab *Limanda limanda* from contaminated sites. Marine Ecology Progress Series 46: 65-69.

Rogers, I.H., Levings, C.D., Lockhart, W.L., and Norstrom, R.J. 1989. Observations on overwintering juvenile chinook salmon (*Oncorhynchus tshawytscha*) exposed to bleached kraft mill effluent in the upper Fraser River, British Columbia. Chemosphere 19:1853-1868.

Ryan, D.E., Thomas, P.E., and Levin, W. 1982. Purification and characterization of a minor form of hepatic microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls. Archives of Biochemistry and Biophysics 216: 272-288.

Scrivener, J.C., Brown, T.G., and Andersen, B.C. 1993. Juvenile chinook salmon (*Oncorhynchus tshawytscha*) utilization of Hawks Creek, a small and nonnatal tributary of the upper Fraser River. Canadian Journal of Fisheries and Aquatic Sciences 51:1139-1146.

Servizi, J.A., Gordon, R.W., Martens, D.W., Lockhart, W.L., Metner, D.A., McBride, J.R., Norstrom, R.J. 1993. Effects of biotreated bleached kraft mill effluent on fingerling chinook salmon (*Oncorhynchus tshawytscha*). Canadian Journal of Fisheries and Aquatic Sciences 50:846-857.

Servos, M.R., Carey, J.H., Ferguson, M.L., Van Der Kraak, G., Ferguson, H., Parrott, J., Gorman, K., and Cowling, R. 1992. Impact of a modernized blached kraft mill on white sucker populations in the Spanish River, Ontario. Water Pollution Research Journal of Canada 27:423-437.

Slaney, T.L., Hyatt, K.D., Northcote, T.G., and Fielden, R.J. 1996. Status of anadromous salmon and trout in British Columbia and Yukon. Fisheries, 21(10):20-35.

Sorensen E.M.B., Ramirez-Mitchell R., Harlan C.W., and Bells, J.S. 1980. Cytological changes in the fish liver following chronic environmental arsenic exposure. Bulletin of Environmental Contamination and Toxicology 25: 93-99.

Stegeman, J.J. 1993. The cytochromes P450 in fish. pp. 137-158 IN: Molecular Biology Frontiers; Biochemistry and molecular biology of fishes, Vol.2. P.W. Hochachka and T.P. Mommsen (eds.) Elsevier Science Publishers. New York.

Stegeman, J.J., Woodin, B.R., and Goksøyr, A. 1988. Apparent cytochrome P-450 induction as an indication of exposure to environmental chemicals in the flounder *Platichthys flesus*. Marine Ecology Progress Series 46: 55-60.

Stehr, C. 1990. Ultrastructure of vacuolated cells in the liver of rock sole and winter flounder living in contaminated environments. Proceedings of the 12th International Congress for Electron Microscopy. Aug. 12-18. Seattle, Washington..

Stein, J.E., Collier, T.K., Reichert, W.L., Casillas, E., Hom, T., and Varanasi, U. 1991. Bioindicators of contaminant exposure and sublethal effects: studies with benthic fish in Puget Sound, Washington. Environmental Toxicology and Chemistry 11:701-714.

Stein, J.E., Hom, T., Collier, T.K., Brown, D.W., and Varanasi, U. 1995. Contaminant exposure and biochemical effects in outmigrant juvenile chinook salmon from urban and nonurban estuaries of Puget Sound, Washington. Environmental Toxicology and Chemistry 14:1019-1029.

Stephen, C., Kent, M.L., and Dawe, S. 1993. Hepatic megalocytosis in wild and farmed chinook salmon *Oncorhynchus tshawytscha* in British Columbia, Canada. Diseases of Aquatic Organisms 16:35-39.

Tysklind, M., Rappe, C., Tillet, D.E., and Eriksson, L. 1995. Inhibition of ethoxyresorufin-O-deethylase (EROD) activity in mixtures of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and

polychlorinated biphenyls (PCBs). Poster presented at Second Society of Environmental Toxicology and Chemistry World Congress (16th Annual Meeting). November 5-9, Vancouver, British Columbia.

Varanasi, U., Stein, J., and Nishimoto, M. 1989. Biotransformation and disposition of polycyclic aromatic hydrocarbons (PAH) in fish. pp. 93-149. IN: Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. U. Varanasi (ed.). CRC Press, Boca Raton.

Yunker, M.B. and Cretney, W.J. 1995. Chlorinated dioxin trends between 1987 and 1993 from samples of crab hepatopancreas from pulp and paper mill and harbour sites in British Columbia. Canadian Technical Report of Fisheries and Aquatic Sciences 2082: 138 p.

Yunker, M.B. and Cretney, W.J. 1996. Dioxins and furans in crab hepatopancreas: use of principal components analysis to classify congener patters and determine linkages to contaminantion sources. IN: Environmental fate and effects of pulp and paper mill effluents. M.R. Servos, K.R. Munkittrick, J.H. Carey and G.J. Van der Kraak (eds.) St. Lucie Press, Delray Beach, Florida.

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Hodson, P.V., Efler, S., Wilson, J.Y., El-Shaarawi, A., Maj, M. and Williams, T.G. 1996. Measuring the Potency of Pulp Mill Effluent for Induction of Hepatic Mixed Function Oxygenase Activity in Fish. Journal of Toxicology and Environmental Health 49(1):83-110.

Presentations:

Wilson, J., Addison, R., Glickman, B., Gordon, R., Martens, D. and Reichert, W. 1996. CYP 1A1 Activity and DNA Adducts in Juvenile Chinook Salmon Overwintering in the upper Fraser and Thompson Rivers in British Columbia. 27th Annual Meeting of the Environmental Mutagen Society, Mar.24 -28, Victoria, BC.

Wilson, J., Parrott, J., and Hodson, P. 1994. Additivity of Polychlorinated Dibenzo-p-dioxin (PCDD) Mixtures for Inducing Ethoxyresorufin-O-deethylase (EROD) Activity in Rainbow Trout. 29th Central Canadian Symposium on Water Pollution Research, Feb. 9, 10, Burlington, Ontario.

Hodson, P., Wilson, J., LeMay, M. and Efler, S. 1994. Factors Affecting the Induction of Mixed Function Oxidase Enzymes in Rainbow Trout Exposed to Pulp Mill Effluents. 29th

Central Canadian Symposium on Water Pollution Research, Feb. 9, 10, Burlington, Ontario.

Parrott, J.L., Wilson, J.Y. and Hodson, P.V. 1993. Potencies of Mixtures of Polychlorinated Dibenzo-p-dioxins (PCDDs) for Inducing Ethoxyresorufin-O-deethylase (EROD) Activity in Rainbow Trout. 20th Annual Aquatic Toxicity Workshop, Oct. 17-21, Quebec City, Quebec.

Poster Presentations:

Wilson, J., Addison, R., Gordon, R., Martens, D., and Glickman, B. 1995. MFO Activity and Contaminant Analysis of Overwintering Juvenile Chinook Salmon in the Fraser River, B.C. Second World Congress (16th Annual Meeting) of the Society of Toxicology and Chemistry, Nov. 5-9, Vancouver, B.C.

Parrott, J.L., Wilson, J.Y. and Hodson, P.V. 1994. Potency of Mixtures of Polychlorinated Dibenzo-p-dioxins (PCDDs) for Inducing Mixed Function Oxygenase in Rainbow Trout (Oncorhynchus mykiss). 2nd International Conference on Environmental Fate and Effects of Bleached Pulp Mill Effluents, Nov. 6-10, Vancouver, B.C.

Hodson, P.V., Efler, S., Maj, M. and Wilson, J.Y. 1994. A Refined Protocol for Measuring the Potency of Effluents, Extracts or Pure Compounds for Inducing MFO Activity of Fish. 2nd International Conference on Environmental Fate and Effects of Bleached Pulp Mill Effluents, Nov. 6-10, Vancouver, B.C.

Efler, S., Hodson, P., Wilson, J. and El-Shaarawi, A. 1994. Protocol for Measuring Activity of Ethoxyresorufin-O-deethylase in Fish Tissue with a Micro-Plate Fluorometer and Spectrophotometer. 29th Central Canadian Symposium on Water Pollution Research, Feb. 9, 10, Burlington, Ontario.

Parrott, J.L., Hodson, P.V., and Wilson, J.Y. 1993. Problems Assessing the Relative Potencies of Polychlorinated Dibenzo-p-dioxins (PCDDs) and Dibenzofurans (PCDFs) for Inducing Ethoxyresorufin-O-deethylase (EROD) Activity in Rainbow Trout. 14th Annual Meeting of the Society of Envrionmental Toxicology and Chemistry, Nov. 14-18, Houston, Texas.

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Title of Thesis: Biochemical Responses Based on Cytochrome P450 Induction in Chinook Salmon (*Oncorhynchus tshawytscha*) Exposed to Bleached Kraft Mill Effluent on the Fraser River.

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